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MORPHOLOGY, SYSTEMATICS, EVOLUTION

Insight into Anopheles (Nyssorhynchus) (Diptera: Culicidae) Species from Brazil

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ABSTRACT Anopheles (Nyssorhynchus) benarrochi s.l., Anopheles (Nyssorhynchus) oswaldoi s.l., and Anopheles (Nyssorhynchus) konderi s.l. collected in Acrelândia, state of Acre, Brazil, were identified based on morphological characters of the male genitalia, fourth-instar larvae, and pupae. Morphological variation was observed in the male genitalia of these species in comparison with specimens from other localities in Brazil. DNA sequence from the nuclear ribosomal second internal transcribed spacer of individuals identified as An. benarrochi s.l. by using male genitalia characteristics showed that the various morphological forms are conspecific but are distinct from An. benarrochi B from Colombia. Anopheles konderi s.l. and An. oswaldoi s.l. both misidentified as An. oswaldoi s.s. (Peryassú) throughout Brazil, may actually comprise at least two undescribed species. Diagnostic morphological characteristics of the male genitalia are provided to distinguish Anopheles benarrochi s.l., Anopheles oswaldoi s.l., and Anopheles konderi s.l. from morphologically similar species. Incrimination of An. oswaldoi s.s. in malaria transmission in Brazil needs further investigation because other undescribed species from Acre may have been confounded with this taxon.

KEY WORDS Anopheles, Nyssorhynchus, male genitalia, identification, internal transcribed spacer 2

In the second half of the 20th century, the control of human malaria transmission was the object of a worldwide campaign to eliminate the vector mosquitoes by using DDT. As a result, malaria transmission was eliminated from developed countries and from developed areas of developing countries. In areas where living conditions were poor and the climate and ecological conditions were ideal for the proliferation of vector mosquitoes, malaria transmission continued to be intense. This is the case in areas in the Brazilian Amazon (Tauil 2006). Because of a continuous migration of susceptible humans from nonendemic areas to the Amazon region and among distinct localities in the Amazon, the annual incidence of malaria has increased. In 1999, the Brazilian federal government and the Amazonian states government adopted an aggressive strategy for malaria control, targeting 32% of the municipalities in the Amazon that accounted for 93.6% of cases (de Castro et al. 2007). Consequently, active monthly searches and immediate treatment of both symptomatic and asymptomatic infections were carried out in communities throughout the Brazilian Amazon (Macauley 2005). As observed by Macauley, the strategy adopted would be effective for the control of malaria if carried out with the following criteria: 1) effective treatment; 2) monitoring of human migration for detection of infected individuals; and, 3) collaboration of the local community. The control measures provided a quick and dramatic reduction in the annual incidence of malaria. However, the program, which was almost exclusively based on the search and treatment of infected individuals, was not continued and the incidence of malaria again began to increase in almost all locations. Unfortunately, other control measures were not adopted, e.g., vector control, human migration control, and improvement of living conditions. Tauil (2006) took into consideration that the rational use of insecticides and larvicides for mosquito control, reduction, elimination, and cleaning of larval habitats, as well as participation of the local community, would be required for the success of any vector control strategy.

