

**Instituto Nacional de Pesquisas da Amazônia – INPA Programa
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**Taxonomia Integrativa, Filogeografia e análise de infectividade por
Plasmodium spp. no complexo Oswaldoi-Konderi (Diptera: Culicidae:
Anophelinae)**

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**Manaus, Amazonas
2019**

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Plasmodium spp. no complexo Oswaldoi-Konderi (Diptera: Culicidae:
Anophelinae)**

Orientadora: Dra. Vera Margarete Scarpassa

Tese apresentada ao Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva do Instituto Nacional de Pesquisas da Amazônia como parte dos requisitos para obtenção do título de **Doutor em Genética, Conservação e Biologia Evolutiva**.

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Sinopse:

Estudou-se a morfologia de todos os estágios de desenvolvimento e a Filogeografia do complexo Oswaldoi-Konderi (Diptera: Culicidae: Anophelinae) da Amazônia brasileira, para a elucidação taxonômica e filogeográfica do complexo.

Palavras-chave:

Anopheles konderi s.s., *Anopheles tadei* n. sp., entomologia médica, espécies crípticas.

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meu maior amigo,

meu amado pai.

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“Biologia é a ciência. A evolução é o conceito que faz a biologia única”

— **Jared Diamond**

“Não é o mais forte que sobrevive, nem o mais inteligente, mas o que melhor se adapta às mudanças”

— **Charles Darwin**

Resumo

Anopheles oswaldoi s.l. (Peryassú) foi durante muito tempo considerado uma única espécie, implicada como vetor secundário de malária em algumas localidades da região amazônica. Estudo subsequente descreveu *Anopheles konderi* Galvão & Damasceno, baseando-se unicamente em diferenças no ápice do edeago das genitálias masculinas. Análises recentes com abordagens moleculares revelaram que as duas espécies são na realidade um complexo de espécies crípticas, com ampla distribuição geográfica e extensas faixas de ocorrência simpática entre elas. Atualmente, o último estudo sugere que cinco espécies (*Anopheles oswaldoi* s.s., *Anopheles konderi* s.s., *Anopheles oswaldoi* A, *Anopheles oswaldoi* B e *Anopheles* sp. nr. *konderi*) existam neste complexo nomeado de Oswaldoi-Konderi. Entretanto, estas espécies nunca haviam sido analisadas morfológicamente e apesar de algumas serem implicadas como vetores secundários, a identidade molecular destas espécies encontradas com *Plasmodium* também não havia sido determinada. No presente estudo foram amostradas 29 localidades na Amazônia brasileira e 71 desovas foram obtidas e estudadas morfológicamente, com o objetivo de explorar a diversidade de espécies no complexo e revelar caracteres morfológicos confiáveis para a sua determinação. Para isto, os caracteres morfológicos de ovo, exúvia da larva, exúvia da pupa, adulto fêmea, adulto macho e genitália masculina foram examinados à procura de variações que possibilitassem sua diagnose. Além disso, dois marcadores genéticos, como o gene Citocromo C Oxidase Subunidade I (*COI*) e um marcador de herança nuclear, o Segundo Espaçador Interno Transcrito (ITS2) foram empregados. Foram inferidos os números de unidades taxonômicas operacionais (OTU's), recuperada as relações filogenéticas entre as espécies com base na Inferência Bayesiana e estimativas do tempo de diversificação do complexo Oswaldoi-Konderi na bacia amazônica. Os três modelos de delimitação de espécies indicaram elevado número de unidades taxonômicas operacionais, entre 14 e 18 OTU's. A morfologia suportou cinco espécies, com base em caracteres da genitália masculina. O neótipo e os parátipos de *Anopheles konderi* s.s. foram reexaminados e os dados indicaram uma mistura de duas formas morfológicas que, posteriormente, foram confirmadas com três marcadores moleculares *COI*, ITS2 e *CAD*. *Anopheles tadei* n. sp. foi descrita baseada nas diferenças morfológicas presentes na genitália masculina, larva de 4º estádio e em dados moleculares. A análise filogeográfica de *Anopheles oswaldoi* A de três localidades indicou forte estruturação genética: Lábrea, Mina Palito e Viruá, situadas nos estados do Amazonas, Pará e Roraima, respectivamente. Futuros estudos serão necessários para elucidar a identidade taxonômica de *An. oswaldoi* A e *An. oswaldoi* B, tendo em vista, que *An. aquacaelestis* Curry, não foi examinado e este encontra-se em sinonímia com *An. oswaldoi* s.s.

Abstract

Anopheles oswaldoi s.l. (Peryassú) was long considered a single species, which would be implicated as a secondary vector of malaria in some localities of the Amazon region. Subsequent study described *An. konderi* Galvão & Damasceno, counting only on differences in the apex of the aedeagus of male genitália. Recent analyzes using molecular approaches revealed that these two species are in fact a complex of cryptic species, with wide geographical distribution and extensive sympatric ranges between them. Currently believed that five species (*An. oswaldoi* s.s., *An. konderi* s.s., *An. oswaldoi* A, *An. oswaldoi* B and *An. sp. nr. konderi*) exist in this named complex of Oswaldoi-Konderi. However, these species had never been analyzed morphologically, and despite being implicated as secondary vectors, the molecular identity of these species found with *Plasmodium* had not yet been determined. In the present study, 29 localities of the Brazilian Amazon were sampled, 71 spawns were obtained and studied morphologically, to explore the diversity of species in the complex and reveal reliable morphological characters for its determination. For this, mitochondrial inheritance genetic markers were used, such as the Cytochrome *c* Oxidase Subunit I (*COI*) gene and a nuclear inheritance marker, Second Transcribed Internal Spacer (ITS2). The numbers of operating taxonomic units (OTU's) were inferred, the phylogenetic relationships recovered using the Bayesian Inference and the time of diversification of the Oswaldoi-Konderi complex in the Amazon basin was determined. Our results indicated a high number of operating taxonomic units (14 - 18 OTU's). The morphology supported five species based on male genitalia characters. The Neotype and Paratypes of *An. konderi* s.s. which were reexamined, indicated a mixture of two morphological forms, confirmed with the molecular data, including another nuclear marker, the *CAD* gene, which is linked to the X chromosome but presents low heterozygosity. *Anopheles tadei* n. sp. was described based on morphological differences between male genitals and two characters in the larva near *An. konderi*. The analysis of a larger region of the *COI* gene (1.211bp) in *An. oswaldoi* A indicated structuring for three populations, Para, Amazonas, and Roraima, respectively. Further studies will be needed to elucidate the taxonomic identity of *An. oswaldoi* A and *An. oswaldoi* B, as *An. aquacaelestis* Curry has not been examined and is synonymous with *An. oswaldoi* s.s.

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1. INTRODUÇÃO GERAL

Os mosquitos da família Culicidae (Ordem Diptera) são exímios vetores de doenças humanas. Dentre as principais doenças destacam-se as Arboviroses como: Dengue, Zika e Chikungunya, e doenças provocadas por protozoários, como o *Plasmodium* spp., que causam a Malária (Service, 1993; Forattini, 2002). A Malária afeta centenas de milhares de pessoas ao redor do mundo, especialmente em países situados nas regiões tropicais e subtropicais, dentre eles o Brasil. Segundo a WHO (2019), no mundo, foram notificados 228 milhões casos e 405 mil óbitos em 2018. No Brasil, a região amazônica representa cerca de ~ 99% dos casos de malária no país (WHO, 2019). Esta região tropical também concentra a maior riqueza de espécies de mosquitos transmissores de doenças, somente o gênero *Anopheles* (vetor da malária) são reconhecidas 69 espécies no Brasil (WRBU, 2019), sendo algumas, de difícil identificação taxonômica.

A delimitação taxonômica é uma tarefa desafiadora quando existem espécies com elevada semelhança morfológica, conhecidas como espécies crípticas (DeSalle *et al.*, 2005), sendo a maior parte destas espécies filogeneticamente relacionadas (Bourke *et al.*, 2010). Desta forma, a taxonomia integrativa consiste na utilização de múltiplas ferramentas, tais como: caracteres morfológicos, marcadores genéticos moleculares, observações ecológicas e comportamentais, para a delimitação de espécies (Seberg *et al.*, 2003; Dayrat, 2005; DeSalle *et al.*, 2005). O emprego de uma abordagem integrativa torna a definição das unidades taxonômicas mais robustas permitindo estimar com maior precisão a diversidade de espécies, e assim incriminar os vetores de doenças (Seberg *et al.*, 2003; Dayrat, 2005; DeSalle *et al.*, 2005; Rubinoff, 2006; Bourke *et al.*, 2013). Entretanto, os complexos de espécies crípticas são muito comuns no gênero *Anopheles Meigen*. Ao menos a metade dos importantes vetores de malária no mundo estão inseridos em complexos, como por exemplo: *An. gambiae*, *An. dirus*, *An. maculatus*, *An. culicifacies*, entre outros (WHO, 2007).

Segundo Mayr (1976), pares ou grupos de espécies que são morfologicamente indistinguíveis ou distingüíveis com dificuldade são chamadas espécies crípticas. O termo é usado inteiramente com base em um conceito morfológico, não significando necessariamente que incluem espécies que são filogeneticamente irmãs (Dobzhansky, 1972). Estudos moleculares durante as últimas três décadas têm revelado que espécies crípticas ou irmãs são muito mais comuns do que anteriormente se pensava (Pfenninger

e Schwenk, 2007). Apesar dessa constatação, a maioria das descrições de espécies ainda é realizada principalmente na análise de caracteres morfológicos. No entanto, sutis diferenças no comportamento de corte durante o acasalamento, sinais químicos (feromônios), fisiologia, preferências de habitat, frequência dos batimentos das asas etc., também podem ser utilizadas na distinção dessas espécies (Bickford *et al.* 2007). Além disso, é importante compreender que o processo de especiação nem sempre é acompanhado com alterações morfológicas, condições ambientais extremas podem impor seleção estabilizadora na morfologia e impedir quaisquer alterações morfológicas durante a especiação, e, portanto, podem resultar em cladogênese morfologicamente estática (especiação sem alteração morfológica) (Krzywinski e Besansky, 2003).

Quando as espécies crípticas são evolutivamente relacionadas (origem recente) à recuperação das relações filogenéticas torna-se complicada por duas razões principais: 1) no geral são espécies de divergência muito recente, e, portanto, podem compartilhar polimorfismo ancestral; 2) as barreiras reprodutivas pós-zigóticas são geralmente incompletas, onde a esterilidade do híbrido (F_1) geralmente aplica-se aos machos (sexo heterogamético), mas não as fêmeas (sexo homogamético), permitindo que esta última possa servir como ponte para potencial introgessão (fluxo de genes entre espécies). No entanto, embora morfologicamente semelhantes e filogeneticamente relacionadas, as espécies crípticas podem apresentar preferências ecológicas, padrões comportamentais, genética, história de vida e graus de susceptibilidade a agentes etiológicos distintos (Neafsey *et al.*, 2015).

