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Studies on *Anopheles* (*Kerteszia*) *homunculus* Komp (Diptera: Culicidae)

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Abstract

The present findings suggest that *Anopheles* (*Kerteszia*) *homunculus* may comprise more than one species. The rDNA ITS2 sequence data corroborate the presence of *An. homunculus l.s.* in Mata Atlântica, southern Brazil, and suggest that specimens from Trinidad may belong to an unnamed morphologically similar species. There is a need for additional studies to establish the geographical distribution of *An. homunculus l.s*. in continental South America and in Trinidad, especially in southern Mata Atlântica, Brazil.

Key words: *Anopheles homunculus*, species complex, morphology, ITS2, malaria

Introduction

The geographical distribution and involvement of *Anopheles* (*Kerteszia*) *homunculus* Komp in human malaria transmission are poorly known. Published literature records show that this species occurs on the eastern slopes of the Andes in Colombia and Bolivia, in Trinidad, Venezuela, the Guianas, Peru and southeastern Brazil (Forattini 1962, 2002; Zavortink 1973; Guimarães 1997). In Brazil, the species was reported in Santa Catarina (Coutinho 1947; Lima 1952; Martins 1958; Rachou 1958; Calado and Navarro-Silva 2005), Paraná (Forattini et al. 1970), São Paulo (Aragão 1964; Ferreira 1964) and Espírito Santo (Sallum et al. 2008).

Anopheles homunculus was described and named by Komp (1937) based on one adult male and three females collected as larvae taken from leaf axils of bromeliads in Restrepo (4° 10' 1.20"S 73° 25' 1.20"W), Department of Meta, Colombia, in September 1935. In the same year Komp named and validated *An.* (*Kerteszia*) *anoplus* from an adult male captured in December 1936 from the same locality as *An. homunculus*. Later, Lane (1953) synonymized *An. anoplus* with *An. homunculus*, and Stone and Knight (1956) designated a lectotype for *An. homunculus*.

Based on morphological characteristics of the adult female, *An. homunculus* is more similar to *An. cruzii* Dyar & Knab than to other species of the subgenus *Kerteszia*. Therefore, separation of these two species using adult female morphological traits can be difficult, especially if the specimens are poorly preserved. In contrast, characters of the male genitalia and the purple color of the immature stages of *An. homunculus* easily separate it from *An. cruzii* (Forattini 1962, 2002). Zavortink (1973) observed morphological variation in specimens of *An. homunculus* from Colombia in comparison with samples from Trinidad. These included wing spot characters, extent of white scaling on midtarsomere 3 and the scale pattern on the maxillary palpomeres. He judged that for an adequate evaluation of the taxonomic importance of those characters more specimens from Colombia were needed. We hypothesize that the morphological variation observed by Zavortink (1973) may be due to the fact that *An. homunculus* represents a species complex.

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The internal transcribed spacer subunit 2 (ITS2) region of the nuclear rDNA cistron has been widely employed in molecular systematic studies of *Anopheles* at the species level (e.g. Li and Wilkerson 2005, Li et al. 2005, Walton et al. 2007, Paredes-Esquivel et al. 2009). Comparisons of the secondary structure of ITS2 across eukaryotes have shown that differences and similarities in a highly conserved region of the 5'side of helix III represent landmark events leading to delimitation of biological species (Coleman 2009). Müller et al. (2007), in a large-scale analysis of ITS2 sequences, suggested that presence of compensatory base changes (CBCs) in helix III of the ITS2 can be used as a marker for species identification. CBCs occur when both nucleotides of a paired site mutate, thus maintaining the pairing and the secondary structure (Coleman 2009). Furthermore, Müller et al. (2007) demonstrated that the presence of a CBC between two organisms predicts with 93.11% accuracy that they belong to different species, whereas the absence of a CBC predicts that they are conspecific 76.57% of the time.

The objective of this study, therefore, was to determine if *An. homunculus* from Mata Atlântica, southeast Brazil, is conspecific with *An. homunculus* from two Colombian localities, Meta, Restrepo (type locality) and Chocó, Charambirá, Istmina, and Trinidad. To accomplish this we compared morphological characteristics of adult males and females, fourth-instar larvae and pupae, and nucleotide sequences of the second internal transcribed spacer (ITS2) of the nuclear rDNA cistron.

Materials and Methods

Mosquitoes for morphological characterization. TYPE SPECIMENS. *Anopheles homunculus*. COLOMBIA, Restrepo, coll. 1935, type specimen (lectotype) no. 53073, USNM, dissected male genitalia mounted on a microscope slide, larval exuviae mounted on a separate microscope slide with 2 other exuviae, exuviae number 3 is associated with the lectotype. *Anopheles anoplus*. COLOMBIA, Restrepo, 1935, coll. E. Osorno Mesa, type specimen no. 53074, USNM, adult male associated with fourth-instar larval exuviae, dissected male genitalia mounted on a separate microscope slide. NON-TYPE SPECIMENS. *Anopheles homunculus*. COLOMBIA, El Retiro, Intendencia de Meta, coll. P. C. A. Antunes, 1 Jan. 1935, det. J. O. Coutinho, June 1946, human landing, forest edge from 1:30 to 5:00 pm, 3 females. Chocó, Litoral del San Juan (formerly Istmina), Charambirá, coll. M. Suarez, 19 April 1984, det. M. Suarez, 1984, intra-domiciliary and peri-domiciliary, 2 females; in forest, human landing, 10 m above ground, 2 females. BRAZIL, Santa Catarina, Brusque, coll. and det. J. O. Coutinho, 1944, 2 male genitalia; coll. and det. J. O. Coutinho, 1946, 1 male genitalia; coll. M. M. Lima, Dec. 1951, det. M. M. Lima, Jan. 1952, 6 larvae; Blumenau, coll. J. O. Coutinho, 1944, det. J. O. Coutinho, July 1946, 1 male, 1 female, male genitalia; coll. and det. J. O. Coutinho, 1943, 6 male genitalia, 1 pupal exuviae; det. O. R. Causey, 1950, 3 male genitalia; Caldas da Imperatriz, coll. J. O. Coutinho, 1944, det. J. O. Coutinho, 1946, 1 male genitalia. Paraná, São Vicente, intra-domiciliary, coll. and det. E. Luz, June 1966, 1 female. São Paulo, Cananéia, Sitio Itapuan (25º 0' 54"S 47º 55' 37"W), coll. and det. M. A. M. Sallum, 15 May 2007, 2 females, 2 males, 1 male genitalia, with associated larval and pupal exuviae on microscope slides, same collection data, 24 Oct. 2007, 4 females, 15 males, 5 male genitalia, with associated larval and pupal exuviae on microscope slides. *Anopheles homunculus s. l.* TRINIDAD, Mosquito Middle America, 1 female and 1 fourth-instar larva, accession number TR1572, Trinidad 65-66.

Mosquitoes for ITS2 characterization. A single adult female specimen from Charambirá, municipality Litoral del San Juan (formerly Istmina), Chocó Department, Colombia, was collected in 1984 by Marco Suarez. This specimen, retained at room temperature in the Coleção Entomológica da Faculdade de Saúde Pública da Universidade de São Paulo, was used to generate ITS2 rDNA sequence. For Trinidad, we used three specimens collected from human bait, all with the following data: St. Andrew/St. David County, near Tamana, 61.16972222º W 10.47388889º N, 8 Aug. 2000, Wilkerson and Chadee coll. For Brazil, larvae and pupae identified as *An. homunculus* were taken from water in leaf axils of bromeliads in two localities: Valsugana, Santa Teresa municipality (19º 57' 58.4" S 40º 34' 45.2" W), Espírito Santo state, and Sitio Itapuan, Cananéia municipality (25º 0' 54"S 47º 55' 37"W), São Paulo state, both situated in the eastern Serra

do Mar, Mata Atlântica, Brazil. Larvae and pupae were raised in the laboratory to obtain adults with associated larval and pupal exuviae. The male genitalia of individuals from Trinidad and Mata Atlântica, Brazil were compared with those of the holotype, and when possible species identification was based on morphological characters of all life stages, except the egg.

DNA extraction, amplification, sequencing. ITS2 sequences were derived from eight individually reared adult males from Brazil (5) and Trinidad (3), and one museum-archived adult female from Colombia (see above). DNA was extracted following the animal tissue DNA extraction protocol provided by the QIAgen DNeasy® Blood and Tissue Kit (QIAgen Ltd., Crawley, UK). The extraction protocol for the Colombian 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 when using museum specimens, DNA was extracted in a separate room in a reverse flow microbiological safety cabinet.