The Brazilian National Program for Malaria Prevention and Control is divided into nine components (Coura et al. 2006). One of these is the selective control of vectors. Thus, any measures adopted should be dependent on which mosquito species are important in malaria transmission in a given locality. Therefore, entomological studies are necessary for both knowing the species composition in a certain location and for understanding ecological and biological aspects of those species that have vector potential. Singer and de Castro (2006) pointed out that malaria transmission in the Amazon over the past 100+ years has been favored by ecosystem transformations caused by human migration, expanding of frontier
lands for agricultural, settlement, cattle ranching, and natural resource extraction resulting in extensive deforestation. Furthermore, Singer and de Castro (2006) considered that the phenomenon defined as frontier malaria operates in three spatial scales in conjunction with a temporal scale, and they discussed some ecological characteristics of An. darlingi Root, the primary malaria vector species in the Amazon. A fundamental point is that human behavior can promote the creation of ecological conditions for the proliferation of vector species and human exposure to mosquito bites. Moreover, Singer and de Castro (2006) observed that the bimodal biting pattern of An. darlingi, at dawn and dusk, may be related to the phase of the deforestation/human incursion process. Vittor et al. (2006) examined the impact caused by deforestation in the population of An. darlingi in an area situated in the Peruvian Amazon. They found that the An. darlingi biting rate was 278 times higher in deforested areas than in predominantly forested areas. It is noteworthy that in highly deforested area in Brazil (Dourado municipality, in São Paulo state), An. darlingi showed bimodal dusk and dawn biting peaks (Forattini 1987), whereas in the Peruvian Amazon, Vittor et al. (2006) observed a unimodal biting peak, from 2100 to 2300 hours. An. darlingi was rare in areas of primary forest, suggesting that the immatures were not ovipositing in the forest habitats. According to Singer and de Castro (2006), malaria transmission rapidly increases in areas of colonization projects where substantial government-sponsored and informal human migration is accompanied by deforestation. After 6 or 7 yr of land occupation, when a more organized urbanization process is established, malaria transmission becomes more stable and malaria rates decrease. Along with the process of land occupation and urbanization, ecological conditions may become inhospitable for An. darlingi; however, they may be adequate for other Anopheles Meigen vector species. Vasconcelos et al. (2006) suggested that a reduction in relative humidity in deforested Amazon areas, the presence of larval habitats and the increase in temperature may be favorable for the proliferation of those Anopheles vector species that have capacity to adapt to those environmental conditions. Boëte and Paul (2006) pointed out the possibility that changes in anopheline species or genotype composition within a population of mosquito vectors, as a result of, for example, vector-control measures, could have a considerable impact on transmission of sympatric parasite species. For example, Póvoa et al. (2003), in a study conducted in the city of Belém, Pará, observed a change in the species composition of Anopheles, with the reappearance of An. darlingi. This was followed by an increase in malaria cases caused by Plasmodium vivax Grassi & Feletti and a decrease of those caused by Plasmodium falciparum. V247, and Plasmodium malariae Feletti & Grassi. The infection rates of An. oswaldoi s.l. for all Plasmodium tested were higher than those observed for An. deaneorum Rosa-Freitas. No samples of An. darlingi and An. triannulatus (Neiva & Pinto) were found positive. Because of these results, An. oswaldoi s.l. was considered to be the main malaria vector in those localities. Later, Branquinho et al. (1996) dissected the midguts and the salivary glands to determine oocyst and sporozoite rates in Anopheles species collected in Senador Guiomard and Plácido de Castro, state of Acre. As a result, only one An. oswaldoi s.l. collected from a Shannon trap was found to be positive for both sporozoites and oocysts. In the same areas, Marrelli et al. (1998) recorded An. oswaldoi s.l. and humans infected with P. vivax-like/P. simiovale Stephens parasites corroborating the importance of An. oswaldoi s.l. as a vector in recently settled areas in the state of Acre. Subsequently, Marrelli et al. (1999a) compared the susceptibility of An. oswaldoi s.l. and An. konderi s.l. to infection with P. vivax by using mosquito populations obtained in Sena Madureira, in the state of Acre, and in São Miguel, in the state of Rondônia, respectively. They demonstrated that An. oswaldoi s.l. was involved in the transmission of P. vivax, whereas An. konderi s.l. developed P. vivax oocysts in the midgut, sporozoites failed to develop in the salivary glands. Incrimination of An. oswaldoi s.l. as a vector of P. vivax in Putumayo, southern Colombia, was shown by Quiñones et al. (2006). The epidemiological importance of An. oswaldoi s.l. may be over or underestimated in the state of Acre as a consequence of species misidentification. Natal et al. (1992) collected 4,588 culicid specimens in the settlement Pedro Peixoto, located in the Purus River Basin. Pedro Peixoto settlement is situated in the municipalities of Rio Branco, Senador Guiomard and Plácido de Castro, Acre. Among 53 species or species groups identified, An. oswaldoi s.l. (as An. oswaldoi) was the most numerous species collected in the region and made up 86% of the anophelines collected (3,156 specimens). However, two males identified as An. oswaldoi s.l. by Natal et al. (1992) that are deposited in Faculdade de Saúde Pública (FSP/USP) collection, it was possible to identify them as An. rangeli Gabaldón, Cova Garcia, and Lopez. Consequently, some individuals of An. rangeli were misidentified as An. oswaldoi s.l.

