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NEW RECORDS OF *ANOPHELES HOMUNCULUS* IN CENTRAL AND SERRA DO MAR BIODIVERSITY CORRIDORS OF THE ATLANTIC FOREST, BRAZIL

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ABSTRACT. Two new records of *Anopheles homunculus* in the eastern part of the Atlantic Forest are reported. This species was found for the first time in Barra do Ouro district, Maquiné municipality, Rio Grande do Sul state, located in the southern limit of the Atlantic Forest. The 2nd new record was in the Serra Bonita Reserve, Camacan municipality, southeast Bahia state. These records extend the geographical distribution of *An. homunculus*, suggesting that the species may be widely distributed in coastal areas of the Atlantic Forest. It is hypothesized that the disjunct distribution of the species may be caused by inadequate sampling, and also difficulties in species identification based only on female external characteristics. Species identification was based on morphological characters of the male, larva, and pupa, and corroborated by DNA sequence analyses, employing data from both 2nd internal transcribed spacer of nuclear ribosomal DNA and of mitochondrial cytochrome *c* oxidase subunit I.

KEY WORDS Atlantic Forest, *Anopheles homunculus*, malaria, entomological surveillance, mosquitoes

INTRODUCTION

The genus *Anopheles* Meigen includes 478 mosquito species subdivided into 7 subgenera (Harbach 2011). In South America, there are 85 species belonging to the subgenera *Anopheles* Meigen, *Kerteszia* Theobald, *Nyssorhynchus* Blanchard, *Stethomyia* Theobald, and *Lophopodomyia* Antunes, with 67 species occurring in Brazil (WRBU 2011). The subgenus *Kerteszia* includes 12 Neotropical species, of which 7 were reported in Brazil (Guimarães 1997). Distribution of the subgenus extends south from state of Vera Cruz, Mexico, through Central and South America to the states of Misiones, Argentina, and Rio Grande do Sul, Brazil (Zavortink 1973). In Brazil, *Anopheles neivai* Howard, Dyar, and Knab was found in Parque Nacional do Jau, Amazonas state (Cerqueira 1961, Hutchings et al. 2005). *Anopheles boliviensis* (Theobald) had been registered in Brazil; however, with no recent record, its identification remains questionable (Collucci and Sallum 2003). Other *Kerteszia* species found in Brazil include: *An. bambusicolus* Komp (Santa Catarina and Paraná states), *An. laneanus* Corrêa and Cerqueira (São Paulo and Rio de Janeiro states), *An. cruzii* Dyar and Knab (Sergipe to Rio

Grande do Sul states), and *An. bellator* Dyar and Knab (in areas along the Atlantic coast) (Marrelli et al. 2007). The 7th species is *An. homunculus* Komp (São Paulo, Paraná, Santa Catarina, and Espírito Santo states). This species was described by Komp (1937) from specimens collected in Restrepo, Meta Department, Colombia. Females of *An. homunculus* are morphologically similar to those of *An. cruzii*, making them difficult to distinguish. Characteristics of the male genitalia and immature stages, however, allow an accurate identification (Sallum et al. 2009).

Species of the subgenus *Kerteszia*, acting as vectors of *Plasmodium*, use bromeliad phytotelmata as larval habitats (Downs and Pittendrigh 1946). In Brazil, human *Plasmodium* transmission is largely confined to the Amazon region, where 99.8% of the cases occur, involving *An. (Nyssorhynchus) darlingi* Root as the primary vector. In the extra-Amazonian region, especially in the states within the range of the Atlantic Forest, *An. cruzii* and *An. bellator* are vectors of autochthonous malaria, in a cycle that likely involves monkeys belonging to the genera *Cebus* and *Allouata* (Oliveira-Ferreira et al. 2010). In southern and southeastern Brazil, *An. homunculus* was found naturally infected with human *Plasmodium* in Santa Catarina, giving it the status of either secondary or potential vector (Smith 1952).

