

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

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Anopheles (Nyssorhynchus) pristinus Nagaki & Sallum, n. sp. of the Myzorhynchella Section is described based on morphological characters of adult females, males, fourth-instar larvae, pupae and male genitalia. *Anopheles (Nyssorhynchus) antunesi* Galvão & Amaral is characterized to fix its identity and distinguish it from *An. pristinus*. The eggs of *An. antunesi* are described for the first time. Molecular characterization employing sequences of the COI mitochondrial gene and the ITS2 region of ribosomal DNA are provided for each species. *An. antunesi* and *An. pristinus* are compared with morphologically similar species of the Myzorhynchella Section. The results of the present study suggest that the new species has been misidentified as both *An. antunesi* and *Anopheles lutzii* Cruz. *An. antunesi* and *An. pristinus* are sympatric, occurring at high altitudes in Serra da Mantiqueira, Southeastern Brazil.

Key words: COI - ITS2 - morphology - Serra da Mantiqueira - new species

The genus *Anopheles* Meigen includes 458 species, which are subdivided into seven subgenera (Harbach 2008). The subgenus *Nyssorhynchus* Blanchard includes some of the most important vector species of human malaria parasites in Central and South America (Sallum & Wilkerson 1997) and is divided into three sections, the Albimanus, Argyritarsis and Myzorhynchella Sections (Harbach 2008). The Albimanus Section includes 19 formally recognized species (Faran 1980), the Argyritarsis 11 species (Linthicum 1988, Rosa-Freitas 1989, Motoki et al. 2009) and the Myzorhynchella four species, i.e., *Anopheles lutzii* Cruz, *Anopheles parvus* (Chagas), *Anopheles nigratarsis* (Chagas) and *Anopheles antunesi* Galvão & Amaral (Galvão 1941). These four taxa were described from specimens collected in Brazil (Belkin et al. 1971) and there is no epidemiological evidence supporting the involvement of any Myzorhynchella species as vectors of pathogens.

Galvão & Amaral (1940) described *An. antunesi* based on morphological characters of all life stages, including eggs. Specimens were from Vila Emilio Ribas, municipality of Campos do Jordão, Serra da Mantiqueira, Southeastern Brazil. Since the description of *An. antunesi*, few studies have been conducted for species of the Myzorhynchella Section. Galvão (1941) described and illustrated the eggs and the male genitalia of specimens of *An. antunesi* collected in Casa Grande, a forest

reserve in the Serra do Mar within the Mata Atlântica, in municipality of Salesópolis, state of São Paulo (SP). More recently, Forattini et al. (1997) described the eggs of *An. antunesi* from specimens collected in Campos do Jordão, Serra da Mantiqueira.

The type specimen of *An. antunesi* was deposited in the Faculdade de Medicina, Universidade de São Paulo (FMUSP) (Galvão & Amaral 1940). The FMUSP collection was transferred to the Faculdade de Saúde Pública (FSP) of USP in the late 1960s; however, except for one adult female paratype (E-2034) (Forattini et al. 1970), no other type specimen of *An. antunesi* was found among the individuals in the FMUSP collection. It is likely that the holotype of *An. antunesi* is either lost or deposited in an unknown collection.

While examining specimens of *An. antunesi* from Serra da Mantiqueira, we observed that they could be separated into two morphological forms based on the pattern of pale and dark wing spots and characters of the larvae and male genitalia. One morphological form corresponds to *An. antunesi*, whereas the second form belongs to an undescribed species. The objective of this paper is to describe the new species of the Myzorhynchella Section and to characterize *An. antunesi* employing both morphological characters and DNA sequence for the ITS2 region of ribosomal DNA and the COI mitochondrial gene.

MATERIALS AND METHODS

Females were collected in a Shannon trap in the vicinities of the Parque Nacional do Itatiaia, municipality of Itatiaia, state of Rio de Janeiro (RJ), Brazil (-22.41630556S 44.62213889W, Datum SAD69) and in Pico do Itapeva, municipality of Pindamonhangaba, SP, Brazil (-22.75847222S -45.51527778W, Datum SAD69). Eggs were collected from two females of each locality. Females were blood-fed and one wing was removed

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48 h later to induce oviposition. Eggs were fixed in Bouin's solution 36 h after oviposition and prepared for scanning electron microscopy (SEM) following the protocol described by Forattini & Marucci (1993). External morphology of the eggs was examined in a JEOL-P15 scanning electron microscope (JEOL, Japan).

Morphological characters of the adult females, males, fourth-instar larvae, pupae and male genitalia were examined. 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 for morphological characters follows Harbach & Knight (1980). The nomenclature of the wing veins follows Belkin (1962), and the terminology for wing spots follows Wilkerson & Peyton (1990). Specimens used in this study are deposited in the Entomological Collection of the FSP-USP.