The Oswaldoi Group (Faran 1980) of the subgenus Nyssorhynchus Blanchard comprises 18 species, five of which have been incriminated as vectors of human Plasmodium and at least four, An. aquasalis Curry, An. benarrochi Gabaldón, Cova Garcia & Lopez, An. oswaldoi, and An. nunezovari Gabaldón are complexes of sibling species. Marrelli et al. (1999b) using nucleotide sequences of the ITS2 of the rDNA showed that An. oswaldoi s.l. consists of a complex of at least four species, one of which may correspond to An. konderi Galvão & Damasceno, recently elevated from synonymy with An. oswaldoi by Flores-Mendoza et al. (2004). There are various internal transcribed spacer (ITS2) sequences of An. oswaldoi s.l. available in GenBank, i.e., Brazil: Acre AF055068, Amapá AF056318,
Amazonas AF056317, Rondônia AF055069 (Marrelli et al. 1999b); Colombia: Putumayo, AY679149-55 (Ruiz et al. 2005); Peru: Yurimaguas, AF055071 (Marrelli et al. 1999b); and from unnamed localities: U92344, U92352-3 (Danoff-Burg & Conn direct submission). A FASTA search revealed that the ITS2 sequences of An. oswaldoi s.s. (n/H1100512) generated from individuals collected in the type locality in Espírito Santo state and also in southern São Paulo state (Espírito Santo EF457228-37; São Paulo EF457238-9) were unique with regard to those deposited in GenBank (Motoki et al. 2007). Consequently, the identification of the specimens mentioned above remains unresolved.

Characteristics of the male genitalia have proven effective for separating species of Culicidae, including those of the genus Anopheles. Anopheles oswaldoi and An. konderi can be easily separated based on morphology of the aedeagus (Flores-Mendoza et al. 2004). Similarly, Bergo et al. (2007) hypothesized that An. goeldii Rozeboom & Gabaldon may be a valid species and that there is an unnamed morphological form that can be misidentified as An. nuneztovari when using adult female characters. In this study, with the primary objective of collecting An. oswaldoi s.l. to verify species identification, we obtained reared-associated specimens of all Anopheles species encountered in Acrelândia municipality, state of Acre, Brazil.

Materials and Methods

Mosquito Collection. Immature collections were conducted in Linha 14 (9°41′03.5″ S, 67°08′05.5″ W), Ramal do Granada, Acrelândia municipality, state of Acre, Brazil (Fig. 1), in the area of the Acre Project (Silva-Nunes et al. 2006). Acrelândia borders the
counties of Senador Guimarães and Plácido de Castro in Acre, and the states of Amazonas and Rondônia and the country of Bolivia. The county is located between the Abunã and Iquiri Rivers in the Acre River Valley. Ramal do Granada is part of the Pedro Peixoto Organized Agricultural Settlement, which was implemented by the National Institute for Colonization and Agrarian Reform in the mid-1970s. Details about the area of the Acre Project are in Silva-Nunes et al. (2006).

In this study, rDNA ITS2 nucleotide sequences were derived from seven individually reared adult male specimens, with associated fourth-instar larval and pupal exuviae, and adult male genitalia kept as vouchers. Species identification was based on the male genitalia characteristics.

DNA Extraction, ITS2 Amplification, Cloning, and Sequencing. 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.