The known distribution of *An. homunculus* is largely restricted to east of the Andes, with records from Colombia, Venezuela, Trinidad, Guyana, Suriname, Peru, Bolivia, and Brazil (Zavortink 1973, Marrelli et al. 2007), with 1 report from west Andes (Chocó Department, Colombia) by Sallum et al. (2009). This species was first reported in Brazil in 1946 in the state of Santa Catarina, in Camboriu, Blumenau,

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Table 1. Details of *Anopheles (Kerteszia) homunculus* included in this study (in bold) as well as sequences downloaded from GenBank (*).¹

Specimen	Country	State/ department	Latitude (°)	Longitude (°)	COI GenBank accession no.	ITS2 GenBank accession no.
BA22-31	Brazil	Bahia	-15.39126	-039.56226	JQ291233	JQ291243
BA22-33	Brazil	Bahia	-15.39126	-039.56226	JQ291234	JQ291244
ES10-2	Brazil	Espírito Santo	-19.966701	-40.578736	JQ291235	FJ176950*
RS20-21	Brazil	Rio Grande do Sul	-29.590194	-50.261667	JQ291236	JQ291245
RS20-33	Brazil	Rio Grande do Sul	-29.590194	-50.261667	JQ291237	JQ291246
RS30-56	Brazil	Rio Grande do Sul	-29.581806	-50.265667	JQ291238	JQ291247
RS30-158	Brazil	Rio Grande do Sul	-29.581806	-50.265667	JQ291239	JQ291248
SP23-1	Brazil	São Paulo	-25.015479	-47.927357	JQ291240	FJ176949*
ST19	Brazil	São Paulo	-25.015479	-47.927357	JQ291241	FJ176945*
ST24	Brazil	São Paulo	-25.015479	-47.927357	—	FJ176946*
ST26	Brazil	São Paulo	-25.015479	-47.927357	JQ291242	FJ176947*
ST29	Brazil	São Paulo	-25.015479	-47.927357	—	FJ176948*
E-10225	Colombia	Charambirá	4.266667	-77.533333	—	FJ176951–FJ176959*
Isolate A	Trinidad	St. Andrew/St. David	10.473889	-61.169722	—	FJ176960*
Isolate B	Trinidad	St. Andrew/St. David	10.473889	-61.169722	—	FJ176961*
Isolate C	Trinidad	St. Andrew/St. David	10.473889	-61.169722	—	FJ176962*

¹ COI, cytochrome *c* oxidase subunit I of mitochondrial gene; ITS2, 2nd internal transcribed spacer of ribosomal DNA.

Brusque, and Palhoça Indaial, municipalities located on the coastal region between the mountains and the coastal Atlantic (Coutinho 1947). Specimens of *An. homunculus* were also found in Santa Catarina: Guaramirim, Nova Trento, and São José (Rachou and Ferraz 1951). The 1st record in São Paulo state was in 1963 (Ferreira 1964), and in Paraná state in 1966, based on material collected in São Vicente in Araruna municipality (Forattini et al. 1970). In 2006, the species was reported in Santa Teresa, Espírito Santo state (Sallum et al. 2008). This was the most northern record of *An. homunculus* in the Atlantic Forest.

The objectives of this study were to: 1) assess the presence of *An. homunculus* in Serra Bonita Forest Reserve, in Camacan, Bahia, and in Barra do Ouro district of Maquiné, Rio Grande do Sul, in Brazil; 2) characterize specimens from both localities based on sequences of the 2nd internal transcribed spacer (ITS2) of ribosomal DNA, and of cytochrome *c* oxidase subunit I (COI) of mitochondrial gene; and 3) compare individuals from Bahia and Rio Grande do Sul with *An. homunculus* from São Paulo and Espírito Santo states, Brazil, Colombia, and Trinidad, based on ITS2 sequences.

MATERIALS AND METHODS

Larvae and pupae of *An. homunculus* were taken from water in *Nidularium* Lemaire and *Vriesea* Lindl bromeliad tanks in 2 localities, Serra Bonita Forest Reserve, Camacan municipality, Bahia state, and in 2 locations in Barra do Ouro district, Maquiné municipality, Rio Grande do Sul state (Table 1). Serra Bonita is located within the Central (or Bahia) Biodiversity Corridor, and Barra do Ouro, Maquiné, is within the southern limit of Serra do Mar Corridor of the Atlantic

Forest (Galindo-Leal and Câmara 2003). In Rio Grande do Sul, the altitude of the collection sites ranged from 80 to 250 m, while in Bahia, sampling was done at 800 m above sea level.