Sequences of the ITS2 of ribosomal DNA and COI barcode subunit of the mitochondrial genome were generated for six specimens of *An. antunesi* and 12 specimens of *Anopheles pristinus*. Genomic DNA was extracted from individual mosquitoes following the insect DNA extraction protocol provided by the QIAgen DNeasy® Blood and Tissue Kit (QIAgen Ltd, Crawley, UK). For one specimen of *An. antunesi*, only the abdomen was used to obtain sequences. The extraction protocol for this specimen was the same used for whole specimens except that the DNA was eluted in 50 µl of buffer AE. Considering that the chance of cross contamination was high, DNA was extracted in a flow microbiological safety cabinet.

The ITS2 was amplified 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). Polymerase chain reactions (PCR) were carried out in a 25 µL reaction mix containing 1 µL of DNA of the first elution; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 2.5 µL DMSO; 5 pM of each primer; 200 µM each dNTPs; 2.5 U New England Biolabs® Taq polymerase (New England Biolabs, Ipswich, MA); the remaining volume of ultrapure H₂O up to 25 µL. Amplification consisted of a 3 min denaturation at 94°C, 34 cycles at 94°C, 60°C and 72°C for 30 s each, followed by a 10-min extension at 72°C.

The primers 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') (Folmer et al. 1994) were used to amplify 658 bp of the COI gene. PCR reactions (25 µL) contained 1 µL DNA; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 2.5 µL DMSO; 5 pM of each primer; 200 µM each dNTPs; 2.5 U New England Biolabs® Taq polymerase (New England Biolabs, Ipswich, MA); the remaining volume of ultrapure H₂O up to 25 µL. Amplification consisted of a 3-min denaturation at 94°C and 35 cycles at 94°C, 55°C and 72°C for 1 min each, followed by a 7-min extension at 72°C. COI and ITS2 PCR products were purified using PEG precipitation (20% polyethylene glycol 8000/2.5 M NaCl). Sequencing reactions were carried out in both directions using the ABI Big Dye Terminator Kit version 3.1 (PE Applied Biosystems, Warrington, England) with the same primers used

for PCR. Sequencing reactions were purified in Sephadex G50® columns (GE Healthcare). Sequences were analyzed on an ABI Prism 3100 - Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequences were edited using Sequencher® for Windows version 4.9 (Gene Codes Corporation, Ann Arbor, USA), aligned in CLUSTAL X 1.8 (Thompson et al. 1997) and optimized manually in MacClade version 4.08 (Maddison & Maddison 2000). 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/>) and documented using PAUP* 4.0 software (Swofford 2003).

Larval and pupal exuviae, male genitalia, wings and legs of the specimens used for DNA extraction are deposited in the FSP-USP as vouchers. Template DNA from this study is retained at -70°C in the FSP-USP for future reference.

An. (Nyssorrhynchus) antunesi Galvão & Amaral
(Figs 1-4)

An. (Nyssorrhynchus) antunesi of Galvão & Amaral, 1940: 150. Galvão 1941: 552 (taxonomy, bionomics); Rodriguez & Varela 1962: 246 (first record in Uruguay); Gorham et al. 1967: 42 (distribution, illustrated key); Forattini 1962: 432 (distribution, taxonomy, identification key); Belkin et al. 1971: four (type information, bionomics); Knight & Stone 1977: 61 (distribution); Forattini 2002: 213 (identification key).

Morphological characterization - *An. antunesi* can be recognized by the following characteristics of the male genitalia: ventral claspette without spicules, apex narrow (Fig. 1A), ventral surface with distinctly swollen, striated lobes; dorsal claspette with three long setae, most dorsal seta narrow, arising subapically, two large, contorted, flattened ventral setae arising apically; aedeagus long, slender, strongly sclerotized laterally, with a pair of subapical leaflets, leaflets well developed, straight, parallel to the longitudinal axis, strongly sclerotized, serrated on dorsal and lateral surfaces; apex of aedeagus somewhat rounded (Fig. 1E); proctiger membranous, without spicules (Fig. 1C). The adult female can be recognized by having abdominal terga II-VII without scales; variable pattern of pale and dark scales on vein R₄₊₅, mostly pale-scaled, with pale scales medially, with small dark spots at proximal and distal ends; vein CuA₂ pale-scaled on proximal 0.5, dark-scaled distally (Fig. 2A); foretarsomeres 1-3 mostly dark-scaled with narrow apical pale rings, foretarsomeres 4,5 entirely dark-scaled; midtarsomere 1 with few pale scales dorsally at apex, midtarsomeres 2-5 dark-scaled; hindtarsomere 1 mostly dark-scaled, with an apical white ring, hindtarsomere 2 dark-scaled on approximately basal 0.5, white-scaled on apical 0.5, hindtarsomeres 3-5 entirely white-scaled. Based on fourth-instar larvae, *An. antunesi* can be recognized by the following combination of characters: seta 2-C weakly aciculate, seta 4-C single, long, aciculate; seta 14-P usually with three long, slender branches (Fig. 3A); seta 1-II-VII palmate, with short pedicel, leaflets ta-