Amplification of the ITS2 region 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 a 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. For the specimen from Colombia, we used 4 µl of the first elution. PCR protocol consisted of a 2-min denaturation at 94ºC, 34 cycles at 94ºC, 57ºC and 72ºC for 30 sec each, followed by a 10 min extension at 72ºC. For the museum specimen from Colombia, the amplicons generated in the first PCR were used as template for a second amplification, employing the same protocols for the fresh specimens. PCR products were visualized in 1% agarose gels stained with ethidium bromide. ITS2 PCR amplicons obtained from one individual of *An. homunculus* from Colombia were purified using PEG precipitation (20% polyethylene glycol 8000/2.5 M NaCl) and cloned into pGem-T Easy Vector (Promega, Madison, WI, USA). Eight positive clones were sequenced plus one direct sequence from PCR products. Sequencing reactions were carried out in both directions using the PCR primers and the Big Dye® Terminator Kit v.3.1 (Applied Biosystems, Warrington, England). Sequences were analyzed in an ABI Prism 377 - ABI Sequencer (Applied Biosystems, Foster City, CA, U.S.A.), and edited using Sequence Navigator® (version 1.0.1, PE Applied Biosystems).

ITS2 sequences were annotated using the HMMer algorithm (Eddy 1998) and optimized in ITS2 Database (Schultz et al. 2006; Selig et al. 2008). Considering that there was no highly similar sequence in the ITS2 Database, the novel ITS2 secondary structure was constructed in the Vienna RNA Package (Hofacker 2003), RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Sequences, including secondary structures were aligned and edited in 4Sale, "A tool for Synchronous RNA Sequence and Secondary Structure Alignment and Editing" (http://4sale.bioapps.biozentrum.uni-wuerzburg.de/index.html) (Siebel et al. 2008). The presence of CBCs in the ITS2 sequences was examined in 4Sale. Additionally, secondary structure prediction was carried out in Sfold (Software for statistical folding and rational design of nucleic acids, available at (http://www.wadsworth.org/) (Ding and Lawrence 2003, 2001; Ding et al. 2004). In Sfold, we used the Srna module that provides tools and statistics to characterize the Boltzmann ensemble of ITS2 structures. Details of the algorithm and its capabilities are presented in Ding and Lawrence (2003). The similarity of the ITS2 sequences generated in this study with those available in GenBank was assessed using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Intraspecific sequence differentiation was assessed using mean uncorrected P distance in PAUP* (Swofford 2003).

Template DNA from this study is retained at -70ºC in the Faculdade de Saúde Pública (FSP-USP), São Paulo, Brazil, for future reference (DNA reference numbers are: Brazil, Mata Atlântica, Cananéia: ST19, ST24, ST26, ST29, SP23-1; Santa Teresa, Espírito Santo: ES10-2; Charambirá, Litoral del San Juan (formerly Istmina), Colombia: E-10225). Microscope slides bearing the larval and pupal exuviae and male genitalia of the specimens from Mata Atlântica used for DNA extraction are deposited in the FSP-USP collection. Both wings and three legs of the Colombian specimen, mounted on a microscope slide, are deposited in FSP-USP collection (accession number E-10225). Vouchers and DNA from specimens collected in Trinidad (St.

Andrew/St. David County, near Tamana: "homunculusA", "homunculusB", "homunculusC") are deposited in the National Museum of Natural History, Smithsonian Institution, in Washington, D.C., USA.

Results

Anopheles (*Kerteszia*) *homunculus* Komp

Anopheles (*Kerteszia*) *homunculus* Komp, 1937: 509. Lectotype male (no. 3) with associated larval skin and male genitalia on separate microscope slides, deposited in the National Museum of Natural History (NMNH), USA. Type-locality: Restrepo, Meta, Colombia.

Anopheles (*Kerteszia*) *anoplus* Komp, 1937: 514. Holotype male with associated larval exuviae and male genitalia on separate microscope slides, deposited in the National Museum of Natural History (NMNH), USA. Type-locality: Restrepo, Meta, Colombia. Lane 1953: 287 (syn. *An. homunculus*).

Anopheles (*Kerteszia*) *homunculus* of Coutinho, 1946: 149 (first record in Brazil, systematics); Coutinho 1947: 13 (distribution); Rachou 1958: 149 (malaria vector status); Lima 1952: 401 (systematics, distribution); Lane 1953: 287 (systematics, distribution); Martins 1958: 429 (adult female identification); Forattini 1962: 441 (M*) (systematics, distribution); Aragão 1964: 73 (distribution, bionomics); Ferreira 1964: 329 (distribution, bionomics); Zavortink 1973: 20 (in part, specimens from Brasil, systematics); Calado and Navarro-Silva 2005: 1128 (PCR-RAPD, PCR-RFLP identification); Sallum et al. 2008: 671 (first record, Espírito Santo state).

Morphological characterization. *Anopheles homunculus* can be recognized as described below. In the male genitalia the ventral claspette is densely spiculose mesally (Fig. 1A,B), possessing a broad, large, lateral expansion that is somewhat sinuous at the lateral edges, curved posteriorly in ventral direction and possessing a long, sharp retrorse point anteriorly (Fig. 1C,D); the lobes of the ventral claspette are separated by a deep funnel-shaped emargination, and are connected at base (Fig. 1E); the dorsal claspette has two groups of long setae, a dorsal group composed of five long, twisted, flattened, swollen at mid-length, then tapering, becoming slender at apical 0.3 and ending in a narrow, pointed apex, and the ventral group is composed of three setae that are narrow, contorted and flattened (Fig. 1C,D); the aedeagus is long and slender with a pair of distinctive subapical leaflets (Fig. 1E) and the apical opening is bordered by two minute, incomplete lateral sclerites (Fig. 1F). Adult females can be recognized by a lack of scales on abdominal terga II-VII, the mesepimeron has both upper and middle patches of scales, the hindtarsomeres 2-5 possess broad apical bands of white scales, the maxillary palpomere 3 has scales slightly to moderately outstanding, scales on the palpomere 4 are decumbent or moderately outstanding, and the abdominal terga are dark, mauve when whole mounted in Canada balsam on a microscope slide. Larvae are distinctly purple with a dorsal pattern of dark pigment on the thorax and abdomen (Fig. 2A). The thorax is entirely bright purple, with dark pigment forming a complex pattern (Fig. 2A). On the abdomen the purple color is more evident on segments III, VI, VII and VIII, and laterally on the remaining segments. The abdominal dark pigment pattern on segments I and II is concentrated dorsomedially, the dark pattern of segment I is connected to that on segment II by a narrow dark bridge, in the abdominal segment III, the dark pattern extends laterally at the level of seta 6, with two large unpigmented areas bordering a central unpigmented area, the dark pigment pattern reaches the posterior border of the segment with a median dark line extending anteriorly into the unpigmented medial portion (Fig. 2B), the dark pattern on segments IV and V are distinct, restricted to the longitudinal anteroposterior portion, the dark pattern on segment IV is connected to that on segment V by a narrow dark bridge, on segments VI, VII and VIII, the dark pattern is distinct, extending to lateral sides (Fig. 2C). The fourth-instar larva can be recognized as follow: seta 4-A usually single; seta 3-C stronger than 2-C (Fig. 2D); 4-C stronger than 2-C, single or forked at apex; 5,7-C long; 6-C moderately long or long, reaching or extending beyond insertion of 4-C; 1-I-VII palmate, small, with pointed or blunt leaflets; 5-II-V branched near base; 6-VI long, plumose; 4a-X usually weakly developed, shorter or slightly longer than anal saddle; pecten alternating long and short spines medially, spicules restricted to basal portion of external edge; and saddle darkly pigmented dorsally. The pupa can be recognized by: possessing a trumpet darkly pigmented at mid-length, somewhat yellow at

base and apex (Fig. 2E); seta 9-V moderately long, pointed, dissimilar to 9-III; paddle obovate, unpigmented or very weakly pigmented, lighter than posterior abdominal segments (Fig. 2F); outer edge of paddle distal to external buttress without spicules, marginal spicules along distal portion of external buttress closely spaced, moderately long, numerous; and, by its distinct purple color.