The immatures collected were reared to the adult stage in the laboratory, resulting in 7 males and 14 females associated with either larval and pupal exuviae or pupal exuviae only. Species identification was based on morphological characters of the larva, pupa, and male genitalia. Molecular characterization was based on both ITS2 rDNA and COI sequences. The ITS2 sequences were generated for specimens from Bahia and Rio Grande do Sul, which were then compared with those downloaded from GenBank (including representative from GenBank DQ364655), based on sequences from individuals collected in the states of São Paulo and Espírito Santo, and also from Colombia and Trinidad (Table 1; data from DQ364655 were not available to be included in Table 1). In addition, COI mitochondrial gene sequences were obtained for specimens from Bahia, Rio Grande do Sul, São Paulo, and Espírito Santo states and used for COI characterization.

Two specimens (male and female) from Bahia state and 4 (males) from Rio Grande do Sul state were used for molecular characterization. Genomic DNA was extracted using QIAgen DNeasy® Blood and Tissue Kit, following the animal tissue DNA extraction protocol provided by the company (QIAgen Ltd., Crawley, United Kingdom). Amplification of the ITS2 region was carried out using the primers 5.8SF (5'-ATC ACT CGG CTC GTG GAT CG-3') and 28SR (5'-ATG CTT AAA TTT AGG GGG TAG TC-3'), following the protocol adopted by Nagaki et al. (2010). 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 ~650 base pairs of COI, following the protocol adopted by Nagaki et al. (2010).

Gene sequence alignments of the ITS2 and mitochondrial COI were constructed in Clustal X version 2.0 (Larkin et al. 2007). Sequence similarities of the ITS2 sequences generated in this study with those of *An. homunculus* previously available in GenBank were assessed using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>). Basic statistics for COI and ITS2, including evolutionary divergence among sequences (nucleotide composition, pairwise distances, and overall mean distances) were assessed using the maximum composite likelihood model in Mega version 5 (Tamura et al. 2011). To estimate pairwise distances and overall mean distances, all positions containing gaps and missing data were excluded from the analyses, resulting in a total of 400 positions in the final ITS2 data set and 605 positions in the final COI data set.

Template DNA from this study is retained at -70°C in the Faculdade de Saúde Pública (FSP-USP), São Paulo, Brazil, for future reference (DNA reference numbers are: ST19, ST26, SP23-1, ES10-2, BA22-31, BA22-33, RS20-21, RS20-33, RS30-56, RS30-158). Larval and pupal exuviae and male genitalia slides of specimens used for DNA extraction were deposited in the FSP-USP collection maintained at the Faculdade de Saúde Pública, Universidade de São Paulo, Brazil.

RESULTS

The COI and ITS2 sequences generated for this study are available in GenBank under the following accessions: COI (JQ291233–JQ291242) and ITS2 (JQ291243–JQ291248). The ITS2 region of the nuclear rDNA cistron was sequenced for 4 individuals of *An. homunculus* from Maquiné, Rio Grande do Sul state, and 2 individuals from Camacan, Bahia state, Brazil, and thus compared with ITS2 sequences of *An. homunculus* downloaded from GenBank (Table 1). The alignment of 25 ITS2 sequences generated a total of 482 positions (including the 5.8S and the 28S flanking regions). A FASTA search using the database, nucleotide collection—Optimize for: Highly Similar (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed that the ITS2 sequences of *An. homunculus* from Bahia and Rio Grande do Sul shared 100% similarity with FJ176945–FJ176949 from Cananéia, São Paulo, FJ176950 from Santa Teresa, Espírito Santo, and FJ176958 from Colombia. Excluding gaps and missing data, pairwise distances among 25 nucleotide sequences, containing a total of 400 positions, varied from 0.000 to 0.0127 (between FJ176956 and FJ176957), with an overall mean distance of 0.0028. Average

nucleotide composition consisted of 18.7% T, 23.0% A, 29.7% C, and 28.6% G. Twelve sequences from Rio Grande do Sul, Espírito Santo, and São Paulo states (excluding DQ364655) revealed a single haplotype comprised of 18.7% T, 23.2% A, 29.6% C, and 28.5% G.