One microliter of the first elution was used as DNA template in the polymerase chain reactions (PCR). Amplification of the ITS2 region was carried out using 5.8SF, 5′-ATC ACT CGG CTC GTG CAT GC-3′, and 26SR, 5′-ATG CTT AAA TTT AGG GGG TAG TC-3′ primers. PCR was carried out in a 25-μl reaction mix containing 1 μl of DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 2.5 μl of dimethyl sulfoxide, 5 pmol of each primer, 200 μM each dNTPs, and 2.5 U of Taq polymerase (New England Biolabs, Ipswich, MA). PCR amplification protocol consisted of a 2-min denaturation at 94°C, 34 cycles at 94°C, 57°C and 72°C for 30 s each, followed by a 10 min extension at 72°C. PCR products were electrophoresed in 1% TAE agarose gels stained with ethidium bromide. ITS2 PCR amplicons obtained from two individuals of An. oswaldoi s.l. were purified directly from bands excised from agarose gel using the QIAquik gel extraction kit and cloned into pGem-T Easy Vector (Promega, Madison, WI). Four positive clones were sequenced.

Sequencing reactions were carried out in both directions using the above PCR primers and the Big Dye Terminator kit, version 1.3 (PE Applied Biosystems, Warrington, England). Sequences were analyzed either on an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA) or on a 377-ABI sequencer (Applied Biosystems) (An. oswaldoi s.l. clones).

Sequence Analysis. Sequences were edited using Sequence Navigator, version 1.0.1 (PE Applied Biosystems), aligned in CLUSTAL X (Thompson et al. 1997) and optimized manually in MacClade, version 4.3 (Maddison and 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/). Intraspecific sequence differentiation was assessed using mean uncorrected P distance in PAUP (Swofford 2003).

Vouchers. Template DNA from this study is retained dry at −70°C in the FSP-USP for future reference (DNA reference nos. are AC18-16, An. konderi s.l.; AC18-102; AC18-107, An. oswaldoi s.l.; AC18-104; AC15-109; AC18-115, An. benarrochi s.l.). Immatures and male genitalia slides of the same specimens used for DNA extraction are deposited in the FSP-USP entomological collection as vouchers, accession numbers from E-12928 until E-12934.

Results and Discussion

In a larval habitat in Linha 14 (9°41′03.5″ S, 67°08′05.3″ W), Ramal do Granada, Acrelândia, Acre, we obtained 32 specimens that were preliminarily identified as either An. oswaldoi s.l. (n = 12) or An. benarrochi s.l. (n = 20) by adult female and fourth-instar larvae. When examining characters of the dissected male genitalia, we identified distinct morphological forms: two similar to An. benarrochi s.l. (Fig. 2A–D), one similar to An. oswaldoi s.l. (Fig. 3A–D), and one similar to An. konderi s.l. (Fig. 4A–D).

Molecular Characterization. The ITS2 was sequenced for seven individuals, four from An. benarrochi s.l. (GenBank accession nos. EU636797–EU636800), two (eight clones) from An. oswaldoi s.l. (EU636902–EU636909), and one from An. konderi s.l. (EU636901). The ITS2 base composition was 20.6% T, 27.7% A, 27.0% C, and 24.2% G for An. oswaldoi s.l.; and 20% T, 27.7% A, 27.0% C, and 24.2% G for An. oswaldoi s.l. and 20% T, 27% A, 28% C, and 25% G for An. konderi s.l.