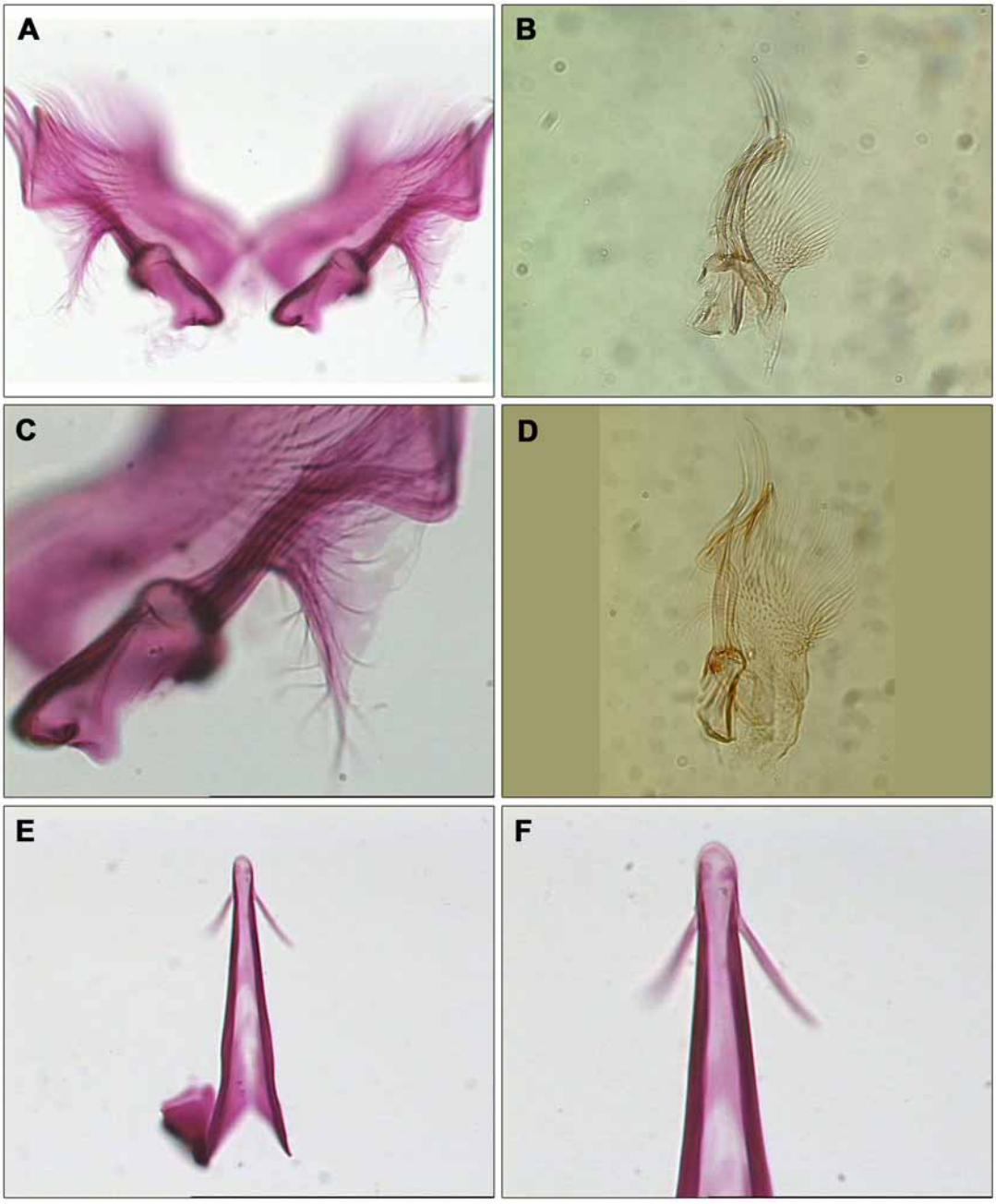


FIGURE 1. Male genitalia of *Anopheles homunculus* Komp (lectotype) from Restrepo, Colombia, and *Anopheles homunculus* from Mata Atlântica, Brazil. A, B, detail of the ventral claspette showing mesal spicules; C, D, detail of the ventral claspette showing the anterior retrorse process; E, aedeagus showing the lateral leaflets; F, detail of the apex of the aedeagus showing the minute apicolateral sclerite. B and D represent the lectotype of *Anopheles homunculus*.

Distribution. *Anopheles homunculus s.l.* has a large, discontinuous distribution in South America. It was reported from localities in Colombia, Venezuela, Bolivia, the Guianas, Peru and Brazil (Zavortink 1973). In Brazil, *An. homunculus* is known from areas in the Mata Atlântica in Santa Catarina, Paraná, São Paulo and Espírito Santo states (Forattini 1962, 2002, Marrelli et al. 2007, Sallum et al. 2008).

Considering the possibility that *An. homunculus* may be misidentified as *An. cruzii*, it would be plausible to suppose that the geographical distribution of the taxon may be more extensive than is reported in the published literature. The species may occur along the Atlantic coast, and also in areas in west Brazil.

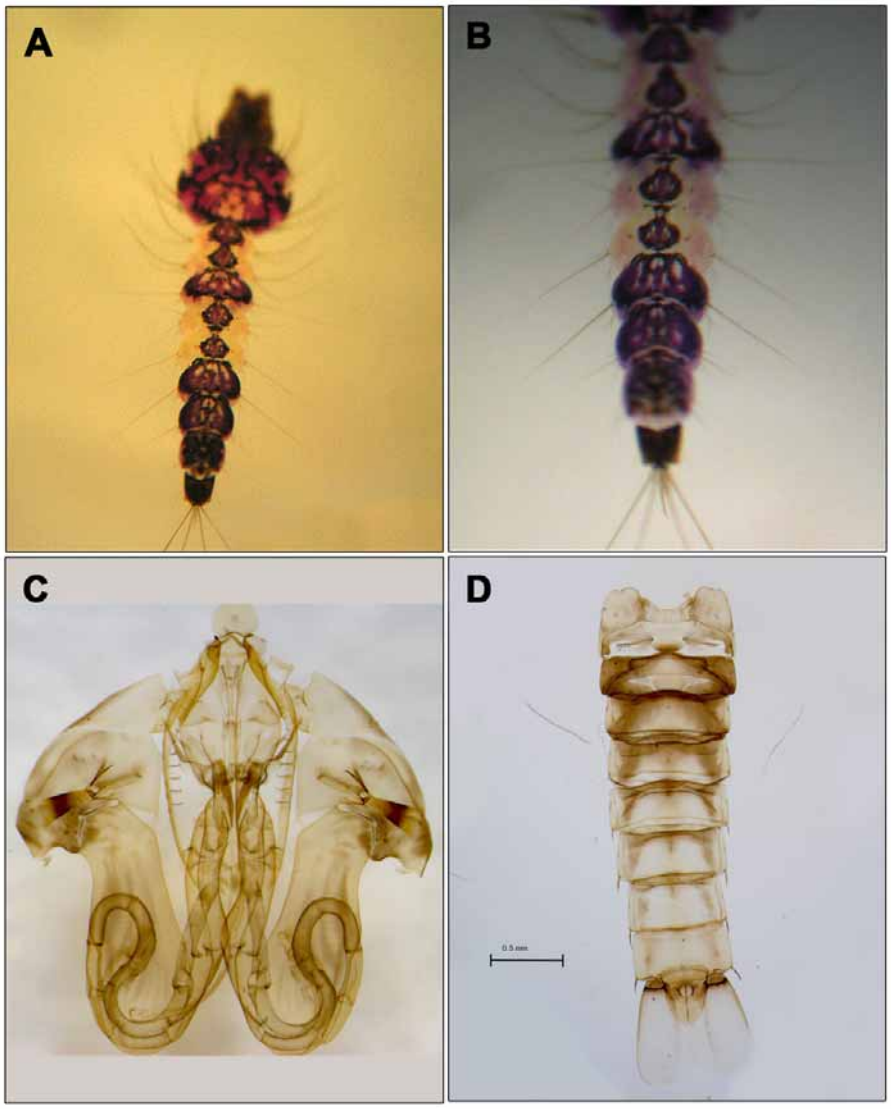


FIGURE 2. Fourth-instar larva and pupa structures of *Anopheles homunculus* from Mata Atlântica, Brazil. A, fourth-instar larva showing the bright purple color in the thorax and abdominal segments I-VIII; B, fourth-instar larva showing the darkly pigmented pattern in the abdominal segments I-VIII; C, cephalothorax of the pupa, showing a dark ring in the middle of the trumpet; D, metanotum and abdomen of the pupa.

Bionomics. Habitats for the immature stages of both forms of *An. homunculus* are the leaf axils of epiphytic and terrestrial bromeliads in areas of dense humid primary forest.