Semelhantemente, o grupo Oswaldoi [uma categoria infra subgenérica descrita em Faran (1980)] do subgênero *Nyssorhynchus* do gênero *Anopheles*, restrito a América do Sul, reúne sete complexos de espécies (Bourke *et al.*, 2010). Neste grupo, está inserido o *An. oswaldoi s.l.*, que tem sido incriminado como vetor local de malária na Amazônia brasileira e Colômbia (Faran, 1980; Quiñones *et al.*, 2006). Recentemente, Ruiz-Lopez *et al.* (2013) e Saraiva *et al.* (2018) observaram que os complexos *An. oswaldoi s.l.* e *An. konderi s.l.*, até então considerados como complexos distintos (Motoki *et al.* 2011), são grupos parafiléticos, sugerindo a existência de um único complexo, com pelo menos cinco espécies (*An. oswaldoi s.s.*, *An. oswaldoi A*, *An. oswaldoi B*, *An. konderi* e *An. sp. nr. konderi*), sendo denominado de Complexo Oswaldoi-Konderi.

Segundo Ruiz-Lopez *et al.* (2013), baseado em registros anteriores de *An. oswaldoi s.l.* infectados e a identificação molecular posterior, indicaram que pelo menos três espécies do complexo Oswaldoi-Konderi poderiam ser as espécies implicadas na

transmissão de malária na região amazônica (*An. oswaldoi* A, *An. oswaldoi* B e *An.* sp. nr. *konderi*). Entretanto, Saraiva et al. (2018) detectaram que nas localidades em que *An. oswaldoi* s.l. foi encontrado infectado, muitas são áreas de simpatria, assim obscurecendo a proposta de Ruiz-Lopez et al. (2013) de que estas três espécies são vetores potenciais de malária. Desta forma, torna-se necessária a utilização conjunta dos métodos de detecção de *Plasmodium* spp. e a identificação molecular/morfológica do espécime para se obter um resultado mais preciso de qual espécie do complexo Oswaldoi-Konderi estaria envolvida na transmissão da malária.

1.1. Classificação taxonômica

O complexo *Oswaldoi-Konderi* está classificado na Ordem: Diptera, Infraordem: Culicomorpha, Família: Culicidae, Subfamília: Anophelinae, Gênero: *Anopheles* e Subgênero: *Nyssorhynchus* (Forattini, 2002). Além desta, Faran (1980) classifica as espécies do Subgênero *Nyssorhynchus* em categorias informais denominadas de infra subgenéricas (Secção, Série, Grupo e Subgrupo), onde *Oswaldoi-Konderi* encontra-se inserido na Secção: Albimanus, Série: Oswaldoi e Grupo: Oswaldoi (**Tabela 1**), juntamente com as demais 16 espécies formalmente descritas e que estão subdivididas em dois Subgrupos Oswaldoi e Strodei (Harbach, 2004, Sallum et al., 2014; Bourke et al., 2013).

Tabela 1. Classificação das espécies do subgrupo Oswaldoi, segundo Faran (1980).

Subgrupo	Espécies	Descrição
Oswaldoi	<i>Anopheles anomalophyllus</i>	Komp (1936)
	<i>An. aquasalis</i>	Curry (1932)
	<i>An. dunhami</i>	Causey (1945)
	<i>An. goeldii</i>	Rozeboom & Gabaldón (1940)
	<i>An. nuneztovari</i> s.l.	Gabaldón (1940)
	<i>An. evansae</i>	(Brèthes, 1926)
	<i>An. galvaoi</i>	Causey, Deane & Deane (1943)
	<i>An. ininii</i>	Senevet & Abonnenc (1938)
	<i>An. oswaldoi</i> s.l.	(Peryassú, 1922)
	<i>An. konderi</i> s.l.	Galvão & Damasceno (1942)
	<i>An. rangeli</i>	Gabaldón, Cova Garcia & Lopez (1940)
	<i>An. sanctielli</i>	Senevet & Abonnenc (1938)
	<i>An. trinkae</i>	Faran (1972)
Strodei	<i>An. albertoi</i>	Unit (1941)
	<i>An. arthuri</i> s.l.	Unit (1941)
	<i>An. benarrochi</i> s.l.	Gabaldón, Cova Garcia & Lopez (1941)
	<i>An. strodei</i> s.l.	Root (1926)
	<i>An. rondoni</i>	(Neiva & Pinto, 1922)

As chaves dicotômicas são utilizadas rotineiramente na determinação de espécies de *Anopheles* vetores de malária, porém, mais importante que o número de vetores é a variabilidade intraespecífica que é comum e pouco caracterizada, limitando a utilidade de chaves taxonômicas quando se têm as principais características utilizadas sobrepostas a duas ou mais espécies (Matson *et al.*, 2008) resultando em identificações errôneas dos vetores. As sobreposições de caracteres morfológicos mais significantes ocorrem entre *An. nuneztovari*, *An. rangeli*, *An. trinkae*, *An. benarrochi*, *An. evansae*, *An. oswaldoi* e *An. konderi*, observadas nas principais chaves de identificação de *Anopheles*, como em Faran (1980) e Faran e Linthicum (1981). Estas espécies estão amplamente distribuídas na Amazônia brasileira e têm sido coletadas em inventários entomológicos no Brasil (Tadei *et al.*, 1988; Póvoa *et al.*, 2001; Rebelo *et al.*, 2007; Motoki *et al.*, 2012; Barbosa *et al.*, 2014), Colômbia (Naranjo-Diaz *et al.*, 2013), Venezuela (Rúbio-Palis *et al.*, 2013) e Guiana Francesa (Vezenehgo *et al.*, 2015). Estudos morfométricos realizados em *An. nuneztovari*, *An. rangeli*, *An. benarrochi* e *An. evansae* demonstraram sobreposições limiares na marcação negra basal do segundo tarsômero posterior com *An. oswaldoi s.l.* (17 a 33%) conduzindo à erros na classificação destas espécies (Calle *et al.*, 2002). Além disso, *An. konderi* também deve ser adicionado a esta lista, pois só é seguramente distinguido de *An. oswaldoi* pelo exame da genitália masculina, tornando necessário o exame de indivíduos machos (Causey *et al.*, 1946; Flores-Mendoza *et al.*, 2004). Desta forma, interpretações erradas nas identificações entomológicas podem ter reflexos no controle dos verdadeiros vetores de doenças como a malária, inviabilizando as medidas de controle vetorial, tendo em vista que cada espécie responde de maneira diferente ao controle e desenvolvem resistência aos compostos químicos utilizados nos inseticidas (Mulamba *et al.*, 2014).

1.2. História taxonômica

Anopheles oswaldoi s.l. foi descrita a partir de espécimes coletados no Vale do Rio Doce, no Estado do Espírito Santo, Brasil, inicialmente foi denominada de *Cellia oswaldoi* por Peryassú (1922). Em seguida, a espécie foi sinonimizada com *An. tarsimaculatus* (Goeldi, 1905) por Dyar (1923), dando início à uma problemática que gerou vários rearranjos no grupo, dentre eles a descrição de *An. konderi* por Galvão e Damasceno (1942). Para esta última espécie, os autores descreveram como uma nova espécie baseando-se na diferença observada no ápice do edeago (ou mesossoma) da

genitália dos machos. Posteriormente, *An. konderi* foi sinonimizada com *An. oswaldoi* por Lane (1953) ao examinar a morfologia externa de todos os estágios de vida. Posteriormente, Flores-Mendoza *et al.* (2004) ressuscitaram *An. konderi* da sinonímia e designaram um neótipo, entretanto, os autores não utilizaram sequências de DNA e, além disso, utilizaram uma ampla amostragem em Rondônia, Acre e Peru, que consequentemente revelaram-se uma mistura de pelo menos três espécies do complexo Oswaldoi-Konderi. Para *An. oswaldoi* s.s. Motoki *et al.* (2009) revisaram a taxonomia e distribuição geográfica, fixando também o seu *status* taxonômico de espécie distinta com sequências de DNA barcode que possibilitam delimitá-la das demais espécies do complexo.

Estudos moleculares no complexo *Oswaldoi-Konderi* foram realizados inicialmente por Marrelli *et al.* (1999a), utilizando o Segundo Espaçador Interno Transcrito (*ITS2*), em sete populações de *An. oswaldoi* s.l. Os autores determinaram quatro grupos: Grupo I (Acre, Amazonas e Rondônia - Brasil), Grupo II (Ocamo - Venezuela e Amapá - Brasil), Grupo III (Espírito Santo /localidade tipo - Brasil) e Grupo IV (Yurimaguas - Peru). Ruiz *et al.*, (2005) determinaram, mais tarde, que o Grupo II correspondia à *An. oswaldoi* B (Quiñones *et al.*, 2006), o Grupo IV, na verdade era *An. benarrochi* B (Quiñones *et al.*, 2001; Ruiz *et al.*, 2005), o Grupo III, procedente da localidade tipo de *An. oswaldoi* s.s. era na verdade *An. evansae* (Marrelli *et al.*, 2006) e o Grupo I *An. oswaldoi* A (Ruiz-Lopez *et al.*, 2013). Scarpassa e Conn (2006), utilizando um longo fragmento do gene Citocromo C Oxidase Subunidade I - *COI* (1.226 pb) analisaram quatro populações de quatro Estados brasileiros: Acre, Amazonas, Pará e Rondônia, e obtiveram quatro grupos fortemente suportados na árvore filogenética, indicando a existência de quatro linhagens ou espécies neste complexo. Posteriormente, estes grupos foram identificados como: *An. oswaldoi* s.s., *An. oswaldoi* A, *An. konderi* e *An. sp. nr. konderi* (nr = near: próximo) (Ruiz-Lopes *et al.*, 2013).

Outros marcadores genéticos também foram utilizados neste complexo, por exemplo, Motoki *et al.* (2011) analisaram três marcadores moleculares (gene *COI*, *ITS2* e gene *White*) em espécimes identificados morfologicamente como *An. konderi*. Os autores sugeriram a existência de um complexo espécies dentro de *An. konderi* s.l., formado por pelo menos três espécies, suportadas pela elevada divergência genética entre populações do Amapá/Acre e Rondônia/Paraná. Ruiz-Lopez *et al.* (2013) estudaram sequências de *COI* barcode e *ITS2* de *An. oswaldoi* s.l. de 33 localidades da América do Sul; neste estudo, foram identificadas três espécies putativas: *An. oswaldoi* A, *An.*

oswaldoi B e *An. sp. nr. konderi*, e mais *Anopheles oswaldoi* s.s. (já descrita), *Anopheles konderi* s.s. foi utilizada com grupo externo. Nas análises filogenéticas estas últimas agrupou dentro do complexo *An. oswaldoi* s.l., levando os autores a sugerir a existência de um único complexo Oswaldoi-Konderi. No entanto, devido à baixa amostragem de *An. konderi* naquele estudo, esta inferência não pode ser claramente concluída. Saraiva *et al.* (2018) analisaram sequências de *COI barcode* e ITS2 de 18 localidades da Amazônia brasileira, nas análises foram incluídas amostras de *An. oswaldoi* s.l. e *An. konderi* s.l. A análise conjunta dos dois complexos por meio de inferências filogenéticas confirmou a parafilia do complexo *An. konderi* s.l. que levaram a concluir a existência de um único complexo e a necessidade de descrever formalmente as espécies observadas.