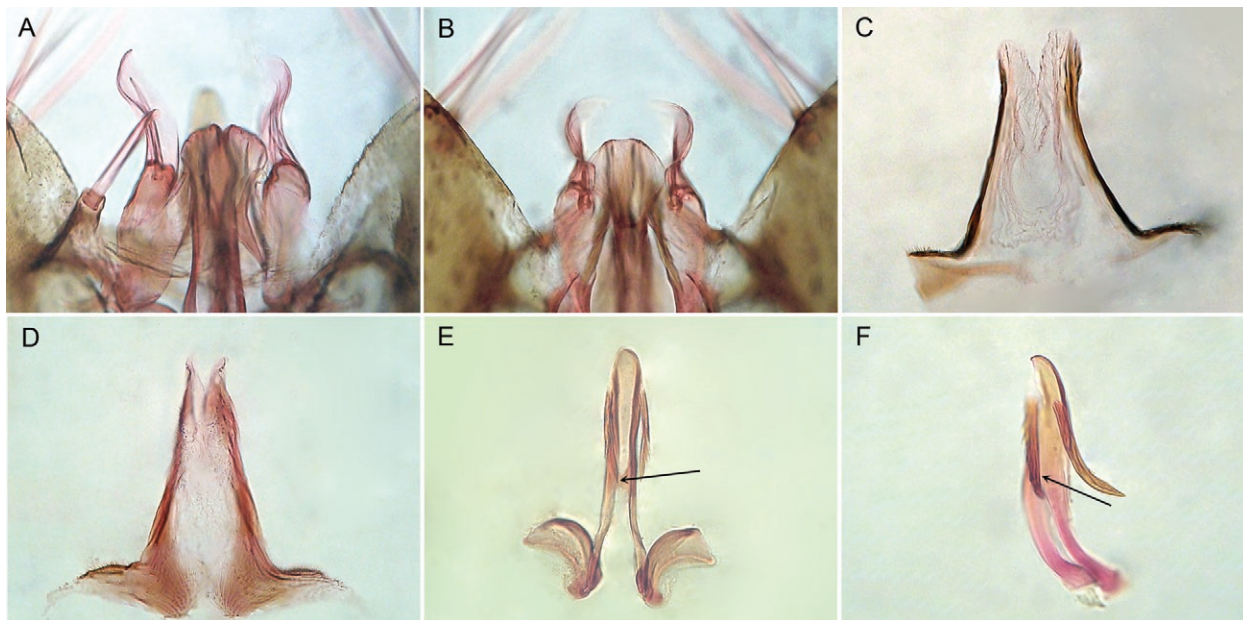


Fig. 1: male genitalia of *Anopheles antunesi* (A, C, E) and *Anopheles pristinus*, n. sp. (B, D, F) from Southern Brazil. A, B: ventral claspette (dorsal aspect); C, D: proctiger; E, F: dissected aedeagus showing the position of the ventromesal subtriangular projection.

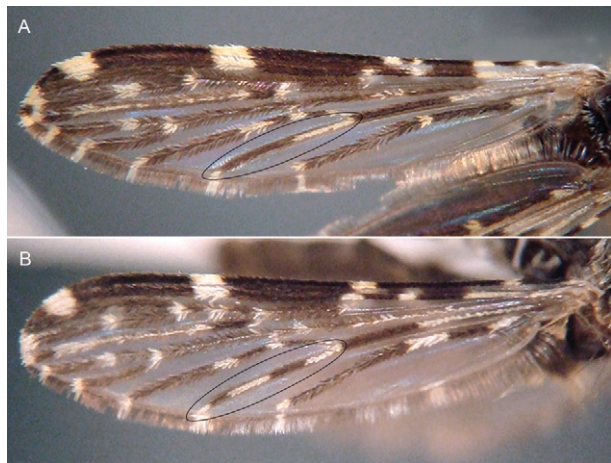


Fig. 2: dorsal surface of the wings of *Anopheles antunesi* (A) and *Anopheles pristinus*, n. sp. (B) from Southern Brazil, showing details of the vein CuA_2 .

pering to apex, ending in a pointed apex (Fig. 3C); 6-IV-VI long, bifid (Fig. 3E). Pupae have the trumpet darkly pigmented at midlength, 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 dorsal surface, ribs weakly divided into lobes (Fig. 4A, D); deck narrow, uniformly covered with irregularly shaped tubercles (Fig. 4C); ventral and lateral plastron with somewhat circular pores (Fig. 4B).