ITS2 sequences of An. benarrochi s.l. are available in GenBank from Brazil (Rondônia state deposited in GenBank (AF462384 and AF462383; Marrelli et al. 2005), unlisted localities (U92325; Danoff-Burg & Conn direct submission), and Colombia (Puerto Asis AY684976–84; La Manuela, Liberia). A FASTA search revealed that the ITS2 sequences of An. benarrochi s.l. from Acrelândia, Acre (n = 4, reported here) are identical, but distinct from those already in GenBank, sharing highest sequence similarity at 97% with An. benarrochi B from Colombia, AY684984–AY684976. Along an ITS2 456 bp alignment, 12 bases varied, including one 1-bp (base 316), one 4-bp (bases 334, 335, 336 and 337), and two 2-bp (bases 370, 371 and 381, 382) indels, and three singleton polymorphic sites (bases 282, 347, and 348) (Fig. 5). Mean uncorrected P distance among sequences of An. benarrochi B (AY684984) and An. benarrochi s.l. ranged from 0.00665 to 0.00671 (AC18-104). In comparison with two An. benarrochi s.l. ITS2 sequences from Rondônia state deposited in GenBank (AF462383 and AF462384) by Marrelli et al. (2005), we observed that these sequences are 97% similar to each other. In addition, ITS2 sequence identity among AF462383–4 (Rondônia) and AY684976–84 (Colombia) is only 86%. Similarly, sequence identity among AF462383, AF462384, and those generated from An. benarrochi s.l. collected in Acrelândia, Acre, is 93 and 91%, respectively.
We cloned and sequenced four copies of the entire ITS2 per individual for two An. oswaldoi s.l. mosquitoes from Acrelândia, Acre. The fragment amplified included 60 bp of 5.8S and 42 bp of the 28S. The cloned sequences were aligned with reference sequences of An. oswaldoi s.s. from Espírito Santo and São Paulo (EF457237 and EF457239; Motoki et al. 2007) and other ITS2 sequences of An. oswaldoi s.l. available in GenBank from Brazil (Acre AF055068, Amazonas AF056317, Rondônia AF055069; Marrelli et al. 1999b). The length of the amplified sequences varied from 488 to 490 bp, with length variation due to a 2-bp indel (Fig. 6). A few base substitutions were observed; however, they were not consistent among the clones. Two cloned sequences of four of each individual were 100% identical; however, there were no identical sequences among the clones generated from samples AC18-107 and AC18-102. We estimated mean sequence divergence of ITS2 variants within individuals by using uncorrected P distance in PAUP (Swofford 2003). Sequence divergence among the clones of AC18-107 ranged from 0.00000 to 0.01435, whereas those of AC18-102 ranged from 0.00000 to 0.00615. By comparing cloned sequences of AC18-107 to AC18-102, we observed that uncorrected P distance ranged from 0.00205 to 0.01435. No diagnostic loci were detected for individuals AC18-107 or AC18-102. All of the distinctive clones sequenced for An. oswaldoi s.l. were deposited in GenBank (accession nos. EU636802–EU636809).