Até o momento, *An. oswaldoi* possui um único sinônimo, o *An. aquacaelestis* Curry (Rozeboom e Gabaldón, 1941). Recentemente, sugeriu-se que *An. oswaldoi* B pode representar *An. aquacaelestis*, com base na ocorrência geográfica. Entretanto, para a ressurreição de *An. aquacaelestis* será necessário o exame do holótipo e a comparação com *An. oswaldoi* B, que consequentemente permitirá também descrever as demais espécies do complexo *An. oswaldoi* (Ruiz-Lopez *et al.*, 2013).

1.3. Distribuição geográfica

Os membros de *Oswaldoi-Konderi* encontram-se distribuídos desde a Costa Rica até o norte da Argentina, mas principalmente nos países da América do Sul (Figura 1) (Peryassú, 1922; Galvão e Damasceno 1942; Rozeboom, 1942; Simmons, 1979; Faran, 1980; Peyton *et al.*, 1983; Rúbio-Palis e Curtis, 1992; Lounibos e Conn, 2000; Laubach *et al.*, 2001; Flores-Mendoza *et al.*, 2004; Scarpassa e Conn, 2006; Bergo *et al.*, 2007; Motoki *et al.*, 2011; Pinault e Hunter, 2011; Ruiz-Lopez *et al.*, 2013 e Saraiva *et al.*, 2018).

Segundo Saraiva *et al.* (2018), a distribuição estimada para cada membro do complexo Oswaldoi-Konderi na América Latina, inferida por meio de dados coletados e dados da literatura, *An. oswaldoi* s.s. e *An. konderi* s.s. apresentam até o momento distribuição disjunta, com populações isoladas na Amazônia e sudeste do Brasil. *An. oswaldoi* B apresenta ocorrência ao longo de todo o extremo norte da América do Sul, incluindo a Amazônia. *An. oswaldoi* A e *An. sp. nr. konderi* ocorrem nas regiões central e oeste da região amazônica, além de uma faixa de simpatria na Amazônia ocidental brasileira. Entretanto, em decorrência de ainda existir grandes *gaps* de amostragens, os

limites desta distribuição geográfica possivelmente podem sofrer alterações com amostragens adicionais, que podem revelar novos registros e novas áreas de simpatria.

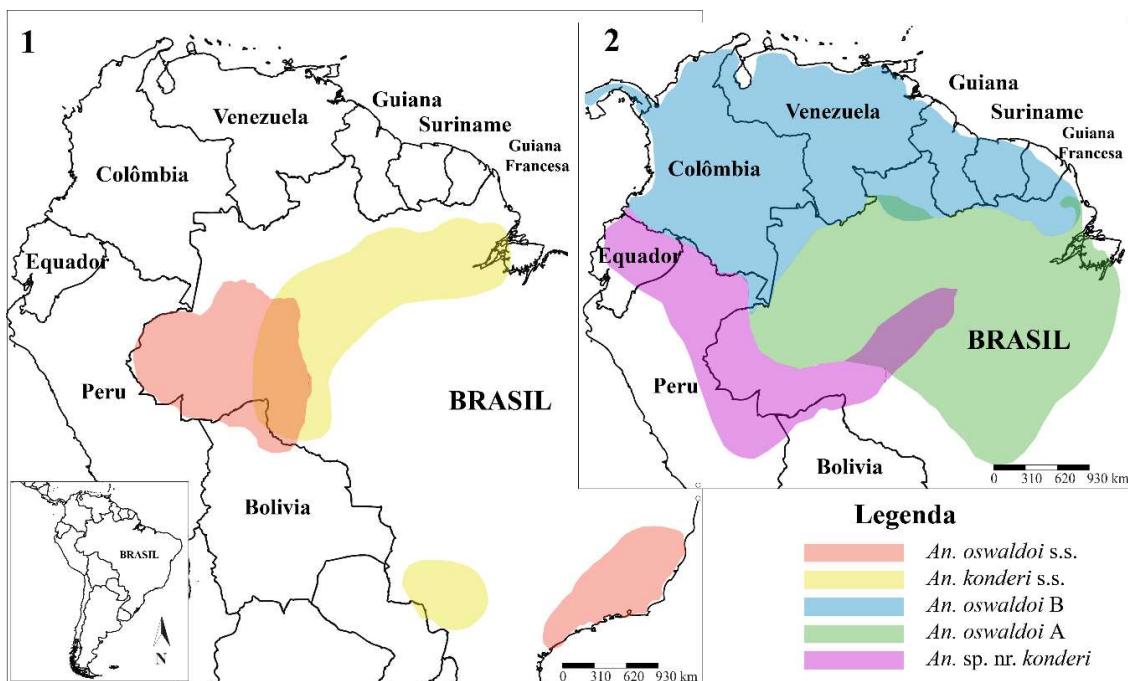


Figura 1. Distribuição geográfica das espécies com complexo Oswaldoi-Konderi na América do Sul, segundo Saraiva et al. (2018). Mapa 1. Distribuição disjuntas (Amazônia e Sudeste brasileiro) de *An. oswaldoi* s.s. e *An. konderi* s.s. Mapa 2. Distribuição restrita à bacia amazônica de *An. oswaldoi* A, *An. oswaldoi* B e *An. sp. nr. konderi*.

Em relação a preferências ambientais, os membros de *Oswaldoi-Konderi* ocorrem principalmente em ambientes de floresta tropical úmida (Floresta de Várzea, Ripárias ou Ciliares) e raramente são encontrados em grandes extensões de campos de Cerrado (Deane *et al.*, 1948; Forattini, 2002). Esta plasticidade para explorar ambientes distintos foi preliminarmente observada por Klein *et al.* (1991) e Saraiva *et al.*, (2018), no entanto, as amostragens foram insuficientes para *An. oswaldoi* B, *An. oswaldoi* s.s. e *An. sp. nr. konderi*, por incluir uma pequena área da sua distribuição geográfica. Estudos recentes têm sugerido que os limites de distribuição entre as populações estão relacionados à paisagem, definida por diversas eco-regiões, em que sua variação foi pouco avaliada (Desrochers, 2010). A permeabilidade da paisagem pode afetar a estruturação genética de certas espécies, como observado em *An. darlingi* por Motoki *et al.* (2012).

1.4. Características morfológicas

Os membros do complexo Oswaldoi-Konderi são idênticos morfologicamente no estágio adulto (fêmea). Estes podem ser reconhecidos pelos seguintes caracteres: 1) segundo tarsômero posterior com marcação escura de 8 à 20% basal, 2) terceiro e quarto tarsômeros posteriores totalmente brancos, 3) quinto tarsômero posterior com anel basal escuro, 4) quarto segmento palpo branco, e as extremidades escuras, 5) mancha preumeral escura menor do que a mancha umeral clara (Gorham *et al.*, 1973).

Segundo Flores-Mendoza *et al.* (2004), a única diferença entre *An. oswaldoi* e *An. konderi* está na região apical do edeago da genitália masculina, sendo em *A. oswaldoi* s.l. de formato arredondado, mais longo do que largo, enquanto que em *An. konderi* s.l. a região apical apresenta o formato triangular, ou seja, mais largo do que longo e com a presença de pequenos folíolos laterais.

1.5. Importância médica

Anopheles oswaldoi s.l. foi encontrada infectada Hayes *et al.* (1987) no Peru, por Rúbio-Palis *et al.* (1992) na Venezuela, Quiñones *et al.* (2006) na Colômbia, Hiwat e Bretas (2011) no Suriname e Dusfour *et al.* (2012) na Guiana Francesa. No Brasil, *An. oswaldoi* s.l. foi encontrada infectada por Arruda *et al.* (1986) no Estado do Pará, por Ferreira *et al.* (1990) em Rondônia e por Branquinho *et al.* (1993) no Acre. Tadei *et al.* (1998) encontraram um espécime em Cachoeira da Porteira (Pará) e Póvoa *et al.* (2001) registraram um espécime no município de Serra do Navio, Amapá. O último relato de infecção foi notificado por Santos *et al.* (2005) no município de Anajás, Pará. A espécie *An. konderi* s.l. nunca foi encontrada infectada (Flores-Mendoza *et al.*, 2004; Motoki *et al.*, 2011). No entanto, este dado pode ser incompleto considerando-se que estas duas espécies, por muito tempo, foram consideradas sinônimas e, também, mesmo como espécies válidas, em decorrência da elevada semelhança morfológica de ambas, *An. konderi* pode ter sido identificada como *An. oswaldoi* s.l. Marrelli *et al.* (1999b) testaram a competência vetorial de *An. oswaldoi* e *An. konderi* em condições de laboratório e obtiveram maior taxa de infecção para *An. oswaldoi* s.l. (13,8%) do que para *An. konderi* (3,3%); entretanto, os autores utilizaram apenas amostras de uma única localidade (Rondônia) para os testes.

2. OBJETIVOS

2.1. Objetivo geral

Estudar a diversidade taxonômica e genética utilizando ferramentas morfológicas e moleculares e testar infecção natural por *Plasmodium* spp. no complexo Oswaldoi-Konderi

2.2. Objetivos específicos

Os objetivos específicos encontram-se contemplados nos três capítulos que compõem a presente tese. Os títulos dos capítulos estão apresentados abaixo com seus respectivos objetivos:

Capítulo 1. Morphological and molecular analyses in the Oswaldoi-Konderi Complex

(*Anopheles: Nyssorhynchus*) of the Brazilian Amazon

- Identificar as espécies do complexo Oswaldoi-Konderi utilizando dados morfológicos e moleculares;
- Inferir as relações evolutivas do complexo Oswaldoi-Konderi;
- Analisar espécies do complexo Oswaldoi-Konderi da natureza que possam estar infectados com *Plasmodium* spp.

Capítulo 2. *Anopheles (Nyssorhynchus) tadei*: A New Species of the Oswaldoi-konderi complex (Diptera, Culicidae, Anophelinae), morphological and molecular comparisons with *An. konderi* sensu stricto.

- Reexaminar neótipo de *Anopheles konderi* s.s. e comparar com espécimes obtidos na localidade tipo para fixar a identidade morfológica e molecular de *An. konderi* s.s.;
- Descrever taxonomicamente a espécie próxima de *An. konderi* s.s.

Capítulo 3. Filogeografia de *Anopheles (Nyssorhynchus) oswaldoi* A (Diptera: Anophelinae) da Amazônia brasileira: uma espécie do Complexo Oswaldoi-Konderi

- Estudar a diversidade genética e Filogeografia das três ‘linhagens’ de *An. oswaldoi* A;
- Verificar se as ‘linhagens’ podem representar espécies.

Capítulo 1

**Morphological and molecular analyzes in the Oswaldoi-Konderi
Complex (*Anopheles*: *Nyssorhynchus*) of the Brazilian Amazon**

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Abstract

Background: The Oswaldoi-Konderi complex involves a set of five species, two formally described (*Anopheles oswaldoi* s.s. and *Anopheles konderi* s.s.) and three putative species (*Anopheles oswaldoi* A, *Anopheles oswaldoi* B and *Anopheles* sp. nr. *konderi*) These species occur in a wide range of the Brazilian Amazon, where previously *An. oswaldoi* s.l. was found naturally infected with *Plasmodium* spp.

Methods: In the present study, mosquitoes from the Oswaldoi-Konderi complex were collected from twenty-nine (29) locations in the Brazilian Amazon region, where they were subjected to morphological analyses and spawning obtained for morphological comparisons of all stages of development (egg, larvae, pupa and adults), in addition to molecular sequences of *COI* and *ITS2*.