Molecular characterization - The amplicon length of the ITS2 sequence was consistent at 472 bp (without the flanking 5.8S and 28S). Six sequences revealed a single haplotype comprised of 19.1% T, 19.3% A, 28.8% C and 32.8% G. A FASTA search using the Database: nucleotide collection - Optimize for: Somewhat Similar (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed that the ITS2 sequence of *An. antunesi* shares 77% similarity with *Anopheles pictipennis* (Philippi) within a query coverage of 84%. Two unique COI haplotypes were detected in the 658 bp (without the primer regions) fragment. Haplotype 1 is comprised of 39.2% T, 28.7% A, 15.8% C and 16.3% G, whereas haplotype 2 is comprised of 39.5% T, 28.6% A, 15.5% C and 16.4% G. The ITS2 and COI sequences generated during this study are available in GenBank under the following accessions: ITS2 (GU989324 - GU989329), COI (GU989342 - GU989347, GU989349).

Distribution - *An. antunesi* was recorded in Brazil (Galvão & Amaral 1940, Galvão 1940, Forattini 1962, 2002, Gorham et al. 1967), Argentina (Darsie 1985, Gorham et al. 1967) and Uruguay (Rodríguez & Varela 1962, Gorham et al. 1967). In Brazil, the species was reported in SP, municipality of Campos do Jordão (Galvão & Amaral 1940, Galvão 1940, Forattini et al. 1997), state of Maranhão (MA), Northeast (Rebêlo et al. 2007), state of Rio Grande do Sul (RS) (Cardoso et al. 2004), municipality of Guaíba (Deane & Neto 1969) and state of Paraná (PR) (Rachou & Ricciardi 1951), municipality of Foz do Iguaçu (Consolim et al. 1993). However, in considering the difficulties of identifying species of the

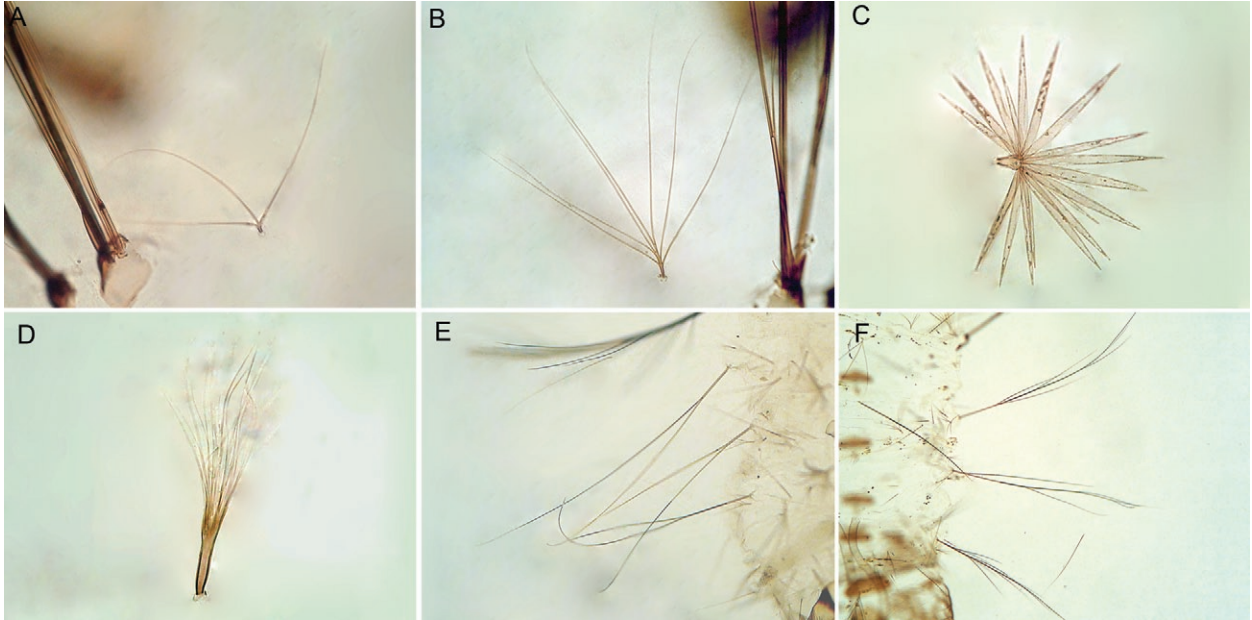


Fig. 3: larval characters of *Anopheles antunesi* (A, C, E) and *Anopheles pristinus*, n. sp. (B, D, F) from Southern Brazil. A, B: seta 14-P; C, D: palmate seta 1-II-VII; E, F: seta 6-IV-VI.