Medical importance. *Anopheles homunculus* was observed to be a highly anthropophilic and endophylic species in Blumenau, Santa Catarina (Coutinho 1947), and a secondary or local vector of human malaria in localities in eastern Mata Atlântica, southern Brazil (Rachou 1958; Forattini 1962, 2002). Females were found naturally infected with both oocysts and sporozoits in localities in Santa Catarina state by Coutinho (1946), Rachou (1946), and Rachou and Ferreira (1947).

The role of *An. homunculus* in *Plasmodium* transmission in Trinidad has been recorded in the literature as either a vector or suspected vector (Pittendrigh 1948; Forattini 1962; Chadee and Kitron 1999; Chadee et al. 1999).

ITS2 molecular characterization. The second internal transcribed spacer (ITS2) of the nuclear rDNA cistron was sequenced for six individuals of *An. homunculus* from Cananéia, Mata Atlântica, Brazil (GenBank accession numbers FJ176945-FJ176950), one direct sequence and eight clones from a single individual from Colombia (GenBank accession numbers FJ176951-FJ176959), and three specimens of *An. homunculus l. s.* from Trinidad (GenBank accession numbers FJ176960-FJ176962). The ITS2 sequences consist of the following base composition: 0.17544% T, 0.20468% A, 0.31871% C and 0.30117% G for Cananéia, Brazil; 0.17289% T, 0.20465% A, 0.32079% C and 0.30168% G for Colombia; and 0.17544% T, 0.19883% A, 0.31823% C and 0.30750% G for *An. homunculus l. s*. for Trinidad.

A single ITS2 sequence of this species was available in GenBank (DQ364655, Brazil, Malafronte et al. 2006, unpublished). The ITS2 sequences of *An. homunculus* from Mata Atlântica, Cananéia, São Paulo (n = 5), and Santa Teresa, Espírito Santo (n = 1) share 100% similarity. However, one individual from Parque Estadual Turístico do Alto Ribeira (Petar Reserve), southern São Paulo state (DQ364655), exhibited only 99% similarity with these specimens. Along a 344 bp alignment, three bases varied, one singleton polymorphic site (position 341), and two 1-bp indels (positions 339 and 344, Fig. 3). Mean uncorrected "P" distance among ITS2 sequences of *An. homunculus* from Cananéia / Santa Teresa and DQ364655 is 0.00292.

Comparing the ITS2 cloned sequences from a single Colombian specimen with those from Cananéia / Santa Teresa, excluding DQ364655, the mean uncorrected P distance ranged from 0.00 to 0.00887. One cloned ITS2 sequence shares 100% similarity with sequences from samples collected in Cananéia / Santa Teresa. Sequences from the Colombian specimen showed superimposed chromatogram peaks apparently due to intragenomic indel variation in a CG repeat at positions 312 and 313, and a polymorphic C or T indel at position 337. Single base pair polymorphism in highly conserved regions may, however, represent sequencing/cloning artifacts. Apparently, there is no fixed difference between the Colombian and Cananéia / Santa Teresa specimens. Two clones showed either a T or C insertion at position 337, whereas the majority showed a deletion in this position. In contrast, sequences from the Cananéia / Santa Teresa specimens showed no polymorphism, and all five individuals were directly sequenced from PCR products, with a T at position 337 in all individuals.

Mean uncorrected P distance among three ITS2 sequences generated from *An. homunculus l. s.* collected in Trinidad is 0.0000. There is a single ambiguity, which may be due to a sequencing artifact (Fig. 3). The mean uncorrected P distance among the ITS2 sequences of *An. homunculus l. s.* from Trinidad and *An. homunculus* from Colombia and Brazil ranged from 0.00298 to 0.01190. ITS2 sequences from Trinidad and from Colombia and Mata Atlântica varied at five sites, including two 2-bp indels (bases 211, 212 and 282, 283) and one singleton polymorphic site at position 190 (Fig. 3). The mean uncorrected P distance among clones from Colombia ranged from 0.00 to 0.01173. The most divergent clone is E10225clone15. The mean uncorrected P distance between this clone and the remaining clones varied from 0.00887 to 0.01173.

Secondary structures were predicted in Sfold by centroids in Botzmann-weighted ensemble, which generated a set of clusters, and from them, the best cluster centroid was determined that reflected the high-frequency base-pair in the structure sample. Sfold also compares the centroids with suboptimal minimum free energy (MFE) structures. Based on sequence of one clone from Colombia (FJ176955), the Srna module of Sfold generated the ensemble centroid shown in Fig. 4A, whereas for FJ176953 the ensemble centroid is shown in Fig. 4B, and for the clone (FJ176945) from Cananéia, Mata Atlântica, Brazil, the ensemble centroid is shown in Fig. 5. For *An. homunculus* B (FJ176961) from Trinidad, the ensemble centroid is shown in Fig. 6. In comparing the ensemble centroids of these three populations of *An. homunculus*, we identified helix I, II, and III. Helix II can be recognized by the characteristic pyrimidine-pyrimidine mismatch (C-U) (Fig. 6). Helix III is the longest helix and can be recognized by having its most conserved region on the 5' side, near the tip (in brackets). Also, a highly conserved pairing (GGU) is in helix III on positions 131-133, and a UGGU motif is at positions 200-203 on the 3'side of the most conserved region of helix III. The indels observed in specimens from Trinidad are in loop regions, i.e., deletion of a UA at position 211-212 is located in a loop between two side arms, and the UG insertion, positions 280-281, is on a loop at the apex of a sidearm of helix III (Fig. 6). This sidearm is absent in sequences from Colombia and Mata Atlântica, Brazil (Fig. 4B, 5), except

in one clone from Colombia (FJ176955, Fig. 4A). No compensatory base changes (CBC) were observed among sequences from the three populations.