Results: A total of 193 specimens were obtained for molecular analysis and 71 spawns for morphological analysis. The characters of the male genitalia revealed significant differences between the species of the complex. In addition, *An. konderi* showed marked differences in the larval stage and male genitalia, which had not yet been observed. The combined molecular analyzes with two markers were consistent for at least six species, including *Anopheles ininii*, in the Oswaldoi-Konderi complex. While the delimitations with only one molecular marker revealed a great diversity of groups in the complex.

Conclusions: The Oswaldoi-Konderi complex is composed of at least six species widely distributed in the Brazilian Amazon. These species are morphologically separated when analyzing the characters of the male genitalia. *An. ininii* is potentially one of the members of the complex that could have been mistakenly classified as *An. oswaldoi* s.l. before.

Key words: *Anopheles oswaldoi* A, *Anopheles oswaldoi* B, *Anopheles* sp. nr. *konderi*, Cryptic species, Male genitalia.

Background

Malaria is a serious disease that still affects thousands of people around the world. According to the World Health Organization, 219 million cases and 435,000 deaths were reported in 2017 [1]. In the same year, Brazil reported an increase of 84% (218,000 cases) compared to 2016. Most cases are registered in the Amazon region, which concentrates ~99% of malaria in the country [1]. In this region, the main malaria vector is *Anopheles darlingi* Root [2]. However, in addition to *An. darlingi*, other species of *Anopheles* Meigen genus play a regional, local and secondary vector role, and many of these species form species complexes, such as *An. albitalis* s.l. [3] *An. nuneztovari* s.l. [4–6], *An. triannulatus* s.l. [7, 8], *An. konderi* s.l. [9] and *An. oswaldoi* s.l. [10–13]. Currently, *An. darlingi* has shown significant genetic structure for three groups in Brazil [14].

Species complexes are relatively common in the Culicidae family [15], which consist of species that are difficult or impossible to identify based only on morphology [16]. In the Oswaldoi group of the Albimanus Section [17], for example, seven of the 15 species formally described, are currently considered to be species complexes [7, 10], in which *An. oswaldoi* s.l. and *An. konderi* s.l. form a species complex with at least five cryptic species separated basically with molecular markers [12].

Anopheles oswaldoi (Peryassú) [18] was described for the Vale do Rio doce, Espírito Santo and, later, *An. konderi* Galvão & Damasceno [19] was described from Coari, state of Amazonas, both in Brazil. These two species are morphologically identical in adult, being distinguishable only based on the structure of the apex of the aedeagus of the male genitalia, where in *An. oswaldoi*, this structure is elongated and narrow, while in *An. konderi* it is short and wide. In addition, this latter has a pair of lateral leaflets. However, these differences were not sufficient to prevent *An. konderi* from being placed in synonymy with *An. oswaldoi* by Lane [20]. Later, based on molecular studies [10],

differences in behavior [21] and infection by *Plasmodium* spp. [22], led Flores-Mendoza et al. [19] to resurrect from the synonym *An. konderi* and designate a neotype. However, these authors did not consider the possibility that other species could coexist in the complex and could obscure the redescription of *An. konderi* if molecular data were not used to establish the identity of *An. konderi* s.s. [12, 13, 23]. As was done in the redescription of *An. oswaldoi* s.s. by Motoki et al. [24], in which the authors also deposited a sequence and designated a lectotype, thus allowing a safe comparison with the other species of the Oswaldoi-Konderi complex.

Currently, the Oswaldoi-Konderi complex consists of five species; *Anopheles oswaldoi* s.s., *An. oswaldoi* A, *An. oswaldoi* B, *An. konderi* s.s and *An. sp.nr. konderi* [12, 13]. Except for *An. oswaldoi* s.s. and *An. konderi* s.s., which have already been redescribed, the other species have not been properly characterized and described.

The present study aimed to perform morphological comparisons in the taxonomic units delimited with the molecular data, to determine: 1) The number of species in the complex, 2) to identify morphological characters that can differentiate them, and 3) to estimate the geographical distribution of the species in the Brazilian Amazon region.

Materials and methods

Mosquito collection and morphological identification

The choice of collection points was based on sample gaps from previous studies [9–13, 25], and records of mosquitoes infected with *Plasmodium* spp. in the Brazilian Amazon region[2, 26–29]. The 29 points sampled covered the six Brazilian states that are in the Amazon biome (Amazon rainforest): Acre (3), Amapá (8), Amazonas (9), Pará (3), Rondônia (2), Roraima (4). The specimens from the state of Acre and Rondônia correspond to the VMS study [11], the specimens from Lábrea (Amazonas) and Serra do

Cachorro (Pará) were provided by collections made by Dr. Ronildo Alencar with CDC traps, and 84 sequences of *COI* were reused from our previous published study. The nomenclature used followed the same one designated by Ruiz-Lopez et al. [12] in *An.* sp. nr. *konderi* (= species close to *An. konderi*), species not described. **Table 1** presents information on sampled sites, geographic coordinates, number of specimens, number of spawns obtained, and number of individuals analyzed for morphology and molecular analysis. Geographic distribution maps of species were generated to present an overview of the distribution of species and sympatric and allopatric populations. All mosquito collections were authorized by the Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (IBAMA), by the Biodiversity Authorization and Information System (SISBIO) granted to VMS (permanent license nº 38440).

Adult (female) mosquitoes were captured using a light trap, model of Shannon [30], installed inside the forest between approximately 18:00 to 22:00 hours. The captured specimens were placed in paraffined cardboard collecting cups and transported alive in polystyrene boxes to the insectary at the Laboratory of Population Genetics and Evolution of Vector Mosquitoes at the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, Brazil. The spawning was obtained from females of the nature and to stimulate oviposition one of the female's wings was removed. From each progeny, specimens from each stage of development were separated for morphological analysis, and a specimen by spawning was sequenced for the *COI* gene, region of the DNA barcode (DNAm) and for the region of the ITS2 - second internal transcribed spacer, of the ribosomal DNA.

Morphological analysis

At each stage of development (E: eggs, Le: larva exuvia, Pe: pupa exuvia, MG: male genitalia, F: adult female and M: adult male), 10 specimens of each spawn obtained from the insectary were separated for morphological analysis and molecular (**Table 1**).

The eggs were preserved in glutaraldehyde (4%) and later, they were prepared for electron microscopy following the protocol by Sallum et al. [23]. The eggs were photographed in a Tescan scanning electron microscopy scanner, available at INPA Laboratório Temático de Microscopia Óptica e Eletrônica (LTMOE). The measurements performed were total length, total width, and diameter of the micropyle. The Le, Pe and MG were mounted on permanent slides with Canada's Balsam, and the structures were designed in a clear camera coupled to a light microscope, brand Nikon Eclipse E200, with increases in objectives from 10 to 40X. The adult specimens (females and males) were fixed in a paper and pin triangle and all external morphology characters were examined.

The male genitalia (MG), as they present the greatest morphological differentiation among the members of the Oswaldoi-Konderi complex, were used as the main character for the initial separation of species in the complex. For this, measurements of the structures of the ventral claspete and the apex of the edeago were performed. These characters of the male genitalia were also used as support for the species tree inferred with the molecular data (mitochondrial and nuclear markers).

Additionally, *Anopheles ininii* belonging to the Oswaldoi group, collected in Mina Palito, municipality of Novo Progresso, State of Pará. It was included in the because showed high morphological similarities to *An. oswaldoi* s.l. in the adult female phase and differing only in the presence of clear marking on the 4th middle tarsomere. Although no spawning was obtained for *An. ininii*, 6F, 1M and 1MG were collected in field, allowing

the morphological comparison of the genitalia (aedeagus and the ventral claspete) with the five species of the Oswaldoi-Konderi complex.

DNA extraction, PCR amplification and sequencing reaction

Genomic DNA was extracted from the legs of adult mosquitoes using the phenol-chloroform protocol [31]. The DNA pellet was resuspended in 30 µL in sterile water. A small aliquot of this DNA was stored at -20° C and then used as a template for the Polymerase Chain Reaction (PCR) amplification for the two markers (*COI* barcode and ITS2). The remaining DNA aliquots are kept frozen as voucher DNA at -80° C in the Laboratory of Population Genetics and Evolution of Vector Mosquitoes, of the Instituto Nacional de Pesquisas da Amazônia - INPA, Manaus, Brazil.

The DNA barcode region of the *COI* gene was amplified using the universal primers named LCO 1490 and HCO 2198 [32]. The amplification conditions used were the same as Motoki et al. [9]. For the nuclear marker, primers 5.8SF and 28SR were used, which amplify the ITS2 region [23]. The amplification profile was as described in Motoki et al. [9]. High fidelity Taq Platinum to DNA Polymerase (Life Technologies) was included in all PCR reactions. The PCR products were checked with 1% agarose gel electrophoresis, stained with GelRed™ (Biotium Inc., Hayward, USA) and observed under UV light. The amplified products were purified using PEG precipitation. Sequencing reactions were conducted for both DNA strands, using the Big Dye Terminator kit for electronic injection in the ABI 3130xl automatic sequence analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

The sequences of *COI* and ITS2 were automatically aligned with ClustalW [33] and edited in BioEdit v.7.2.5 [34], with the help of Chromas Lite (Chromas Lite® Software). The consensus sequences were translated into amino acids to verify stop

codons, pseudogenes or *Numts*. These sequences were compared to those available on GenBank, using the BLAST Tool [35] available at the National Biotechnology Information Center (NCBI) website (<http://blast.ncbi.nlm.nih.gov/blastn>). Estimates of the number of haplotypes for each marker were verified in the DnaSP v.6.0 program [36]. A sequence, corresponding to each haplotype recovered, will be deposited on the public platform of GenBank.

Species Delimitation

The species were previously identified based on morphology, especially characters of the male genitalia (aedeagus and ventral claspette) and, subsequently, the Operational Taxonomic Units (OTU's) were determined according to the reciprocal monophyly criterion, based on the inference trees. Bayesian (IB) and maximum likelihood (MV). This criterion considered monophyly and high supports of posterior probability (BPP: 0.95/1) and bootstrap (95/100). Then, with the OTU's identified, the genetic distance was calculated using the Kimura 2-Parameters model in the MEGAX program v.10 [37].

The delimitation of species, based on molecular data, was inferred with three delimiters: ABGD strict, mPTP and bGMYC. ABGD (Automatic Barcode Gap Discovery) partitions the molecular data set into groups of similar taxa, establishing a range of maximum intraspecific divergence values ($P_{min} = 0.005$ to $P_{max} = 0.1$), without an a priori hypothesis of species [38]. The mPTP (multi-rate Poisson Tree Process) uses a "Phylogenetic" method based on the different mutation rates in a phylogenetic tree to resolve the interspecific and intraspecific diversity. Unlike the previous method, it does not depend on a priori distance limits, but requires a forked non-ultrametric phylogenetic tree of entry. For the latter, a maximum likelihood tree (ML) was generated using

RAxML-HPC BlackBox 8.2.10 [39] at CIPRES Science Gateway (Stamatakis, 2014) using codon gene partitioning (with the second position of the codon excluded due to invariability) and using the GRT + G model, with a bootstrap of 1,000 replicates. bGMYC (General Mist of Yule Coalescent Bayesian) employs an identification approach taking the limits of uncertainty between species, according to the changes in branching rates in the phylogenetic tree, when different populations contain several species [40]. All species delimitation analyzes were performed using the SPLITS package [41], implemented for R v.3.0.1 (R Foundation for Computational Statistics, Vienna, Austria). The ultrametric trees for Bayesian analyze were generated with BEAUTi and BEAST v.1.10.4 [42]. The parameters used consisted of two independent runs with 100 million generations each, resampling of trees every 1,000 generations and a burning of 25% of the initial trees.