Myzorhynchella Section based on morphological characteristics of the adult females, it is likely that the records of *An. antunesi* in Argentina (Darsie 1985, Gorham et al. 1967), Uruguay (Rodriguez & Varela 1962, Gorham et al. 1967) and Brazil, in MA (Rebêlo et al. 2007), RS (Cardoso et al. 2004), municipality of Guaíba (Deane & Neto 1969) and PR (Rachou & Ricciardi 1951), municipality of Foz do Iguacu (Consolim et al. 1993) may refer to other species of the Myzorhynchella Section. Consequently, further studies are needed to clarify the distribution of *An. antunesi* in South America.

Type data - Holotype female (370), allotype male (371), originally deposited in the Collection of the Departamento de Parasitologia da FMUSP, Brazil, both probably lost.

Medical importance - *An. antunesi* is not known to be of medical importance.

Bionomics - Immature stages of *An. antunesi* were collected in Vila Emilio Ribas, Campos do Jordão, SP, Serra da Mantiqueira, approximately 1.570 m above sea level. Larvae were taken from ground pools, small streams and rock holes along the edges of the Capivari River. The habitat was shaded; the water was fresh, clean and cool, with little or no vegetation. No other species of the *Anopheles* were found associated with *An. antunesi* (Galvão & Amaral 1940). In Casa Grande, on the River Claro shores, situated in Serra do Mar, Salesópolis, Galvão (1941) collected immature stages in backwaters of small streams inside forest. Larvae of *An. antunesi* were also found in a locality in the vicinity of the Parque Nacional do Itatiaia, Serra da Mantiqueira, approximately 1.100 m above sea level, in backwaters of small

streams. The water was fresh, stagnant, cool, well oxygenated, pH approximately 5, with decomposing leaves and little vegetation in partial shade. *An. antunesi* was considered to be zoophilic by Galvão & Amaral (1940) because during the fieldwork specimens of this species did not bite the collectors. Forattini (1962) made a similar observation about the other Myzorhynchella species. In Casa Grande, Salesópolis, adults were captured at night on horses (Galvão 1941).

Material examined - The specimens of *An. antunesi* used for morphology were collected in Brazil, SP, municipality of Campos do Jordão, Vila Emilio Ribas, Galvão & Amaral coll., 1940, Galvão & Amaral, 1940 det.: E-2034 [Fwing, paratype], E-2038 [MG], E-2042 [FLePe], E-2044 [MLePe], E-2047 [MG], E-2048 [FLe], E-2049 [MG]. SP, municipality of Campos do Jordão, Sallum & Wilkerson coll., 20-Nov-2001, Sallum, 2001 det.: E-12438 [LePe], E-12449 [LePe], E-12461 [LePe], E-12462 [LePe], E-12463 [LePe], E-12464 [LePe]. SP, municipality of Pindamonhangaba, Pico do Itapeva, Fazenda Saint Claire, Sallum et al. coll., 27-July-2006, Sallum det., 2006: VP09-17 [LePeG]. RJ, municipality of Itatiaia, Parque Nacional do Itatiaia (22°24'58.7"S 44°37'19.7"W), Motta coll., July-2007: RJ-0 [FPe]. Motta & Nagaki coll., 25-March-2008, Sallum & Nagaki det., 2008, adults were collected in a Shannon trap: RJ03(11) [E], RJ03(12) [E], RJ03(13) [E]. SP, municipality of Salesópolis, Casa Grande, Galvão coll., 1940, det. Galvão, 1940: E-2037 [MG], E-2039 [MLe], E-2041 [MG], E-2050 [MG].

The specimens of *An. antunesi* used for molecular analysis were collected in Brazil, SP, municipality of Pindamonhangaba, Pico do Itapeva, Fazenda Saint Claire,