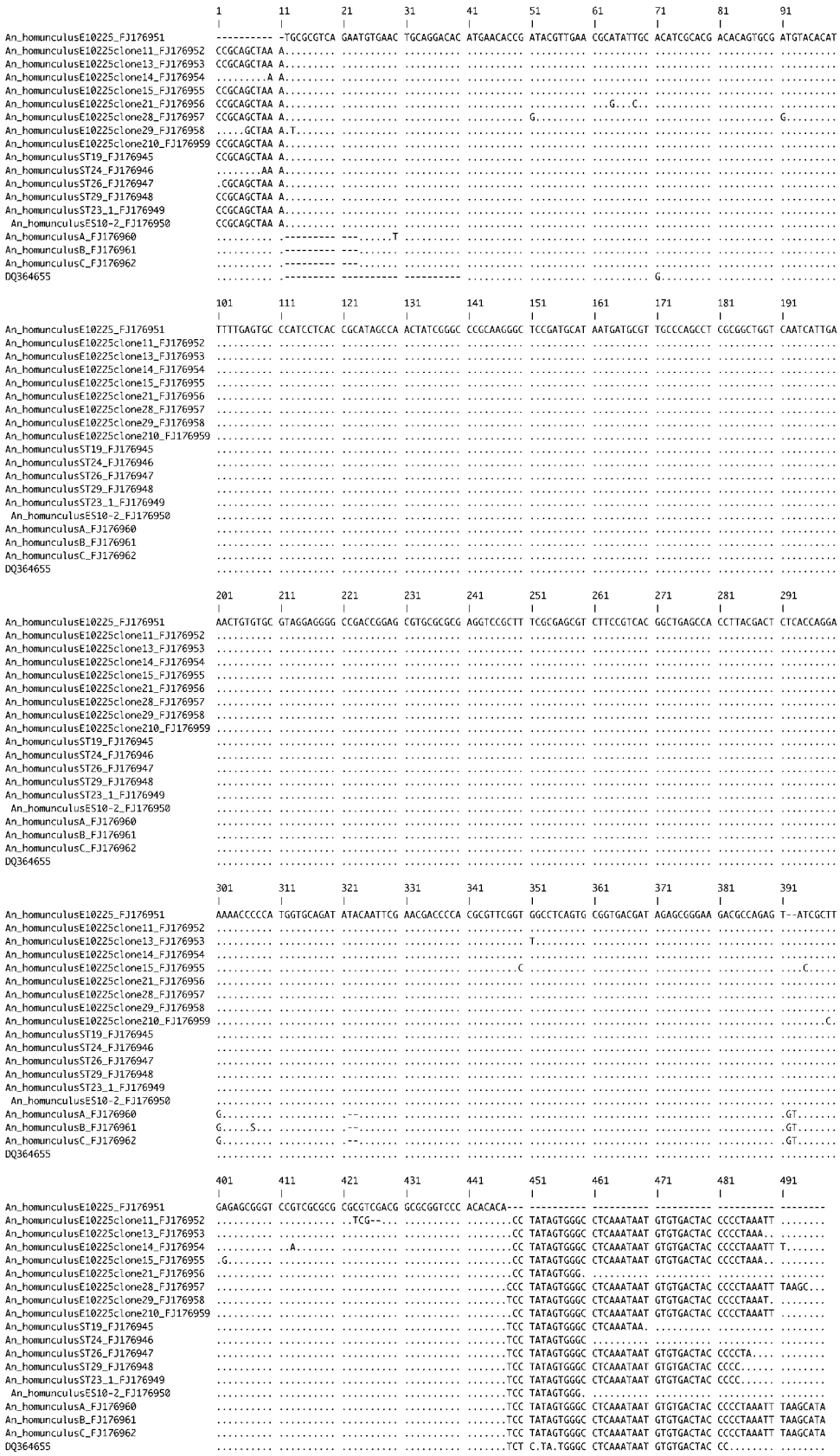


FIGURE 3. A 498 bp 5.8S, ITS2 and 28S sequence alignment of *Anopheles homunculus* from Brazil, Colombia and Trinidad. (-) indicates either an indel or missing data at the 5' and 3' ends.

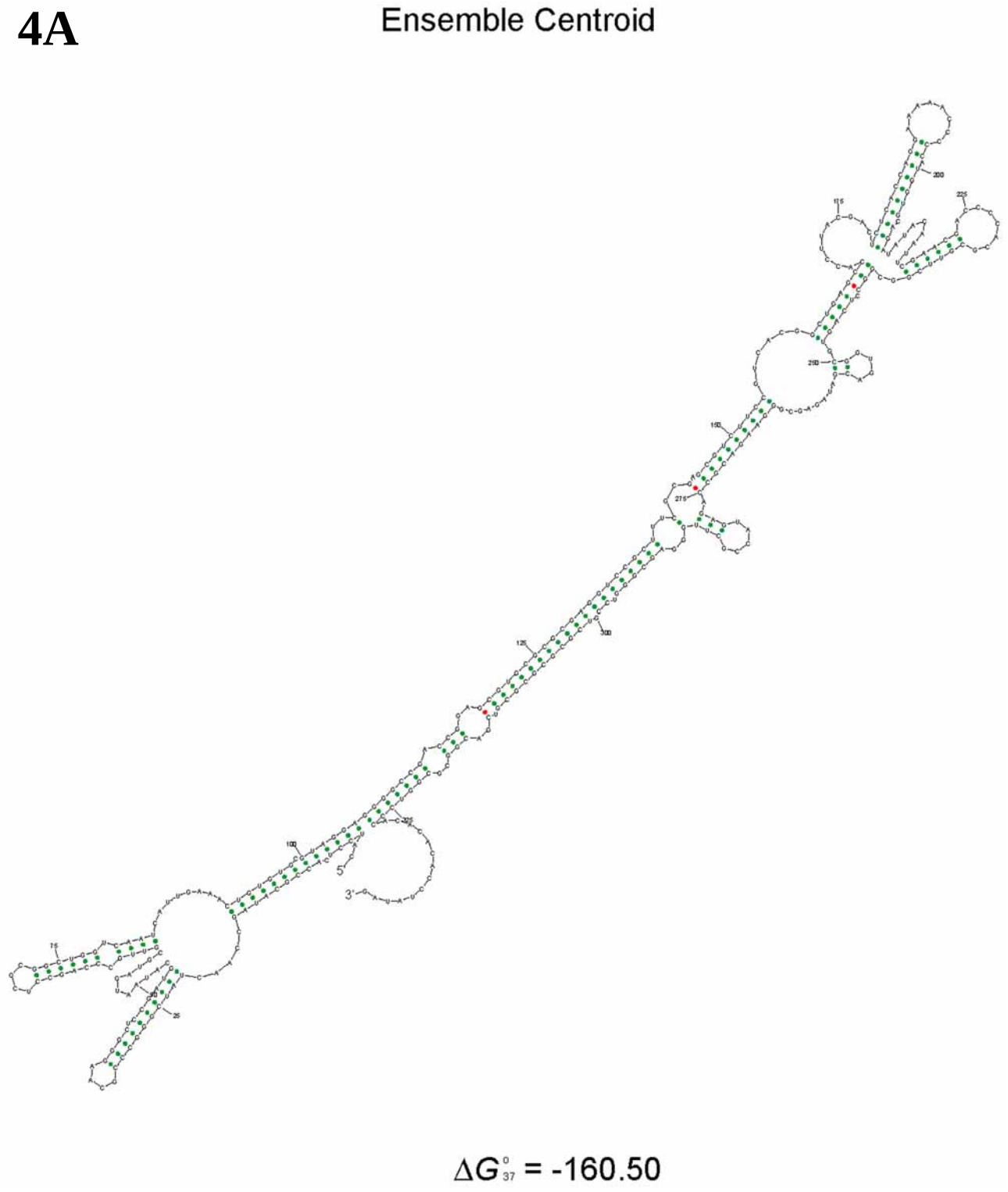
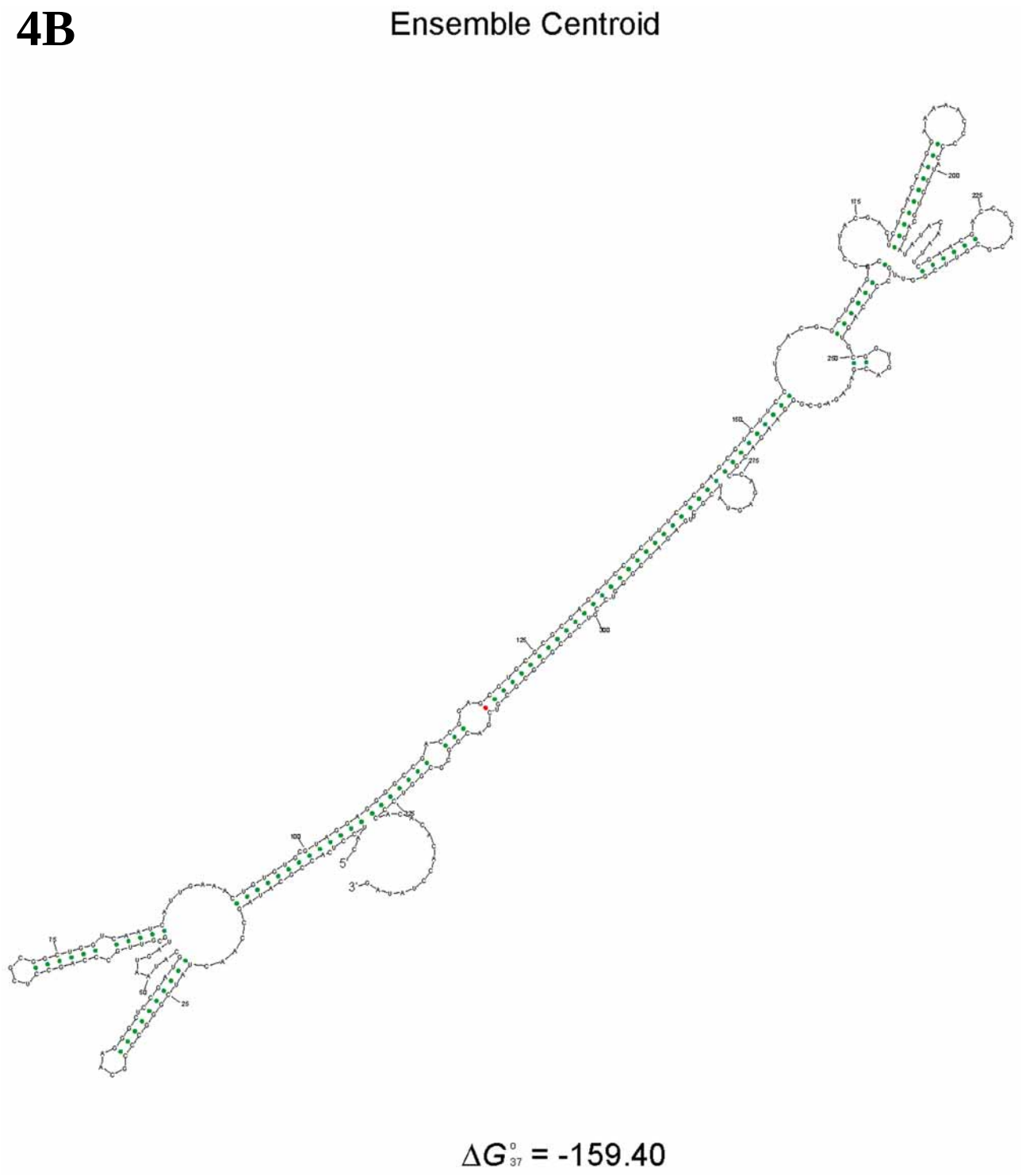


FIGURE 4. A, ITS2 ensemble centroid structure diagram for clone FJ176955 from a specimen of *Anopheles homunculus* from Colombia, generated in Sfold software (http://sfold.wadsworth.org/srna.pl), using Botzmann weighted ensemble features of RNA secondary structures. Folding temperature 37ºC, ionic conditions 1M NaCl. Δ°_{37} = -160.50; B, clone FJ176953, Δ°_{37} = -159.90.