Results

Morphological diagnosis

Anopheles (Nyssorhynchus) oswaldoi Sensu Stricto (Peryassú, 1922)

Adult female: *Anopheles oswaldoi* s.s. can be identified with the combination of morphological characters: Foretarsomere 2th and 3th, with white scale markings between 0.28-0.33 and 0.52-0.84 at the apex, respectively. Foretarsomere 4th with dark scales and narrow clear apex marking. Midtarsomere 4th completely dark and 5th with white scales at the apex. Hindtarsomere 2th with small basal dark marking (up to 20% of the total length of tarsomere 2th). Hindtarsomere 3th and 4th completely white. Hindtarsomere with 5th ring of dark scales at the base and apex with light scales (pale) (**Fig. 1A**). *Wing:* dark pre-humeral spot smaller than the light pre-humeral spot. Light subcostal spot half the

length of the dark costal spot (**Fig. 1B**). *Male genitalia*: basal lobe of the ventral claspete laterally expanded and vertically elongated, with long setae on the basal margin, pre-apical plate of the large ventral claspete, weakly sclerotized, refringent structure with triangular shape, *Aedeagus*: narrow and elongated, apex rounded and without apicolateral leaflets (**Fig. 1C**). *Pupa*: setae 9-V-VIII short, seta 6-II with length equal to or greater than 7-II, seta 2-I with 3-9 long branches, dendritic. *Larva of 4th instar*: setae 2-C spaced, index (1.1-1.4), thin secondary branches starting from a setae central, short split setae 4-C (**Fig. 1D**), setae 1-X implanted outside the selin, anal papilla larger than the X segment. *Spiracular apparatus*: spiracular plate with short whites and triangular outline (**Fig. 1E**).

***Anopheles (Nys.) oswaldoi* Specie B (Not described)**

Adult female: does not have characters that would allow the diagnosis, in this development phase, with *An. oswaldoi* s.s. (**Fig. 2A**), *Wing*: similar to *An. oswaldoi* s.s. (**Fig. 2B**) *Male genitalia*: it presents significant differences that allow the separation with the other species. *Ventral claspete*: broad basal lobe, margin setae large, sclerotized preapical plaque and wide, refringent structure round. *Aedeagus*: apex rounded and sclerotized contour and, wider than that observed in *An. oswaldoi* s.s. (**Fig. 2C**). *Pupa*: marked variations in the lateral spines and similar to that observed in *An. oswaldoi* s.s. *Larva 4th instar*: two structures showed variations corresponding to the species *An. oswaldoi* B and *An. oswaldoi* s.s. *Clypeal setae*: fine branches starting from the main (central) seta, similar to *An. oswaldoi* s.s. (**Fig. 2D**) and *Spiracular apparatus*: conformation of the triangular spiracular plate also similar to *An. oswaldoi* s.s. (**Fig. 2E**). *Eggs*: floats cover a large part of the ventral surface, with emphasis on the posterior region of the float, which has a wide margin (**Fig. 6-B**). *Micropyle*: non-variable (**Fig. 6-B1**). *Chorionic cells*: character not variable to the other species of the complex (**Fig. 6-B2**).

Anopheles (Nys.) oswaldoi Specie A (Not described)

Adult female: dark yellowed integument, in the chest and abdominal segments. Leg markings are like *An. oswaldoi* s.s. and *An. oswaldoi* B (**Fig. 3A**). *Wing*: pattern like *An. oswaldoi* s.s. and *An. oswaldoi* B (**Fig. 3B**). *Male genitalia*: basal lobe flattened and broad, apical lobe narrow and elongated, strongly sclerotized seta, strongly sclerotized preapical plate, refringent structure with rounded apex. *Aedeagus*: apex triangular and narrow, without apicolateral leaflets (**Fig. 3C**). *Pupa* and *Larva*: like *An. oswaldoi* s.s. (**Fig. 3D, 3E**). *Eggs*: the floats cover a large part of the ventral surface, the posterior margin of the narrow float (**Fig. 6-A**). *Micropyle*: not variable (**Fig. 6-A1**). *Detail of the tubercles deck*: this structure did not variable between the species studied (**Fig. 6-A2**).

Anopheles (Nys.) konderi Sensu Stricto (Neotype)

Adult female: dark brown integument, hindtarsomere 2th with variation of (0.18 - 0.20), which showed a slightly higher average (average = 0.19) than the average observed for the other species of the Oswaldoi-Konderi complex (average = 0.14) (**Fig. 4A**). *Wing*: dark preumeral spot smaller than the light preumeral spot, but this difference was not very evident because it was very variable even within the same progeny (**Fig. 4B**). *Male genitalia*: abdominal sternite VIII with evident lateral protuberances, genitalia with basal lobe of the wide ventral claspete and with long setae present, outer margin of the basal lobe with very evident protuberances, sclerotized preapical plate, narrow apical lobe, rounded refringent structure. *Aedeagus*: broad, flat apex, basal sclerotization of the U-shaped aedeagus, very evident apicolateral shales (**Fig. 4C**). *Pupa*: posterolateral spines long in the abdomen, except in segments (II to IV) and segment V facing outwards, but this observation was very variable, even within the same progeny. *Larva 4th instar*: clypeal seta with branches that start from the middle of the length of the seta, as secondary

branches are present and not very evident (**Fig. 4D**). *Spiracular apparatus*: spiracular plate with evident side hollows (**Fig. 4E**). *Eggs*: the floats cover a large part of the ventral surface, the posterior margin of the narrow float (**Fig. 6-C**). *Micropyle*: not variable (**Fig. 6-C1**). *Detail of the tubercles deck*: did not variable between the species studied (**Fig. 6-C2**).

Anopheles (Nys.) sp. nr. konderi (Not described)

Adult female: dark brown tegument, tarsomere markings like *An. oswaldoi* s.s., *An. oswaldoi* A and *An. oswaldoi* B (**Fig. 5A**). *Wing*: very variable, as well as *An. konderi*. Dark preumeral sport smaller than the light preumeral sport, but it is not very evident. Subcostal spot slightly larger than the other species of the Oswaldoi-Konderi complex (**Fig. 5B**). *Male genitalia*: basal lobe broad, without lateral protuberances, broad apical lobe and with short spines, sclerotized and narrow preapical plaque, triangular refringent structure, like *An. oswaldoi* s.s. in this aspect. *Aedeagus*: flattened apex, basal V-shaped sclerotization and apicolateral shales present, but smaller than that of *An. konderi* s.s. (**Fig. 5C**) *Pupa*: Long and narrow lateral spines, except for segments II to IV. *Larva 4th instar*: clypeal setae with ramifications starting at half the length of the bristle, did not show secondary ramifications like those observed in *An. konderi* s.s. (**Fig. 5D**). *Spiracular apparatus*: short spiracular arms, spiracular plaque very wide and without concavity (**Fig. 5E**).

Molecular analyses

A total of 193 specimens belonging to the Oswaldoi-Konderi complex were sampled. Of these, 107 adult females were morphologically examined, and 71 progenies were obtained from nine locations (Table 1). Among the spawning, the following were

examined respectively: *An. oswaldoi* s.s. Coari, AM (1F, 1M, 3MG); *An. konderi* s.s. Coari, AM (1E, 3Le, 3Pe, 3F, 1M, 3MG), Coari AM Neotype material (4Le, 6Pe, 1M, 6MG), Borba, AM (12 Le, 12 Pe, 12F, 12M, 12MG) and Autazes, AM (2E, 6Le, 6Pe, 6F, 3M, 6MG); *An. sp. nr. konderi* Coari, AM (3Le, 3Pe, 3F, 1M, 3MG) and Novo Remanso (4F); *An. oswaldoi* A from Pitinga, AM (1E, 3Le, 3Pe, 3F, 2M, 3MG), Viruá National Park, RR (3E, 15Le, 15Pe, 15F, 10M, 15MG) and Cantá, RR (1Le, 1F, 1M, 1MG) and *An. oswaldoi* B, from Maracá Ecological Station, RR (10E, 22Le, 22Pe, 22F, 22M, 22MG).

For molecular analyses, 193 sequences were obtained for the *COI* gene region of the DNA barcode and only 33 for ITS2, due to the low quality of the sequences of this nuclear marker (**Table 1**). The length of the *COI* barcode gene fragment was 663bp, with 116 haplotypes recovered, of which five haplotypes correspond to *An. ininii* (**Supplementary Table 1**). One hundred, third and three variable bases (20.1%) and 105 information sites (parsimony) were observed for this marker. For the nuclear marker ITS2, the length obtained was 474bp, with 11 haplotypes (H). *Anopheles oswaldoi* s.s. (H = 2), *An. oswaldoi* A (H = 2), *An. oswaldoi* B (H = 1), *An. konderi* s.s. (H = 2) and *An. sp. nr. konderi* (H = 4). Twenty-one variable bases were observed (4.4%), variations in bases were concentrated between sites 157 and 460 sites. Indels (insertions / deletions) were observed in bases 288, 314, 434-460 (**Supplementary Table 2**).

Species delimitation based on molecular data

The genetic distance, estimated from data from the *COI* gene barcode region, revealed mean interspecific values equal to or greater than 4%. The shortest distance (0.04), intriguingly, was obtained between *An. oswaldoi* s.s. and *An. konderi* s.s., which were phylogenetically more distant in the IB tree. The highest genetic distance value was

between *An. oswaldoi* A and *An. oswaldoi* B (0.068), which reflects the result found in the IB tree. The average intraspecific values found within the Oswaldoi-Konderi complex ranged between 0.006 to 0.018. *Anopheles oswaldoi* A and *An. konderi* showed the highest intraspecific values (0.018). Comparing *Anopheles ininii* with the species of Oswaldoi-Konderi complex, *An. ininii* showed a lower interspecific value with *An. konderi* s.s. (0.061) and higher values with *An. oswaldoi* B (0.076), *An. sp. nr. konderi* (0.072) and *An. oswaldoi* A (0.071). These values indicate strong support for the six species within the Oswaldoi-Konderi complex (**Table 2**).

The database generated 111 haplotypes for the five species characterized with morphological data: *An. oswaldoi* s.s. (05 haplotypes), *An. oswaldoi* B (41 haplotypes), *An. oswaldoi* A (39 haplotypes), *An. konderi* s.s. (18 haplotypes) and *An. sp. nr. konderi* (08 haplotypes). Based on this dataset, the five species, previously separated, also formed disconnected networks, indicating that they represent separate species. In addition, subgroups were also observed in *An. oswaldoi* A (three subgroups), *An. oswaldoi* B (seven subgroups) and *An. sp. nr. konderi* (two subgroups). *Anopheles ininii*, included to represent one of the taxa of the outgroup, also formed, as expected, an independent network (**Fig. 7**).