ITS2 sequences of An. oswaldoi s.l. are available in GenBank from Brazil (Acre AF055068, Amazonas AF056318, Amazonas AF056317, Rondônia AF055069; Marrelli et al. 1999b), Colombia (Putumayo, U92352-3; Danoff-Burg & Conn direct submission), and of An. oswaldoi s.s. (Espírito Santo EF457228-37, São Paulo EF457238-9; Motoki et al. 2007). A FASTA search revealed that all cloned sequences of An. oswaldoi s.l. from Acrelândia are distinct from those already in GenBank. In comparing the two identical cloned sequences from AC18-102 with Fig. 2. Male genitalia of An. benarrochi s.l. from Acrelândia, state of Acre and from Dourado, state of São Paulo, Brazil. Form 1: ventral claspette (ventral aspect) (A) and detail of ventral claspette (ventral aspect) (from Acrelândia, AC15-109) (B). Form 2: ventral claspette (ventral aspect) (C), detail of ventral claspette (ventral aspect) (from Acrelândia, AC18-115) (D), dissected ventral claspette (ventral aspect) (E), and detail of dissected ventral claspette (ventral aspect) (from Dourado) (F).
AF056317, AF055068, and AF055069 (excluding 6 and 7 bp from 5’/H11032 and 3’/H11032 end, respectively, because both ends represent nucleotide contamination by insertion of a restriction site that are included in the sequence), we observed that sequence similarity ranged from 98 to 99%, the uncorrected P distance between AF056317 and AC18-102_F3 is 0.00. The variation consisted of a 1-bp indel that may be due to a PCR/cloning error in AF056317. All cloned sequences of AC18-107 are distinct from those already in GenBank with sequence similarity ranging from 97 to 99% compared with AF056317, AF055068, and AF055069. In comparing all distinctive cloned sequences of An. oswaldoi s.l. from Acrelândia, Acre, we observed that the query coverage is 75% only. Along an ITS2 356-bp alignment (U92342, U92348-9) with that from Acrelândia, Acre, we observed that the query coverage is 75% only. Along an ITS2 356-bp alignment (U92342), sequence similarity is 96%, 11 bases varied, including one 2-bp, one 1-bp, two 2-bp indels (bases 222–223, 257, 261–262, and 272–273, respectively), and four singleton polymorphic sites (bases 300, 313, 336, and 344) (Fig. 6). Furthermore, An. konderi s.l. from Acrelândia, Acre, shares 98% similarity with An. oswaldoi s.s. from São Paulo (EF457238-39; Motoki et al. 2007) and Espírito Santo (EF457228-25; Motoki et al. 2007). Along an ITS2 464-bp alignment, nine bases varied, including one 1-bp, one 2-bp indels (bases 361 and 428–429, respectively), and six singleton polymorphic sites (bases 272, 294, 324, 401, 437, and 445, respectively).

**Morphological Characterization.** Anopheles benarrochi was described by Gabaldón et al. (1941) from specimens collected in La Ceiba, Trujillo, Venezuela. Faran (1980) included An. benarrochi in the Strодеi

![Fig. 3. Male genitalia of Anopheles oswaldoi s.l. from Acrelândia and Plácido de Castro, state of Acre and An. oswaldoi s.s. from Linhares, state of Espírito Santo, Brazil. (A) Ventral clasper (ventral aspect, AC18-112). (B) Ventral clasper (ventral aspect, AC18-107) (from Acrelândia). (C) Ventral clasper (ventral aspect). (D) Aedeagus, showing detail of the aedeagal apex (ventral aspect) (from Plácido de Castro). (E) Ventral clasper (ventral aspect). (F) Aedeagus, showing detail of the aedeagal apex (ventral aspect) [from Linhares, ES8(20)-14].](image)
Complex of the Albimanus Section, and reported the distribution of the species to the Orinoco basin and eastern versant of the Andes, including the llanos plateau of Colombia, parts of upper Amazonas in Brazil and Loreto in Peru. Later, Sallum et al. (1997) reported the species in the state of São Paulo, Brazil.

Preliminarily, two morphological forms of An. benarrochi were found in Acrelândia, designated An. benarrochi Form 1 (Fig. 2A and B) and An. benarrochi Form 2 (Fig. 2C and D). Anopheles benarrochi Form 1 (AC15-109 and AC18-117) is morphologically similar to An. benarrochi s.s. of the original description (Galbaldón et al. 1941) of Faran (1980) and that reported in Dourado, state of São Paulo, by Sallum et al. (1997) (Fig. 2E and F). Anopheles benarrochi Form 2 (AC18-115 and AC18-104) can be distinguished from Form 1 by the apex of the ventral claspette of the male genitalia, which is moderately expanded laterally, with apicodistal margin sharply angled and pointed in Form 1, whereas it is somewhat rounded and truncate in Form 2. In addition, the apical margin of ventral claspette has a deep median sulus and the preapical plate of ventral claspette is small, circular, homogeneus and heavily sclerotized in Form 1, whereas the apical margin is somewhat straight and the preapical plate is small, circular, and not homogeneously sclerotized in Form 2.