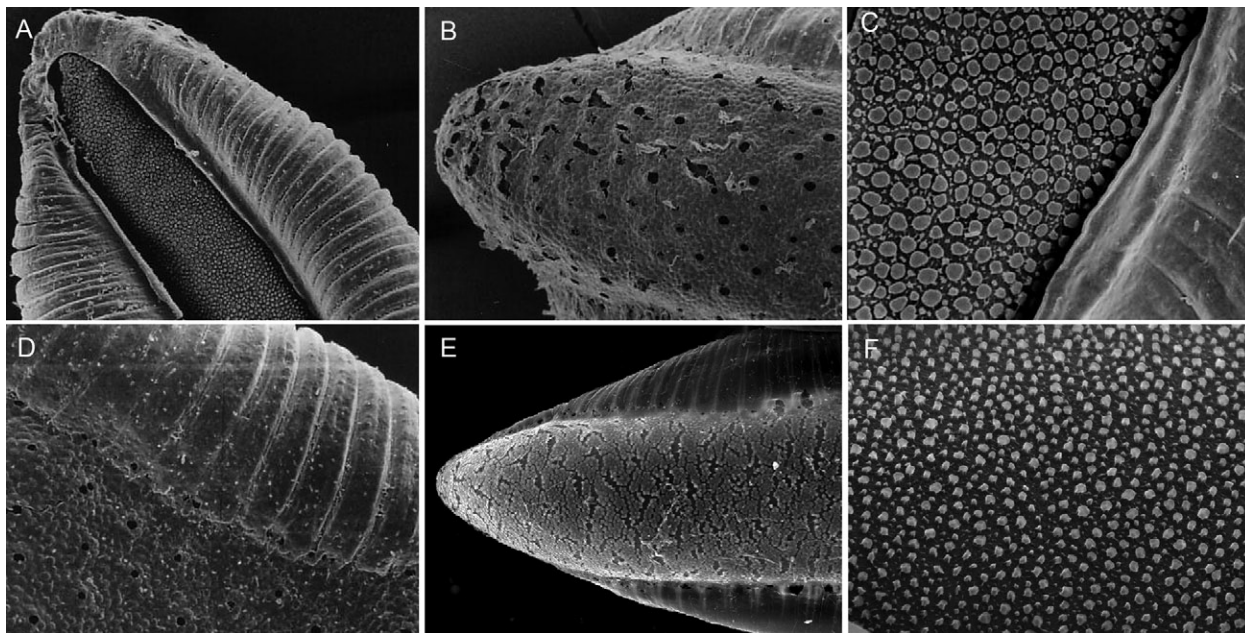


Fig. 4: eggs of *Anopheles antunesi* (A-D) and *Anopheles pristinus*, n. sp. (E, F) from Southern Brazil. A: posterior end (ventral view); B, E: posterior end (dorsal view); C, F: deck tubercles and float; D: lateral view of float.

Sallum et al., coll., 27-July-2006, Sallum det., 2006: VP09-17 [M]; adult collected in Shannon trap: VP11b [F], VP11c [F]. RJ, municipality of Itatiaia, Parque Nacional do Itatiaia (22°24'58.7"S 44°37'19.7"W), Motta & Nagaki coll., 25-March-2008, Sallum & Nagaki det., 2008, adults collected in Shannon trap: RJ03(6) [F], RJ03(11) [F], RJ03(12) [F], RJ03(13) [F].

An. (Nyssorhynchus) pristinus Nagaki & Sallum,
new species
(Figs 1-4)

An. (Nyssorhynchus) antunesi of Forattini et al. 1998:
120 (SEM eggs).

Morphological characterization - *An. pristinus* can be recognized by the following characteristics of the male genitalia: ventral claspette without spicules, apex somewhat rounded in shape (Fig. 1B), ventral surface with distinct lobes, median sulcus somewhat V-shaped; dorsal claspette with a narrow dorsal seta arising subapically, two large, contorted, flattened ventral setae arising from apex; aedeagus long, slender, strongly sclerotized laterally, a pair of subapical leaflets, leaflets well developed, strongly sclerotized, serrated, forming an angle of 25° with aedeagus (Fig. 1F); proctiger membranous mesally, with minute spicules laterally (Fig. 1D), strongly sclerotized at base. The adult female can be recognized by the absence of scales on abdominal terga II-VII; vein R_{4+5} mostly pale-scaled, with small dark spots at proximal and distal ends; vein CuA_2 with two dark spots (Fig. 2B); foretarsomeres 1-3 with narrow, pale apical rings, foretarsomeres 4 and 5 dark-scaled; midtarsomeres 1 and 2 mostly dark-scaled, with few apical pale scales, midtarsomeres 3-5 dark-scaled; hindtarsomere 1

with apical white ring, hindtarsomere 2 dark-scaled at approximately basal 0.5, white-scaled apically, hindtarsomeres 3-5 white-scaled. Based on characters of the fourth-instar larvae, *An. pristinus* can be recognized by the following combination of characters: seta 2-C single, seta 4-C single, aciculate; seta 14-P multibranched, with thin branches (Fig. 3B); abdominal seta 0-II-VII min, seta 1-III-VII palmate, with long, narrow pedicel supporting thin, hyaline leaflets (Fig. 3D), seta 6-IV-VI long, with few long branches (Fig. 3F). The pupae can be distinguished by having the trumpet darkly pigmented at midlength, paddle obovate, lightly pigmented, lighter than posterior abdominal segments, outer edge distal to external buttress with spicules. Eggs with floats lateral in position, long, covering part of dorsal surface, ribs weakly divided into lobes; deck narrow, uniformly covered with fine tubercles (Fig. 4F); ventral and lateral plastron with irregular cracked areas (Fig. 4E).