The Bayesian inference tree (IB) with data from the *COI* gene, separated the five species, and another clade representing *An. ininii*. All species were recovered in reciprocally monophyletic clades, with high support for posterior probability ($BPP \geq 0.99$). This result was congruent with the morphological differences observed in the male genitalia between the species. *An. oswaldoi* s.s. and *An. oswaldoi* B were recovered as sister-taxa, whereas *Anopheles oswaldoi* A was recovered as a sister-taxon of *An. konderi*, *An. sp. nr. konderi* and *An. ininii*. The clade represented by *An. ininii* was recovered within

the Oswaldoi-Konderi complex, as a sister-taxon of *An. konderi* and *An. sp. nr. konderi* (**Fig. 8**).

The delimitation using the ABGD strict algorithm, separated the *COI* gene database into 14 MOTUs (Molecular Operational Taxonomic Units). In addition, this estimate was the most conservative among the delimiters used. With mPTP, the database was separated into 16 MOTUs and with bGMYC 18 MOTUs were recognized, indicating geographical sub-structures (**Fig. 8**).

Based on the *COI* database, *An. oswaldoi* s.s. and *An. konderi* s.s. were included into two main clades. *Anopheles oswaldoi* A was subdivided into three subgroups and *An. ininii* in only one divergent group, with all congruent delimiters (ABGD, mPTP and bGMYC). Boundary differences were obtained for *An. sp. nr. konderi* and *An. oswaldoi* B. *Anopheles* sp. nr. *konderi* was subdivided into two subgroups by the ABGD and mPTP delimiters and into three subgroups by bGMYC. *Anopheles oswaldoi* B, with the highest haplotype number, was the most incongruous. This species was subdivided into five subgroups by ABGD, six subgroups by mPTP and seven subgroups by bGMYC (**Fig. 8**).

The species tree (**Fig. 9**) using the concatenated data (*COI* + ITS2), presented a topology different from that recovered only with the *COI* gene, with the exception of *An. oswaldoi* s.s. and *An. oswaldoi* B that remained as sister-taxa (BPP = 72). The main difference in topology was observed in relation to *An. oswaldoi* A. This species was recovered as a sister-taxon of *An. konderi* (BPP = 78), whereas *An. sp. nr. konderi* was recovered as a sister-taxon of *An. oswaldoi* A + *An. konderi* s.s. (**Fig. 8**).

Molecular dating estimated a divergence time of ~ 2.8 million years for the first diversification event, with more recent radiation (~ 1.2 million years) for *An. oswaldoi* s.s., *An. oswaldoi* B, *An. oswaldoi* A and *An. konderi* s.s. Finally, *Anopheles ininii*, was recovered as a sister-taxon of the clade formed by *An. oswaldoi* A + *An. konderi* + *An. sp.*

nr. *konderi*, but only with data from the *COI* gene (**Fig. 9**). Based on this data, the Oswaldoi-Konderi complex is paraphyletic, if we do not consider *An. ininii* within the Oswaldoi-Konderi complex.

Discussion

Anopheles oswaldoi sensu stricto was recently redescribed Motoki et al.[24], and the ITS2 sequences were provided by Motoki et al. [24]. This redescription allowed morphological comparisons and the association with *COI* barcode sequences provided by the present study.

Anopheles oswaldoi B probably may correspond to *An. aquacaelestis* Curry (Panama), the only synonym for *An. oswaldoi* s.s. In the present study, the characters of the genitalia were compared with the illustrations provided by Curry [43]. Although few characters were analyzed in the present study, they showed morphological similarities with *An. oswaldoi* B. However, we emphasize that, to validate this species, resurrecting from synonymy, it is necessary to examine the type of *An. aquacaelestis* material, which we did not have access for examination in this study.

The specimens collected in the type locality of *An. konderi* (Coari - AM) showed strong morphological variations, which also reflected in divergent sequences for the *COI* and ITS2 markers. These variations were also observed in the material deposited by Flores-Mendoza et al. [19], which suggest that this material was formed by a miscellany of at least two distinct forms, them named of *An. konderi*. To compare the identity of *An. konderi* sensu stricto, the characters of the only deposited neotype, which fixes the species identity, were considered. The material consisted of the adult male, male genitalia, exuvia pupa and exuvia larva, all corresponding to the same individual, the neotype of *An.*

konderi. Based on these morphological comparisons, it was possible to establish the identity of *An. konderi* s.s. and the species near to *An. konderi*.

The six species were delimited based on the morphological criteria, whereas from 14 to 18 MOTUs were recovered based on the *COI* and *ITS2* markers in the Oswaldoi-Konderi complex. Despite the under-structuring of MOTU's, with molecular data from the *COI* marker, we observed that these data were congruent with the morphological data between species; *A. oswaldoi* s.s., *An. oswaldoi* B, *An. oswaldoi* A, *An. konderi* s.s., *An. sp. nr. konderi* and *An. ininii*, as other studies have observed [12, 13]. The morphological differences presented in the present study had not yet been analyzed in the redescription of *An. konderi* s.s. [19] and *An. oswaldoi* s.s. [24], and therefore, provide additional characters to these studies.

Anopheles oswaldoi s.l. and *An. konderi* s.l., although they were morphologically separated, the differences were minimal, and restricted to the characters of the male genitalia. In the present study, in addition to characters in the male genitalia, characters from the immature phase, such as, clipeal setae and 4th stage larvae. The latter also observed among *An. konderi* s.s. and *An. sp. nr. konderi*. Adult females of the five species of the complex are isomorphic, and therefore it is not possible to separate them at this stage, except for *An. Ininii*, being considered cryptic species [16].

The morphological characters of the male genitalia, especially the differences in the ventral claspette, refringent structure and apex of the aedeagus between *An. oswaldoi* B and *An. oswaldoi* s.s. were studied by Sallum et al. [23]. However, these characters have only now been analyzed for the five species of the Oswaldoi-Konderi complex, enabling the diagnosis of these species.

Little is known about the bionomics of *An. ininii*, particularly in the adults. Information on larval habitat was recorded by Reinert [44] in specimens collected in state

of Pará [44]. The adult females of *An. oswaldoi* and *An. ininii* are similar in all characters, except in the 4th palpomere and dark band markings in the anterior and middle tarsomere. The difference between *An. ininii* and *An. oswaldoi* s.l. occurs by a single step in the dichotomous key of adult females, being similar for the other morphological characters, including the markings of the wings [45]. On the other hand, the characters of the male genitalia, especially the ventral claspete of *An. ininii* are fused and tapered at the apex, while in *An. oswaldoi* s.l. and *An. konderi* s.l. the ventral claspete is truncated and does not merge at the apex, forming a medial groove [46]. Another species close to *An. ininii* is *An. sanctielii* Senevet & Abonnec [47], which was described in French Guiana and never was registered in Brazil. This species differs in that the ventral claspete is strongly truncated and fused at the apex and the large lobes at the base [46].

Within the Oswaldoi-Konderi complex, the differences in the male genitalia were also evident when analyzing the ventral claspete and the apex of the aedeagus. Previous studies [19, 24] of description and redescription of *An. konderi* s.s. and *An. oswaldoi* s.s. indicated differences in the presence and absence of lateral leaflets and the ratio between length and width at the apex of the aedeagus. For example, *An. konderi* presents the lateral leaflets and the apex of the aedeagus with a width greater than the length [19], while in *An. oswaldoi* s.l. the apex is longer than wide and without lateral leaflets [24].

The male genitalia have been widely used in the taxonomic determination of species of the genus *Anopheles*. For example, Sallum et al. [48] resuscitated from the synonym *An. albertoi* and *An. arthuri*, from the Strodei complex, based on differences in the male genitalia. Sant'Ana and Sallum [49] described *An. striatus* (formerly = *Anopheles* form CP) of the Strodei complex from the individuals collected in Foz do Iguaçu, Paraná. These authors, initially revealed differences in the male genitalia and later these differences were confirmed with molecular markers *COI* and *White* genes [50].

Anopheles goeldii, belonging to the Nuneztovari complex, is also identified, and differentiated from other species (*An. nuneztovari* s.s., *An. dunhami* and *An. nuneztovari* A) through the male genitalia [51–53]. Recently, dos Santos et al. [53] proposed that morphological differences in the length of the lateral leaflets of theedeago, can determine the four species of the Nuneztovari complex.

Male terminalia has been widely used for species recognition and for separating categories at the generic and subgeneric levels, providing a strong basis for phylogenetic information in cladistic analyses within Diptera [54] and also in other groups of insects [55]. The fact is that the male genitalia provides intuitive morphological bases to support mechanisms of reproductive isolation, through incompatibility between the genitalia, based on the "key-lock" hypothesis [56]. On the other hand, other hypotheses have also been used to explain these divergences, such as pleiotropy [16, 57], gametic competition [58], sexual conflict [59, 60] and sexual selection [60, 61]. However, more recent studies suggest that these differences in genital morphology may contribute to reproductive isolation in less obvious ways, through interactions with sensory mechanisms that result in less reproductive fitness in heterospecific mating [56]. In Lepidoptera, forced laboratory crossings between several related genera have generally shown no evidence in favor of the "Key-Lock" structural hypothesis preventing successful insemination, despite the fact that the male genital has drastically different morphology between species [62]. For example, among the species *Erebia nivalis* × *E. cassioides*, which form a hybrid zone along a gradient in the Alps, at the specific crossing, the male, courts the female before, and the copulation lasts ~ 18 to 30 minutes, while in heterospecific crosses, when the female remains receptive to the crossing, copulation lasts ~ 0.5 to 7 minutes [62]. On the other hand, more recent studies have highlighted the bias in the study only of male genitalia, the results highlight the speed with which the female genital traits can evolve

quickly and with a complex coevolutionary dynamic between the two sexes, shaping male genital structures [63].

Regarding molecular data, the delimitation inferences were congruent, despite the recovery of a high number of operational taxonomic units (OTU) delimited by the three methods - ABGD strict, mPTP and bGMYC. These values were consistent with the number of species supported by morphology (**Fig. 8**) for the five species. Given the rapid and recent divergence inferred for the complex (**Fig. 9**), it is not surprising that more possible strains have been inferred (ABGD strict = 14 OTU's; mPTP = 16 OTU's; bGMYC = 18 OTU's), than the five recognized species. Although species delimitation analyses are widely used to discover enigmatic species, it is not uncommon for these delimiters to generate irrational delimitations with wide confidence intervals and without reflecting relationships with entities in molecular sampling [64]. However, these models are important in the discovery of cryptic species. In addition, the distance-based methods are more conservative, compared to the method based on the Bayesian model (bGMYC) which recovered 18 OTU's, a flexible estimate of alleged OTU's. This result may reflect the high rates of substitutions (mutations), uneven sampling (inclusion of identical sequences), variation in the size of populations between species, gene flow and trees with unresolved topology [65–67].

The Oswaldoi-Konderi complex, as shown in this study and in previous studies [12, 13] is widely distributed in the Amazon basin. Except for *An. oswaldoi* A and *An. konderi* s.s. which had occurrences both in the north and south of the Amazon River, a more restricted pattern was observed in the other species. *An. oswaldoi* s.s. it occurred just west of the Amazon; *An. oswaldoi* B was restricted to the far north, in addition to being registered in other countries, such as Colombia [12, 68] and Venezuela [69]. *Anopheles* sp. nr. *konderi* was registered mainly to the south of the Amazon River, except

for a single population to the north (Novo Remanso - AM). Finally, *Anopheles ininii*, which was included in the Oswaldoi-Konderi complex, was collected only in Novo Progresso (PA).