Ten unique COI haplotypes were detected in the 669-bp (without the primer regions) fragment. Average nucleotide composition comprised 41% T, 27.5% A, 15.1% C, and 16.4% G. Pairwise distance among 10 nucleotide sequences containing a total of 605 positions (excluding missing data) varied from 0.002 (between RS30-56 and RS20-21, and ST26 and RS30-56) to 0.015 (between ES10-2 and BA22-31). The overall mean distance of COI sequence was 0.008.

DISCUSSION

Results of morphological comparisons of characters of male genitalia, 4th-stage larva, and pupa confirmed the presence of *An. homunculus* in Central and in Serra do Mar biodiversity corridors (Galindo-Leal and Câmara 2003) of the Atlantic Forest hot spot. Additionally, statistical analyses of ITS2 data set corroborated morphological identification and also confirmed that *An. homunculus* from the Atlantic Forest was likely to be conspecific with that from Charambirá, Litoral del San Juan, Colombia. Moreover, ITS2 data confirmed previous finding of Sallum et al. (2009), which showed that *An. homunculus* from Trinidad might belong to an unnamed species. Furthermore, results of analyses of COI sequences, generated only for specimens from 3 locations in the Atlantic Forest, indicated that individuals from Serra do Mar Corridor were probably conspecific with those from Central Corridor. The levels of COI divergence among individuals from Serra do Mar and Central corridors were lower than the 3% threshold advocated by Herbert et al. (2003) for defining species limits.

Females of *An. homunculus* are morphologically similar to those of *An. cruzii*, which may explain why there are few records of the species in South America, including in Brazil (Sallum et al. 2009). The new distribution records of *An. homunculus* corroborate the Sallum et al. (2009) hypothesis of a larger geographic distribution for the species than that proposed by Marrelli et al. (2007). The presence of *An. homunculus* in Serra Bonita Reserve in Central Biodiversity Corridor, and in Barra do Ouro, Maquiné, in the southern limit of the Serra do Mar Biodiversity Corridor, extends both north and south the range of occurrence of the species in the eastern Atlantic Forest. However, the disjunct distribution (Marrelli et al. 2007) of *An. homunculus* needs to be confirmed with more intensive sampling along the distribution range of the species where bromeliads are

distributed. Culicidae fauna from bromeliads have been poorly sampled in most areas of the Atlantic Forest, with the majority of the studies (Müller and Marcondes 2006, 2007; Marques and Forattini 2008; Mocellin et al. 2009; Marques et al. 2011) in areas of Serra do Mar Corridor. Considering the 3 major corridors (Serra do Mar, Central, and Northeast corridors) of the Atlantic Forest carrying 671 bromeliad species with a total of 83.6% of all species from the Atlantic Forest domain (Martinelli et al. 2008), it would be important to increase sampling in distinct highly diverse corridors and also in different bromeliad species. It is noteworthy that Marques et al. (2011) found *An. homunculus* to be a common mosquito species in plants of the genera *Vriesia* and *Nidularium* in the southeastern Atlantic Forest of São Paulo state. Unfortunately, bromeliad species were not identified, and thus, plant-mosquito associations were not assessed. However, plants of *Vriesia* and *Nidularium* and other genera should be sampled to confirm the disjunct distribution and to establish a more realistic geographic distribution of *An. homunculus* in the Neotropical region.

Anopheles homunculus should be included in the list of species that are monitored by entomological surveillance programs for malaria control in areas of the Atlantic Forest domain. Moreover, the vector status of this species should be assessed by testing *Plasmodium* infection in field-collected individuals. Additionally, infection studies should include *An. bellator* and *An. cruzii* in order to define vulnerable areas for human malaria (Brasil 2009) and assess the role of each species in the dynamics of malaria transmission in the Atlantic Forest. Laporta et al. (2011) showed that different areas of Vale do Ribeira in the southeastern Atlantic Forest possess distinct risk for malaria transmission. The risk depends on various ecological determinants but also on the *Anopheles* species that occur in the region. Consequently, the design of mosquito control should incorporate data from the species that may represent risk and also be adequate for the region, causing a minimum damage for the natural ecosystem.

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