In spite of morphological differences on the male genitalia, the ITS2 sequences generated from specimens identified either as An. benarrochi Form 1 or An. benarrochi Form 2 are 100% identical, suggesting that these forms are conspecific. This observation raises a question about male genitalia polymorphism and sections of those anatomical structures commonly used to distinguish species of the subgenus Nyssorhynchus. Hribar (1994) demonstrated polymorphism in the male genitalia of An. nuneztovari cytopotypes A, B, and C, and Faran (1980) reported variation in the ventral claspette of the male genitalia of An. benarrochi s.l. Morphological variation in the ventral claspette of An. benarrochi Form 1 and Form 2 from Acrelândia, Acre, could be caused by distortion of the structures during the dissection and mounting on a microscope slide. Thus, special care should be taken when dissecting and mounting male genitalia. Conversely, we can also question into the utility of ITS2 sequences for separating morphologically similar species. ITS2 has been shown to be a useful tool for separating closely related species of Anopheles (Marrelli et al. 2006). However, some differences (herein observed) were found within microsatellite regions showing that not all nucleotide variation may be important for distinguishing among different taxa.

Quinones et al. (2006) by comparing ITS2 sequence with male genitalia characteristics of vouchers specimens found that An. benarrochi s.l. from Peru comprises two morphological forms; one form matches the original description of the species and a second form corresponds to An. benarrochi of Ruiz et al. (2005). Interestingly, the ITS2 sequences of An. benarrochi Form 1 and Form 2 from Acrelândia share the highest similarity (97%) with An. benarrochi B from Colombia. This difference suggests that the individuals of An. benarrochi s.l. from Acrelândia are distinct from An. benarrochi B and may belong to either An. benarrochi s.s. or a distinct species. Supplemental studies are

Fig. 4. Photographs depicting the morphological differences between the male genitalia of Anopheles konderi s.l. from Acrelândia, state of Acre and An. konderi from Macapá, state of Amapá, Brazil. (A) Aedeagus (ventral aspect, AC18-16). (B) Aedeagus (ventral aspect, AC-103) (from Acrelândia). (C) Aedeagus (ventral aspect). (D) Aedeagus, showing detail of the aedeagal apex (ventral aspect) (from Macapá).
necessary to establish a morphological and molecular definition for An. benarrochi s.s. and thus solve the taxonomic status of the populations found in Colombia, Peru, and Brazil. Additionally, it will be worthwhile to examine populations of An. benarrochi s.l. across Brazil for a better evaluation of which forms may occur in the country and to ascertain their taxonomic identity.

In the same larval habitat of An. benarrochi s.l., we collected immatures of An. oswaldoi s.l. While examining characters of the male genitalia, it became evident that the specimens could be separated into two morphological forms designated An. oswaldoi s.l. (Fig. 3A and B), and An. konderi s.l. (Fig. 4A and B). Anoph eles oswaldoi s.l. (Fig. 3A and B) is similar to An. oswaldoi s.s. (Fig. 3E and F). A similar morphological form was found in Plácido de Castro, Acre (Fig. 3C and D). Based on male genitalia characteristics, An. oswaldoi s.l. can be separated from An. oswaldoi s.s. by the apex of the aedeagus (Fig. 3D and F, respectively). Morphological and ITS2 sequence differences suggest that specimens of An. oswaldoi s.l. from Acrelândia, Acre, belong to a new species. It is noteworthy that the ITS2 sequences from two specimens from Acrelândia are 99% similar to those generated by Marrelli et al. (2005) from individuals identified as An. oswaldoi s.l. collected in Rondônia (AF055069) and Acre (Plácido de Castro) (AF055068). Finally, the identity of those specimens from Acre and Rondônia used by Marrelli et al. (2005) remains a problem to be solved by further studies and field collections in the same localities to obtain associated specimens, adult males and females and immatures.