Retrospective studies have notified *An. oswaldoi* s.l. infected in Brazil [2, 27–29, 70–73], in Colombia [68], in Peru [74], in Venezuela [75] and in French Guiana [76]. Ruiz-Lopez et al. [12], based on occurrences of infection by *Plasmodium* spp. and the distribution of the species of the Oswaldoi-Konderi complex, indicated that *An. oswaldoi* A, *An. oswaldoi* B and *An. sp. nr. konderi* could be involved in the transmission of malaria. Later, Saraiva et al. [13], sampled a wider area in the Brazilian Amazon and revealed five areas of sympathy where *An. oswaldoi* s.l. was found to be infected, further obscuring the incrimination of the vector within the Oswaldoi-Konderi complex. In this study, this result was maintained, where the samples collected in locations where *An. oswaldoi* s.l. has been reported infected allow to suggest that only *An. oswaldoi* B and *An. oswaldoi* A may be the species involved in the transmission of the malaria parasite.

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Contributions

VMS conceived the study. JFS performed the specimen collection and laboratory experiments. JFS and VMS performed the statistical analysis. JFS and VMS, wrote the paper. All authors read and approved the final manuscript.

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Ethics declarations

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

List of tables:

Table 1. Sampling sites, number of mosquitoes analyzed and geographical coordinates of the species of the Oswaldoi-Konderi complex in the Brazilian Amazon.

Locality	Morphology	DNA Barcode	ITS2	Coordinates	
				Latitude	Longitude
Acre					
Rio Branco		5	3	-9.981839	-67.782761
Rio Branco, Transacreana		2	2	-10.487778	-68.096372
Sena Madureira		1	1	-9.058347	-68.648772
Amapá					
Calçoene, Lourenço	1*	1	1	2.453581	-51.270192
Ferreira Gomes, Paredão	1*	3		0.835556	-51.208722
Macapá, Farm. Santa Barbara	1*	2	1	0.291222	-50.902000
Macapá, Mata Fome	1*	4	3	0.212639	-50.972694
Mazagão, Vila Carvão	6*	6		-0.199603	-51.363583
Santana, Ilha	3*	3		-0.082264	-51.183981
Serra do Navio, Pedra preta	2*	4	3	0.897622	-52013100
Tartarugalzinho	1*	2	1	1.516417	-50.917139
Amazonas					
Autazes, Sítio Alencar	6**	11	1	-3.699167	-59.131861
Borba, Vila Gomes	12**	2	2	-4.434569	-59.878103
Coari, Igarapé do Isidoro		5	3	-0.083054	-63.133049
Coari, Sítio Tarésio	9**	6	6	-0.083056	-63.133056
Lábrea, Aldeia Palmari	8*	12	1	-7.664528	-65.069750
Manacapuru, AM070	1*	1		-3.268331	-60.653878
Nova Olinda do Norte, AM254	1*	1	1	-3.836944	-59.021500
Novo Remanso, Correnteza	4**	5		-1.002000	-57.126556
Pres. Figueiredo, Pitinga	3**	4	3	-0.782389	-60.060472
Pará					
Anajás, Açaítuba	1*	1		-0.978544	-49.936642
Novo Progresso, Mina Palito	12*	26		-6.434492	-55.951000
Oriximiná, Serra do Cachorro	6*	7	7	-1.002000	-57.126556
Rondônia					
Porto Velho, Parque municipal		2	1	-8.682467	-63.857994
São Miguel		15	1	-0.143333	-63.816667
Roraima					
Amajari, ESEC Maracá	22**	45		3.381431	-61.489319
Canta, Carvoeiro	1**	1		2.575461	-60.633992
Caracaraí, Parna do Viruá	15**	15		1.482811	-61.110656
Caracaraí, Serra da Mocidade	1*	1		1.499906	-61.864533
Total		107	193	41	

* Morphological analysis of a nature specimen (Le, MG or F); ** represents the number of spawns.

Table 2. Values of Kimura 2-parameter (K2P) intra and interspecific genetic distance (K2P) for the *COI* gene, Barcode DNA region, for morphologically separated groups.

Species	1	2	3	4	5	6
1. <i>An. oswaldoi</i> s.s.	0.006					
2. <i>An. oswaldoi</i> A	0,053	0.018				
3. <i>An. oswaldoi</i> B	0,041	0,064	0.013			
4. <i>An. konderi</i> s.s.	0,040	0,050	0,057	0.018		
5. <i>An. sp. nr. konderi</i>	0,047	0,053	0,058	0,041	0.015	
6. <i>An. ininii</i>	0,065	0,071	0,076	0,061	0,072	0.011

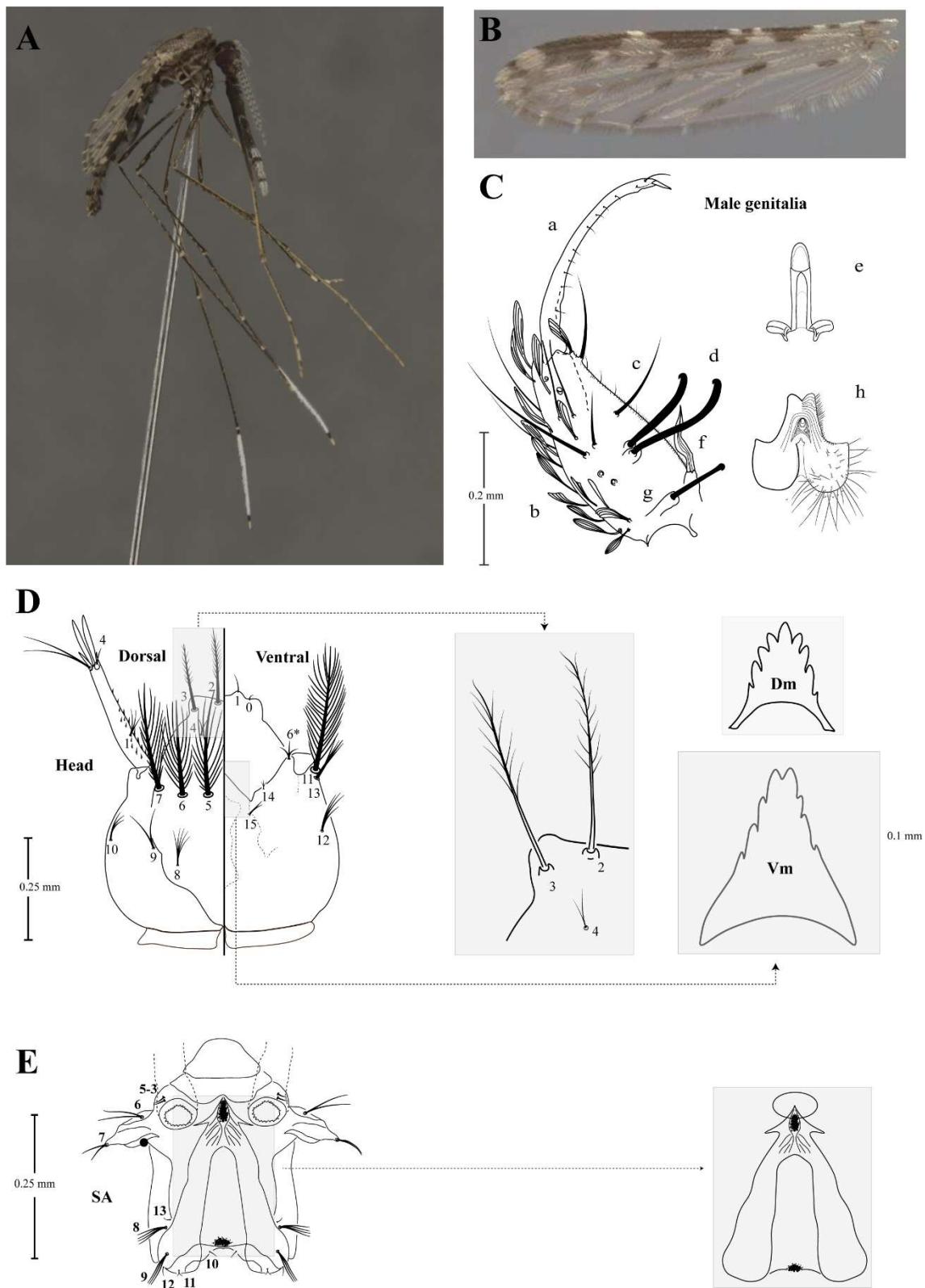


Figure 1. Diagnostic characters for *Anopheles oswaldoi* Sensu Stricto. A) Adult female habitus, B) Female left wing, C) Male genitalia, **a**; gonostylus, **b**; gonocoxite, **c**; internal seta, **d**; accessory setae, **e**; aedeagus, **f**; dorsal claspete, **g**; tubercle of parabasal spine, **h**; ventral claspete, D) 4th larva head, details for diagnosis, clypeal setae and **Vm**; ventromentum and **Dm**; dorsomentum, E) SA; spiracular apparatus, diagnostic details on the contour of the spiracular plate.