Discussion

Difficulties in morphological separation of *An. homunculus* and *An. cruzii* based on adult female characteristics may be one of the causes for the apparent discontinuous distribution of *An. homunculus* in South America. According to the morphological keys of Zavortink (1973) and Forattini (2002), adult females of *An. homunculus* and *An. cruzii* are differentiated by the scales on the maxillary palpomeres and the color of the integument of the abdomen. In *An. homunculus* maxillary palpomere 3 possesses slightly to moderately outstanding scales, the scales are decumbent or moderately outstanding on palpomere 4, there are white scale-

patches at the apices of palpomeres 3-5, palpomeres 4 or 5 or palpomere 4, the patch is largest on 4 when present on more than one segment, and the abdominal segments are dark mauve when specimens are mounted in Canada balsam. In *An. cruzii* the maxillary scales are entirely or mostly decumbent on palpomeres 3 and 4,

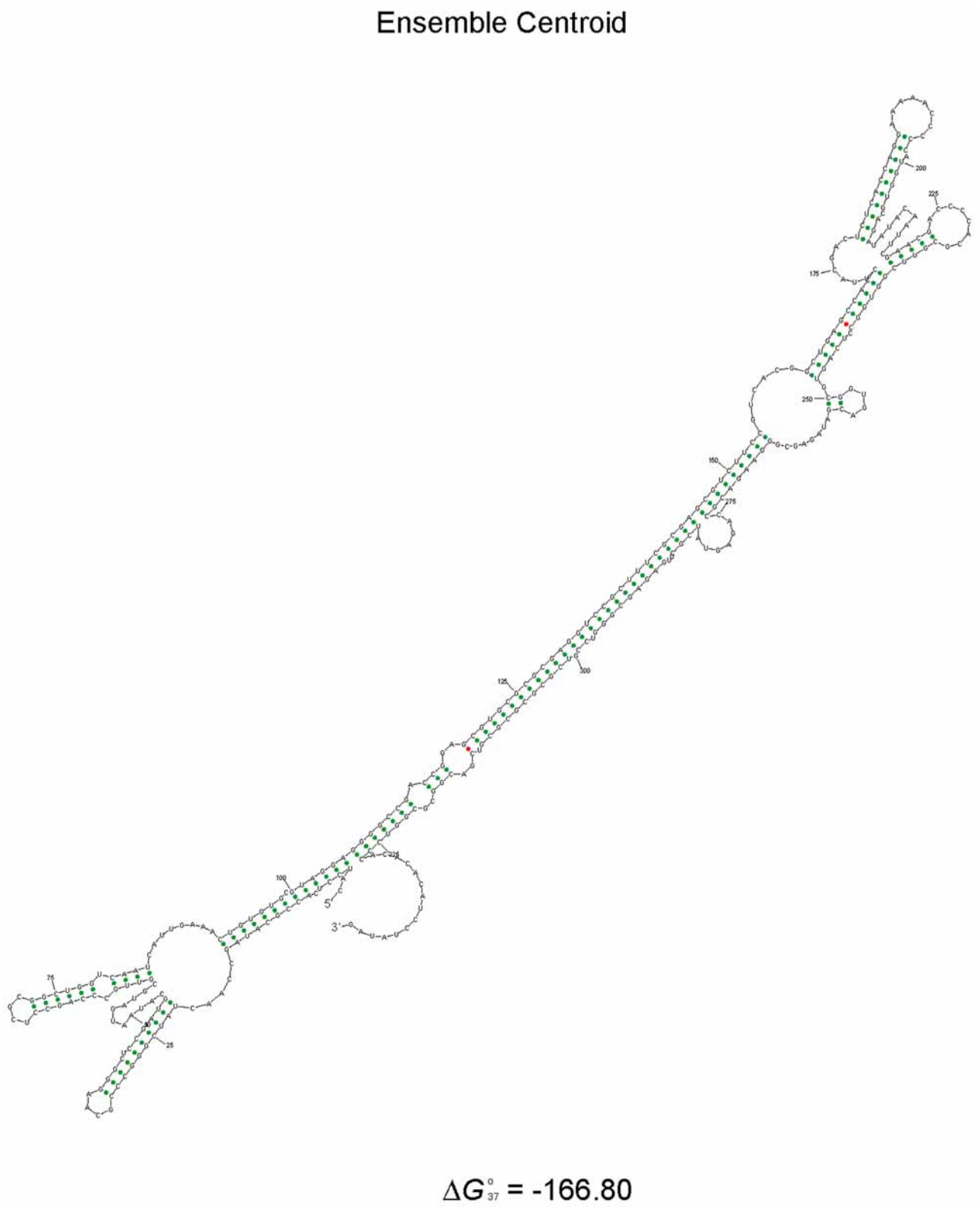


FIGURE 5. ITS2 ensemble centroid structure diagram for specimen of *Anopheles homunculus* ST19 (FJ176945) from Cananéia, eastern Mata Atlântica, Brazil, generated in Sfold software (http://sfold.wadsworth.org/srna.pl) using Botzmann weighted ensemble features of RNA secondary structures. Folding temperature 37ºC, ionic conditions 1M NaCl. Δ°_{37} = -167.80.