Flores-Mendoza et al. (2004) elevated An. konderi from the synonymy of An. oswaldoi and designated the neotype. Unfortunately there is no ITS2 identity available in the GenBank of An. konderi from the type locality of Coari, Amazonas state, generated from the same set of specimens used by Flores-Mendoza et al. (2004). Morphological comparisons of samples of An. konderi collected in Coari, state of Amazonas, Macapá, state of Amapá, and Acrelândia, state of Acre, strongly suggest that specimens from Amapá (Fig. 4C) and Coari (Motoki et al. 2007) are identical, whereas those from Acre can be distinguished by the apex of the aedeagus (Fig. 4A and B). In An. konderi s.s. the subapical, collarlike, subtriangular sclerotization of the aedeagus is somewhat U-shaped, whereas it is V-shaped in An. oswaldoi s.s. from Acre. Additionally, in dorsal aspect, the longitudinal dorsomedial cleft extends apical to the subtriangular sclerotization and the apex is short in An. konderi s.s. (Fig. 4C and D), whereas in An. konderi s.l. the longitudinal dorsomedial cleft does not extend beyond the level of the subtriangular sclerotization and the apex is somewhat longer (Fig. 4A and B).

Scarpassa and Conn (2006) used COI sequences to examine the intra- and interpopulational variability in An. oswaldoi s.l. Results of the maximum parsimony analysis identified four major clusters of the COI haplotypes that may correspond to distinct species. According to their study, male genitalia characteristics...
Fig. 6. A 490-bp ITS2 sequence alignment of *An. oswaldoi* s.l. (AC18-107, AC18-102). *An. oswaldoi* s.l. in GenBank from states of Amazonas, Acre, and Rondônia, Brazil (AF056317, AF055068-9) and *An. oswaldoi* s.s. from state of Espírito Santo and São Paulo, Brazil (EF457237, EF457239). (-) indicates indel events. The letter number after the sample code indicates a clone.
suggest that one group may correspond to An. oswaldoi s.s., a second group to An. konderi, whereas the two other clusters could constitute different lineages or species within the An. oswaldoi complex. Considering the results obtained by Scarpassa and Conn (2006), we observed that the specimens from the states of Rondônia and Acre clustered in Groups I and II. Group I comprises specimens from Rondônia (São Miguel) and Acre (Sena Madureira), whereas Group II is formed by individuals from São Miguel. Parsimony bootstrap support for both Groups I and II is strong (100%). This result suggests that there are at least two distinct species that may be confounded with An. oswaldoi s.s. in those areas. In considering that the sequences in GenBank (AF055068 and AF055069) generated from specimens of An. oswaldoi s.l. (Marrelli et al. 2005) are dissimilar and were collected in the same localities in Rondonia and Acre of those specimens used by Scarpassa and Conn (2006), we hypothesize that they could belong to the same taxon.

Fig. 7. A 469-bp ITS2 sequence alignment of An. konderi s.l. (AC18-16) and An. konderi s.l. in GenBank from unspeciﬁed localities from Brazil (U92342, U92348-9). (-) indicates either indel events or missing data at 5' and 3' ends.
If this scenario is correct, the specimens from Acrelândia would be representative of two other distinct taxa that are largely misidentified as An. oswaldoi s.l. It is also plausible to speculate that one of the groups identified by Scarpassa and Conn (2006) may be co-specific with An. oswaldoi s.l. from Acrelândia, whereas the other may be genetically similar to one individual used by Marrelli et al. (2005) from either Acre or Rondônia. Moreover, our results strongly suggest that at least one of the specimens from Acrelândia (AC18-102) belong to the same species of the specimen represented by AF056317 (from Amazonas state) because the uncorrected P distance is zero and the sequence difference is a single indel. It is evident that further investigation will be necessary to establish a correspondence among distinct morphological forms and the phylogenetic groups found by Scarpassa and Conn (2006), the identity of the specimens of Marrelli et al. (2005) and the correspondence with those from Acrelândia. This is especially relevant because An. oswaldoi s.l. was considered to be involved in malaria transmission in the state of Acre. Finally, variation in male genitalia characteristics within An. oswaldoi s.l. and An. benarochi s.l. seems to have a correspondence with genetic variation that is worthy of further investigation.

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