Molecular characterization - The ITS2 region was sequenced for 12 individuals from Campos do Jordão and Pindamonhangaba. The amplicon length was consistent at 470 bp (without the flanking 5.8S and 28S genes) and the 12 sequences revealed a single haplotype. The ITS2 haplotype comprised 20% T, 19.6% A, 27.4% C and 33.0% G. A FASTA search using the Database: nucleotide collection - Optimize for: Somewhat Similar revealed that the ITS2 sequences of *An. pristinus* share 81% similarity with *An. pictipennis*. The fragment of COI was sequenced for 11 individuals, all were same specimens used to obtain ITS2 sequences. Six mtDNA haplotypes were detected in approximately 658 bp (excluding the primer regions). The base compositions were similar for all haplotypes, comprising

38% T, 29% A, 16% C and 17% G. The ITS2 and COI sequences are available in GenBank under the following accessions: ITS2 (GU989330 - GU989341), COI (GU989348, and GU989350 - GU989359).

Distribution - *An. pristinus* is known only from Serra da Mantiqueira, Southern Brazil. Further studies will be necessary to establish the geographical distribution of *An. pristinus* and the range of sympatry with *An. antunesi*.

Medical importance - *An. pristinus* is not known to be of medical importance.

Bionomics - *An. pristinus* is a sylvatic species that occurs at high altitudes in cloud forests in Serra da Mantiqueira, Southern Brazil. Field collections were made at Fazenda Saint Claire, Pico do Itapeva, in the border area of municipalities of Pindamonhangaba and Campos do Jordão, at approximately 1.781 m above sea level. Larvae and pupae were taken from small, deeply shaded ground pools covered with grass. The larval habitat was in an open area outside the forest. The water from a small, slow running stream was fresh, well oxygenated and cool.

Etymology - The name *pristinus* is Latin for *pristine*, meaning pure, undamaged, fresh and clean and refers to the habitat where the immature stages were found.

Material examined - *Holotype* - Pinned adult male with associated larval and pupal exuviae and dissected male genitalia mounted on microscope slides. Brazil, SP, border of municipalities of Pindamonhangaba/Campos do Jordão, Pico do Itapeva, Fazenda Saint Claire, -22,75847222S, -45,51527778W, Datum SAD69, 27-January-2009, Nagaki et al. coll., specimen SP53-2, FSP-USP coll., E-13162. Paratypes: same collection data as the holotype, specimens SP53-1 [FLePe], SP53-3 [FLePe], SP55(1) [F, one wing removed from adult and mounted on microscope slide], SP55(3) [F, one wing removed from adult and mounted on microscope slide], SP55a [F], SP55b [F]. The holotype and paratypes are deposited in the Coleção Entomológica da FSP-USP.

Other specimens examined - Brazil, SP, municipality of Campos do Jordão, Sallum & Wilkerson coll. 20-Nov-2001, Sallum det., 2001 [identified as *An. near antunesi*, FSP-USP E-12370, LePe]; Fazenda Saint Claire (-22,75847222S, -45,51527778W, Datum SAD69), Nagaki et al. coll., 27-Jan-2009, Nagaki & Sallum det., 2009: SP51-100 [Pe mounted on microscope slide], SP53-4 [Le mounted on microscope slide], SP53-5 [Le mounted on microscope slide], SP53-100 [PeMG mounted on microscope slide], SP53-101 [FPe, one wing and three legs removed from adult and mounted on microscope slide].

Specimens of *An. pristinus* used to generate COI and ITS2 sequences were collected in Brazil, SP, municipality of Campos do Jordão, Sallum & Wilkerson. coll., 20-Nov-2001, Sallum det., 2001: E-12370 [identified as *An. near antunesi*, only the abdomen]; Fazenda Saint Claire (-22,75847222S, -45,51527778W, Datum SAD69), Sallum et al. coll., 27-July-2006, Sallum det., 2006, in Shannon trap: VP11a [F], VP11d [F], Nagaki

et al. coll., 27-Jan-2009, Nagaki & Sallum det., 2009: SP51-100 [F], SP53-100 [M], SP53-101 [F], SP53-4 [P], SP53-5 [P]; in Shannon trap: SP50a [F], SP50b [F], SP55(2) [F], SP55(4) [F].

DISCUSSION

Morphological studies of anophelines from Pico do Itapeva showed that specimens could be separated into two morphotypes based on the pattern of pale and dark wing spots, male genitalia and fourth-instar larvae. Detailed comparisons of specimens from both localities showed that individuals from Itatiaia belong to *An. antunesi* and that there was a second species in Pico Itapeva, which is herein formally named and described as *An. pristinus*. The ITS2 and COI sequences corroborated the morphological hypothesis of two species.