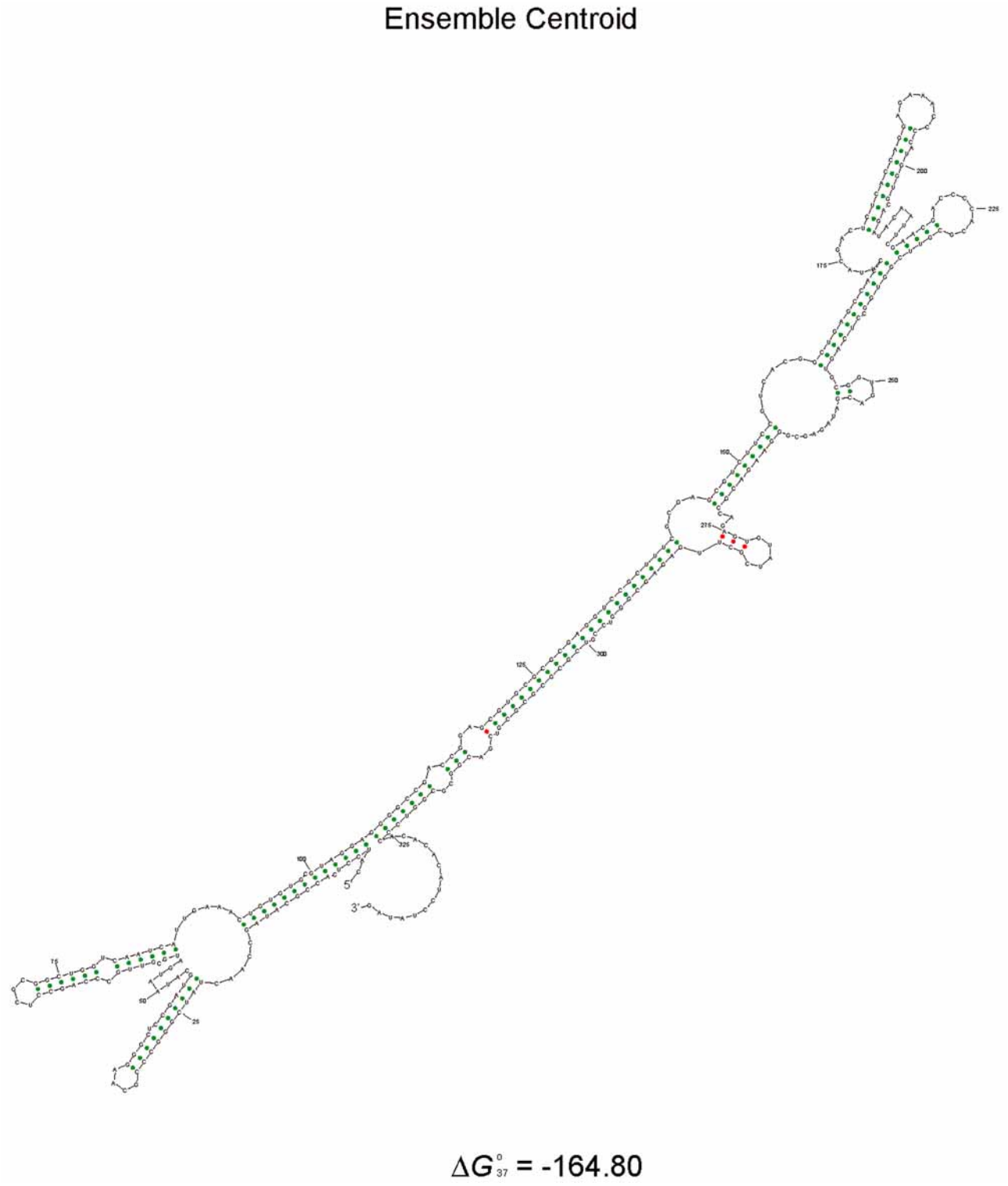


FIGURE 6. ITS2 ensemble centroid structure diagram for a specimen of *Anopheles homunculus* from Trinidad, generated in Sfold software (http://sfold.wadsworth.org/srna.pl) using Botzmann weighted ensemble features of RNA secondary structures. Folding temperature 37ºC, ionic conditions 1M NaCl. Δ°_{37} = -164.50.

or slightly outstanding at the base of 3, there is a white patch at the apices of palpomeres 3–5, that on 3 is subequal in size or larger than the patch on 4 (Zavortink 1973), and the abdominal segments are reddish (Forattini 1962, 2002). This shows that the correct separation of *An. homunculus* from *An. cruzii* is problematic, especially in poorly preserved specimens.

Zavortink (1973) employed specimens from Trinidad to redescribe and illustrate *An. homunculus*. He noted that female specimens from Colombia differ from those obtained in Trinidad by the reduced size or absence of the presector, sector and subcostal pale spots, the usual absence of a light fringe spot at the apex of vein CuA_2, the usual absence of extensive white scaling on midtarsal segment 3 and the usually more decumbent scales on maxillary palpomeres 3 and 4. In the fourth-instar larva, *An. homunculus* from Colombia differ from those from Trinidad by the finer and less conspicuously barbed seta 3-C and the longer seta 4a-X. Similar to specimens from Colombia, those from Brazil can be distinguished from those from Trinidad by the characteristics reported by Zavortink (1973) except for the pale fringe spot at the apex of vein CuA_2, which is always present in specimens from Mata Atlântica and also from Charambirá, Chocó, Colombia. Male genitalia characteristics do not distinguish *An. homunculus* from Colombia, Mata Atlântica or Trinidad. A single male genitalia of *An. homunculus* from Colombia, the lectotype, was available for study (Fig. 1 B, D). In this specimen, the ventral claspette possesses a short, blunt retrorse point anteriorly (Fig. 1B), whereas in specimens from Mata Atlântica (Fig. 1A, C) and Trinidad (Fig. 7A, B), the retrorse point is long and sharp. Komp (1937) described and distinguished *An. anoplus* from *An. homunculus* based on the absence of aedeagal leaflets in *An. anoplus* (Fig. 7C), whereas leaflets are present in the *An. homunculus* (Fig. 1E, F). Lane (1953) synonymized *An. anoplus* with *An. homunculus*. Comparing the sharp retrorse process of the ventral claspette, it is evident that in *An. anoplus* it is long and sharp (Fig. 7D), similar to that of *An. homunculus* from Mata Atlântica (Fig. 1A, C) and Trinidad (Fig. 7A, B), but distinct from that of the lectotype of *An. homunculus* (Fig. 1D). Evaluation of the importance of the retrorse process of the ventral claspette to separate *An. homunculus* from Colombia from *An. anoplus* and *An. homunculus* from Mata Atlântica and Trinidad needs further study, especially because the difference we observed may be an artifact caused by the dissection and mounting process.

Because immature stages were not available from Colombia, and because the ITS2 sequence suggest that individuals from Mata Atlântica are conspecific with *An. homunculus* from that country, we compared specimens from Mata Atlântica with those from Trinidad only. Fourth-instar larvae of *An. homunculus* from Mata Atlântica are distinguished from those from Trinidad by exhibiting the characteristic purple color of the thorax and abdominal segments, which is more evident on segments III, VI, VII and VIII, and the pattern of dark pigment in the dorsal surface of the abdomen (Figs. 2A, B; 8A, B). The dark pigment spots are in a somewhat hexagonal pattern on tergum I which is connected to the pattern of spots on tergum II by a narrow dark bridge. Tergum III has the dark pattern extending to lateral areas at the level of seta 6 with two large unpigmented areas bordering a central, unpigmented area, the dark pigment pattern reaching the posterior border of the segment with a median dark line extending anterior into an unpigmented medial portion. The dark pattern on sterna IV and V are distinct, restricted to the longitudinal anterior posterior area, dark pattern of tergum IV connected to that on tergum V by a narrow dark bridge. Terga VI, VII and VIII are entirely dark (Fig. 2B, C). In *An. homunculus* from Trinidad, the dark pattern on tergum I is somewhat rectangular and on tergum II it is restricted to a narrow dorsal longitudinal area. Tergum III is entirely dark, terga IV and V have sparse dark areas, not forming a pattern, and terga VI, VII and VIII are entirely dark (Fig. 8A, B). Additionally, *An. homunculus* from Brazil can be distinguished from that from Trinidad by possessing a darkly pigmented trumpet, which is yellowish at the base and apex (Fig. 2E), whereas in specimens from Trinidad, the trumpet is entirely lightly pigmented (Fig. 8C).

The ITS2 sequences generated from *An. homunculus* from Mata Atlântica, Brazil and Colombia show differences in comparison to those from Trinidad. As mentioned before, some cloned sequences from Colombia share 100% similarity with those from Mata Atlântica, however, the sequences from Trinidad are distinct. For the specimen from Colombia, the mean uncorrected P distance is greater among the clones (P varied from 0.00 to 0.01173) than among specimens from Colombia and Mata Atlântica (P varied from 0.00 to 0.00887), but lower in comparison with specimens from Colombia and Trinidad (P varied from 0.00298-0.01190). The presence of divergent cloned copies in the ITS sequences may represent PCR, sequencing or cloning artifacts, polymorphisms or the presence of parologous *loci* in non-homologous chromosomes (Mayol

and Rosselló 2001). Interestingly, the only specimen from Colombia, which was a museum-preserved individual, showed variation in the ITS2, i.e., both sparse singleton mutations and C or T insertion/deletion at 3' end (Fig. 3). Mayol and Rosselló (2001) discussed the presence of high divergent ITS copies within several species of the genus *Quercus* (Fogaceae). Among other hypotheses, they discussed the presence of large indels at the 3' end, which could be caused by PCR or sequencing artifacts. Similarly, the presence of a C/T insertion/deletion at the 3' end of *An. homunculus* from Colombia may be caused either by PCR or sequencing artifacts, or represent polymorphism. Furthermore, the DNA extracted from the museum specimen from Colombia was of poor quality that necessitated cloning PCR amplicons, which revealed intra-individual ITS2 polymorphism.

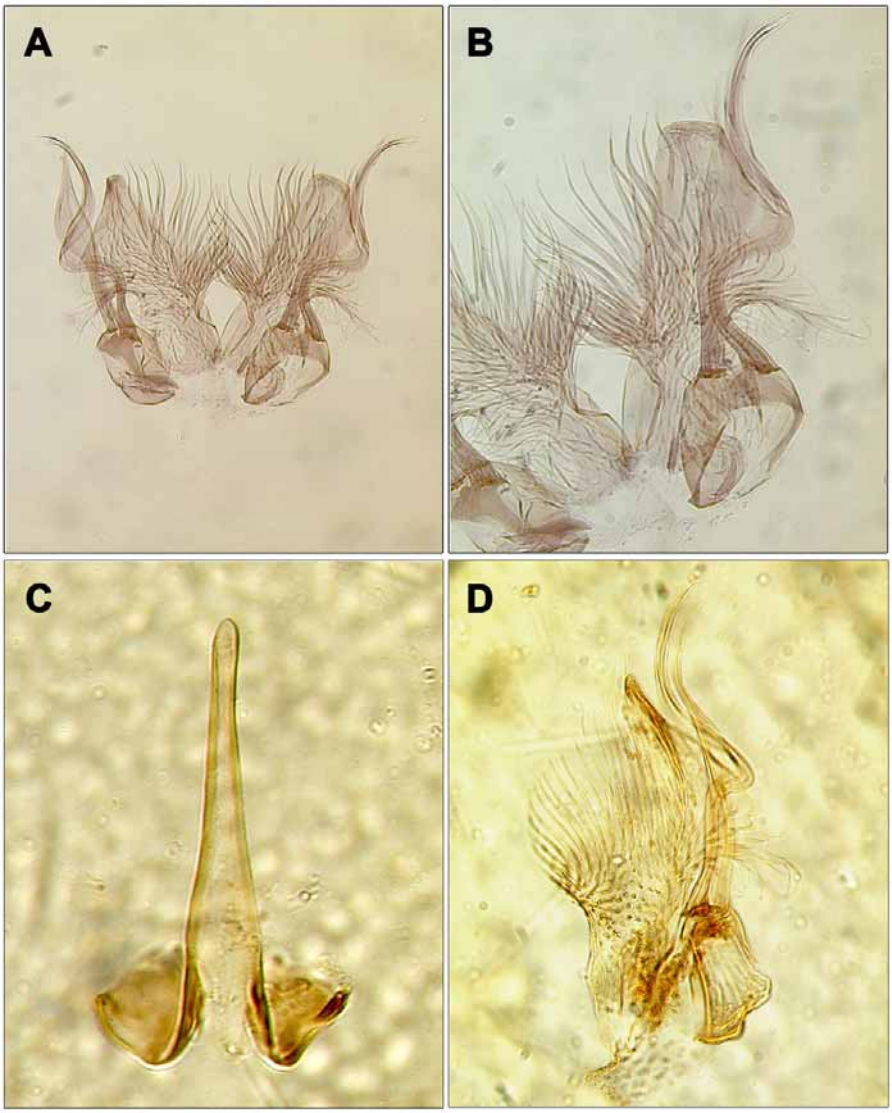


FIGURE 7. Male genitalia of *Anopheles homunculus* from Trinidad, and *Anopheles anoplus* Komp (holotype) from Restrepo, Colombia. A, detail of the ventral claspette showing mesal spicules; B, detail of the ventral claspette showing the anterior retrorse process and dorsal claspette; C, aedeagus, showing the absence of aedeagal leaflets; D, detail of the ventral claspette showing the anterior retrorse process and dorsal claspette. C and D represent the holotype of *Anopheles anoplus*.

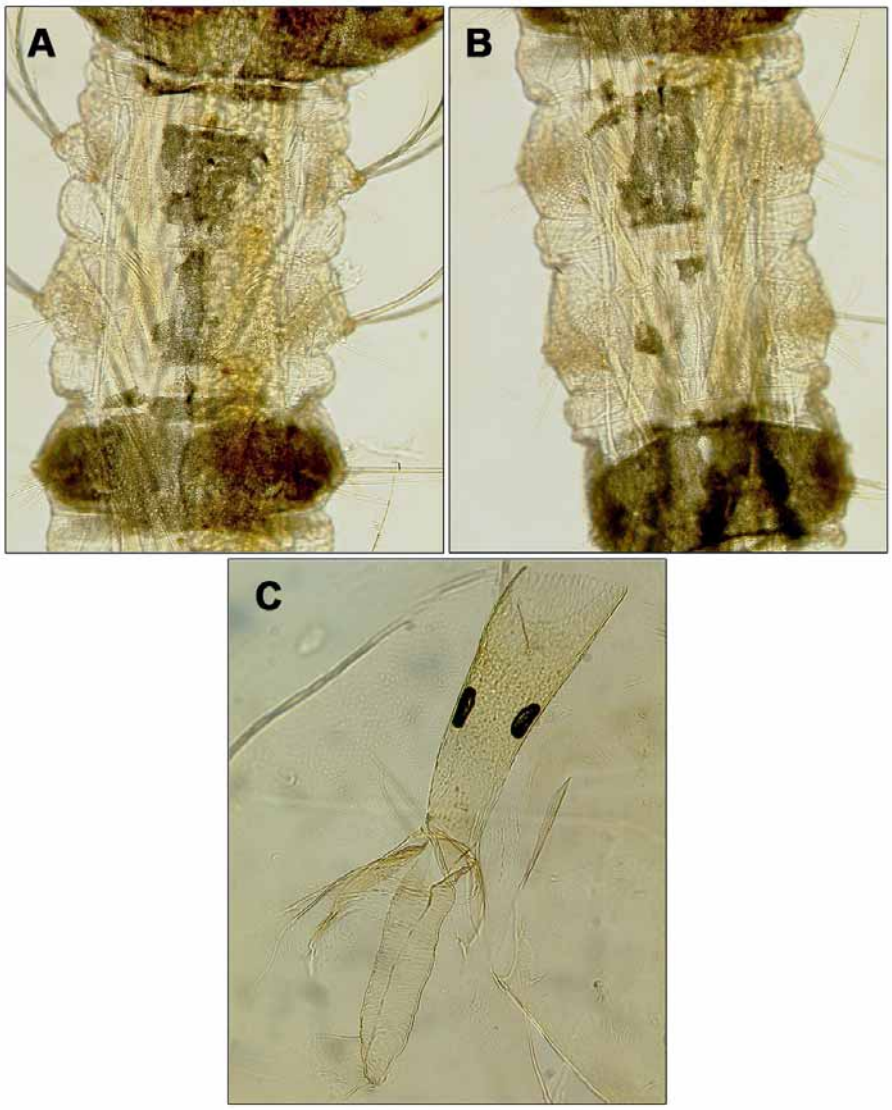


FIGURE 8. Fourth-instar larval and pupal structures of *Anopheles homunculus* from Trinidad. A, detail of the darkly pigmented pattern in the abdominal segments I-III; B, detail of the darkly pigmented pattern in abdominal segments IV-VI; C, detail of pupal trumpet, showing the absence of a dark ring.

Comparing the secondary structure of the ITS2 of specimens from Trinidad, Brazil and Colombia, we noticed that thermodynamic stability is generally maintained for the conserved motifs. A major difference among specimens from Trinidad and Brazil is in the helix III of the ensemble centroid structures, in which specimens from Trinidad showed a sidearm arising from a loop region at the 3' end (Fig. 6). This specific sidearm is absent in sequences from *An. homunculus* from Brazil (Fig. 5). Ensemble centroid secondary structure of cloned sequences from the specimen from Colombia was either similar to those from Brazil or from Trinidad. In this case, the secondary structure of one clone from Colombia (Fig. 4A) showed a sidearm arising from a loop region in the helix III that is also present in Trinidad (Fig. 6), with some minor differences. It is noteworthy that differences among the clones did not cause major changes in the integrity of ensemble secondary structure, and that distinct allele copies of the specimen from Colombia showed secondary structures that were either similar to those from Brazil or Trinidad. Both indels and base differences detected in the alignments of linear ITS2 sequences have been largely used as an indication of the presence of distinct species in the genus *Anopheles* (Ruiz et al. 2005, Motoki et al. 2007, Sallum et al. 2008). However, the value of these differences, especially when observed in loop regions of the secondary structure, needs further

investigation. Consequently, evaluating the extent of ITS2 sequence variation, as well as examination of the secondary structure in additional specimens of *An. homunculus* from Trinidad, Colombia and Brazil, should provide valuable information for understanding the differences observed in the present study. Furthermore, the recovery of ITS2 sequences with secondary structure that is similar to either Mata Atlântica or Trinidad among the clones from Colombia needs further investigation by sequencing ITS2 from a larger sample size. Finally, morphological and molecular evidence shows that *An. homunculus* occurs in Brazil and a second morphological form, here designated *An. homunculus* Trinidad Form, is present in Trinidad. In considering ITS2 sequence identity observed among specimens from Charambirá, Chocó, Colombia and southern Mata Atlântica, and differences between these populations and Trinidad specimens, it would be important to verify the ITS2 sequences of *An. homunculus* from the type locality of El Retiro, Intendencia de Meta, Colombia.

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