In the larval stage, *An. antunesi* and *An. pristinus* can be easily recognized as members of the Myzorhynchella Section by having abdominal seta 6-IV-VI branched; in the adult stage by the absence of both scales and posterolateral scales tufts from all abdominal segments and hindtarsomeres 3-5 white-scaled; by the male genitalia in having the dorsal claspette with two setae arising from the apex and one subapical seta and the accessory setae ending in a sharply acute apex (Galvão 1941). *An. antunesi*, *An. pristinus* and *An. lutzii* can be distinguished from *An. nigratarsis* in the adult stage by the absence of dark scales on hindtarsomeres 3 and 4 (these hindtarsomeres are white-scaled with a dark basal band in *An. nigratarsis*). Furthermore, *An. antunesi*, *An. pristinus* and *An. lutzii* can be separated from *An. parvus* by characters of the aedeagus of the male genitalia. In *An. parvus* the apex of the aedeagus is hook-like medially, with the lateral edges hyaline, whereas in *An. lutzii*, *An. antunesi* and *An. pristinus* the apex is flat, somewhat rounded, never hook-like. *An. antunesi* and *An. pristinus* can be separated from *An. lutzii* by having the aedeagal leaflets borne laterally, approximately parallel to the longitudinal axis of the aedeagus, whereas in *An. lutzii* the aedeagal leaflets arise laterally forming a 45° angle with the longitudinal axis of the aedeagus. Additionally, characteristics of the dark and pale wing spots can distinguish *An. antunesi* and *An. pristinus* from *An. lutzii* and *An. parvus*. *An. parvus* has three dark spots on vein R_{4+5} separated by two well-defined white spots, whereas in *An. lutzii* R_{4+5} is mostly dark-scaled with one preapical and one postbasal white spot. In *An. antunesi* and *An. pristinus*, vein R_{4+5} is mostly pale-scaled with one preapical and one postbasal dark spot. However, specimens of *An. antunesi* from Itatiaia have vein R_{4+5} either mostly dark or mostly pale-scaled. Galvão (1941) considered that *An. antunesi* and *An. lutzii* could be misidentified because of the polymorphisms of vein R_{4+5} , with individuals of *An. antunesi* having this vein mostly dark-scaled.

An. antunesi and *An. pristinus* can be distinguished by the pattern of dark and pale scales on vein CuA_2 . In *An. antunesi*, CuA_2 is white-scaled on the proximal 0.5 and dark on the distal 0.5, whereas in *An. pristinus* CuA_2 has a proximal pale spot, a dark spot, a pale spot and a distal dark spot (Fig. 2A, B). Consequently, the pattern

of pale and dark spots on CuA₂ can be used to identify *An. antunesi* and *An. pristinus*. In the male genitalia, *An. pristinus* can be separated from *An. antunesi* by having the apex of the ventral claspette moderately rounded apically (Fig. 1B), the proctiger spiculate (Fig. 1C) and the ventromesal subtriangular projection positioned approximately at midlength of the insertion and the apex of the leaflets (Fig. 1F). In *An. antunesi*, the apex of ventral claspette is somewhat straight (Fig. 1A), the proctiger is bare (Fig. 1C) and the ventromesal subtriangular projection is positioned distally, approximately at the apex of the leaflets (Fig. 1E). Fourth-instar larvae of *An. antunesi* can be distinguished from those of *An. pristinus* by having seta 1-II-VII palmate with a short pedicel (Fig. 3A) and seta 6-IV-VI bifid (Fig. 3E), whereas in *An. pristinus* 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 lateral branches (Fig. 3F). Characters of the eggs as seen by SEM can also distinguish *An. antunesi* and *An. pristinus*. The deck tubercles of the *An. antunesi* egg are larger and closer together than they are on the *An. pristinus* egg (Fig. 4C, F). The outer chorion of the ventral surface is covered with circular pores in *An. antunesi* (Fig. 4B, D), whereas it has several irregular cracked areas in *An. pristinus* (Fig. 4E). Based on these observations it seems likely that Forattini et al. (1998) described the eggs of *An. pristinus*.

The hypothesis for a new, species sympatric with *An. antunesi* in Pico do Itapeva, Serra da Mantiqueira as observed by morphological differences in few specimens was corroborated by the ITS2 rDNA and COI sequences. ITS2 rDNA and COI sequences reveal that *An. antunesi* and *An. pristinus* are distinct species. The ITS2 sequences of different individuals of each species are identical; however, comparisons between the two species show indels among individuals. ITS2 differences between *An. antunesi* and *An. pristinus* are consistent, suggesting that these differences represent inter-specific variation. The COI sequence variation between *An. antunesi* and *An. pristinus* also corroborates the morphological and ITS2 sequence data, confirming *An. pristinus* as a new species.

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