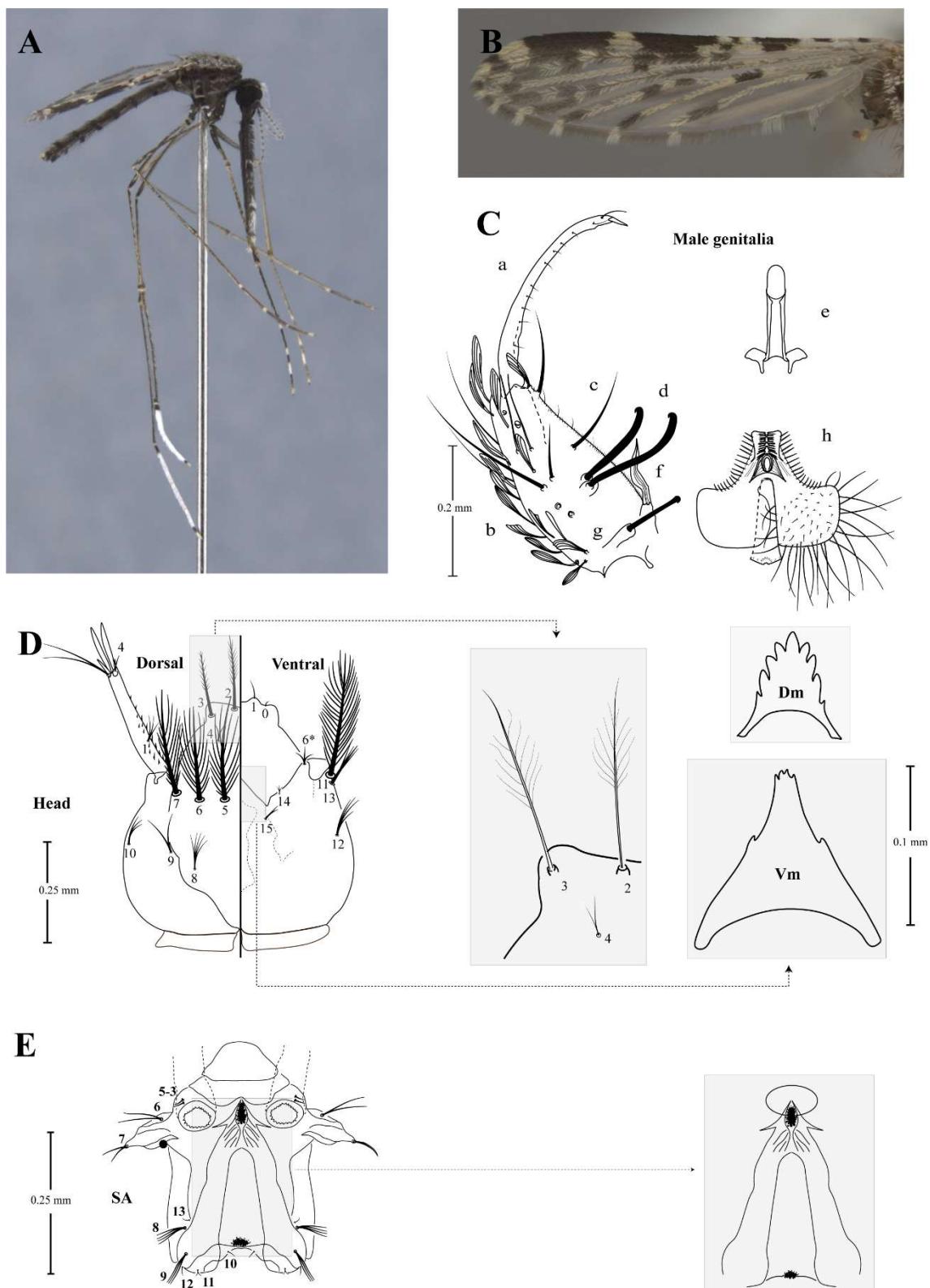


Figure 2. Diagnostic characters for *Anopheles oswaldoi* B. A) Adult female habitus, B) Female left wing, C) Male genitalia, a; gonostylus, b; gonocoxite, c; internal seta, d; accessory setae, e; aedeagus, f; dorsal claspete, g; tubercle of parabasal spine, h; ventral claspete, D) 4th larva head, details for diagnosis, clypeal setae and Vm; ventromentum and Dm; dorsomentum, E) SA; spiracular apparatus, diagnostic details on the contour of the spiracular plate.

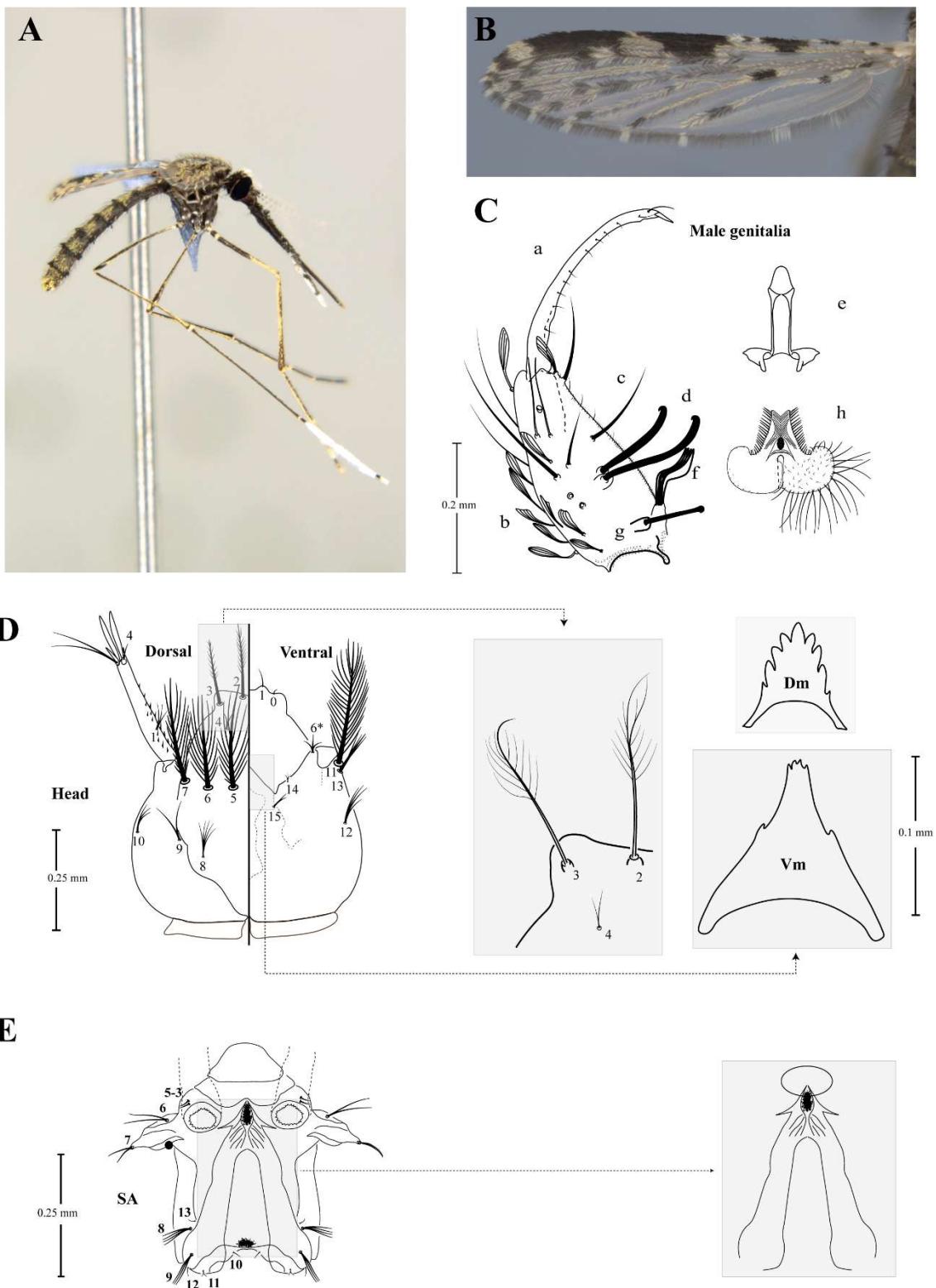


Figure 3. Diagnostic characters for *Anopheles oswaldoi* A. A) Adult female habitus, B) Female left wing, C) Male genitalia, **a**; gonostylus, **b**; gonocoxite, **c**; internal seta, **d**; accessory setae, **e**; aedeagus, **f**; dorsal claspete, **g**; tubercle of parabasal spine, **h**; ventral claspete, D) 4th larva head, details for diagnosis, clypeal setae and **Vm**; ventromentum and **Dm**; dorsomentum, E) SA; spiracular apparatus, diagnostic details on the contour of the spiracular plate.

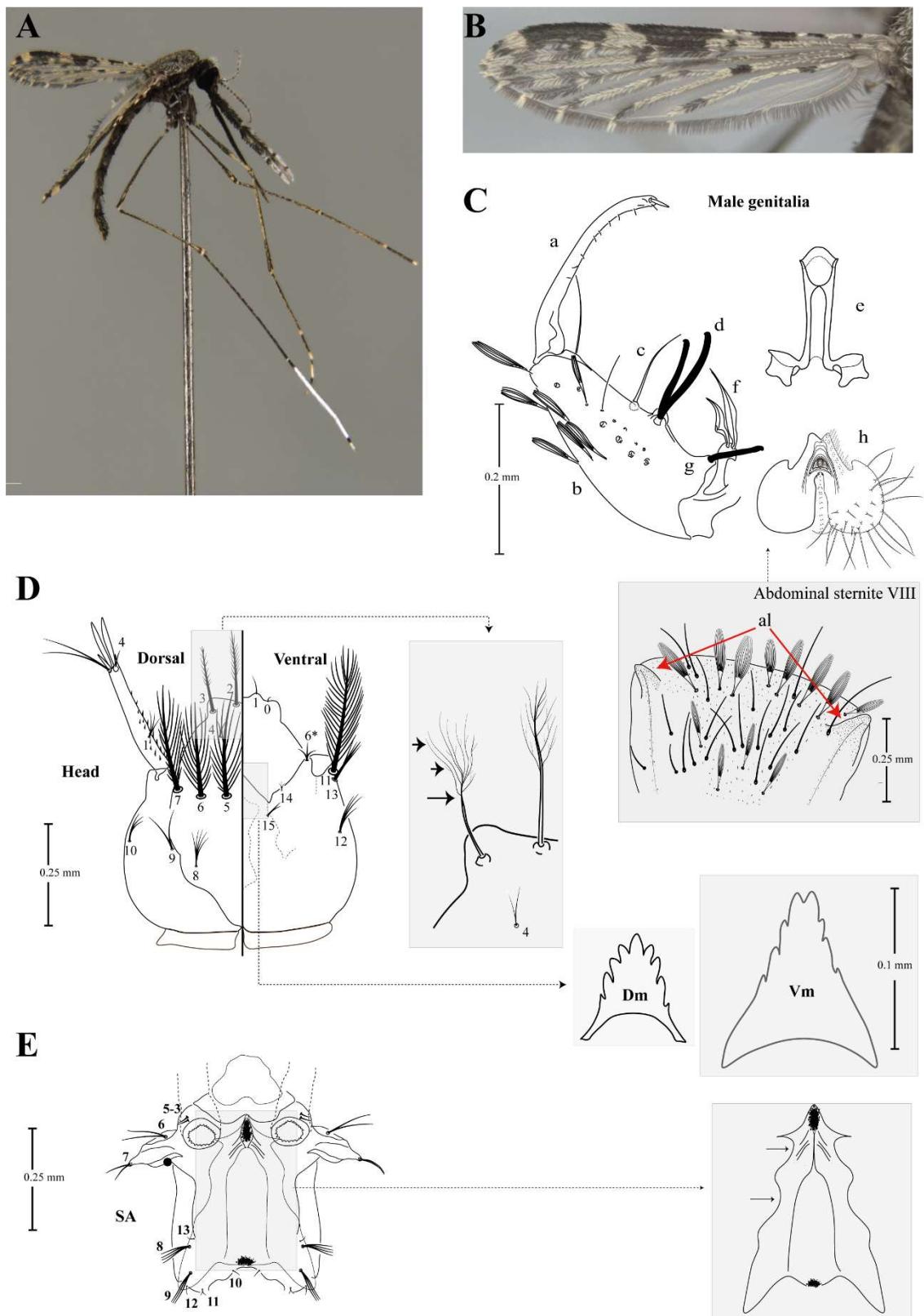


Figure 4. Diagnostic characters for *Anopheles konderi* Sensu Stricto. A) Adult female habitus, B) Female left wing, C) Male genitalia, a; gonostylus, b; gonocoxite, c; internal seta, d; accessory setae, e; aedeagus, f; dorsal claspete, g; tubercle of parabasal spine, h; ventral claspete, Abdominal sternite VIII, al; abdominal lobo. D) 4th larva head, details for diagnosis, clypeal setae and Vm; ventromentum and Dm; dorsomentum, E) SA; spiracular apparatus, diagnostic details on the contour of the spiracular plate.

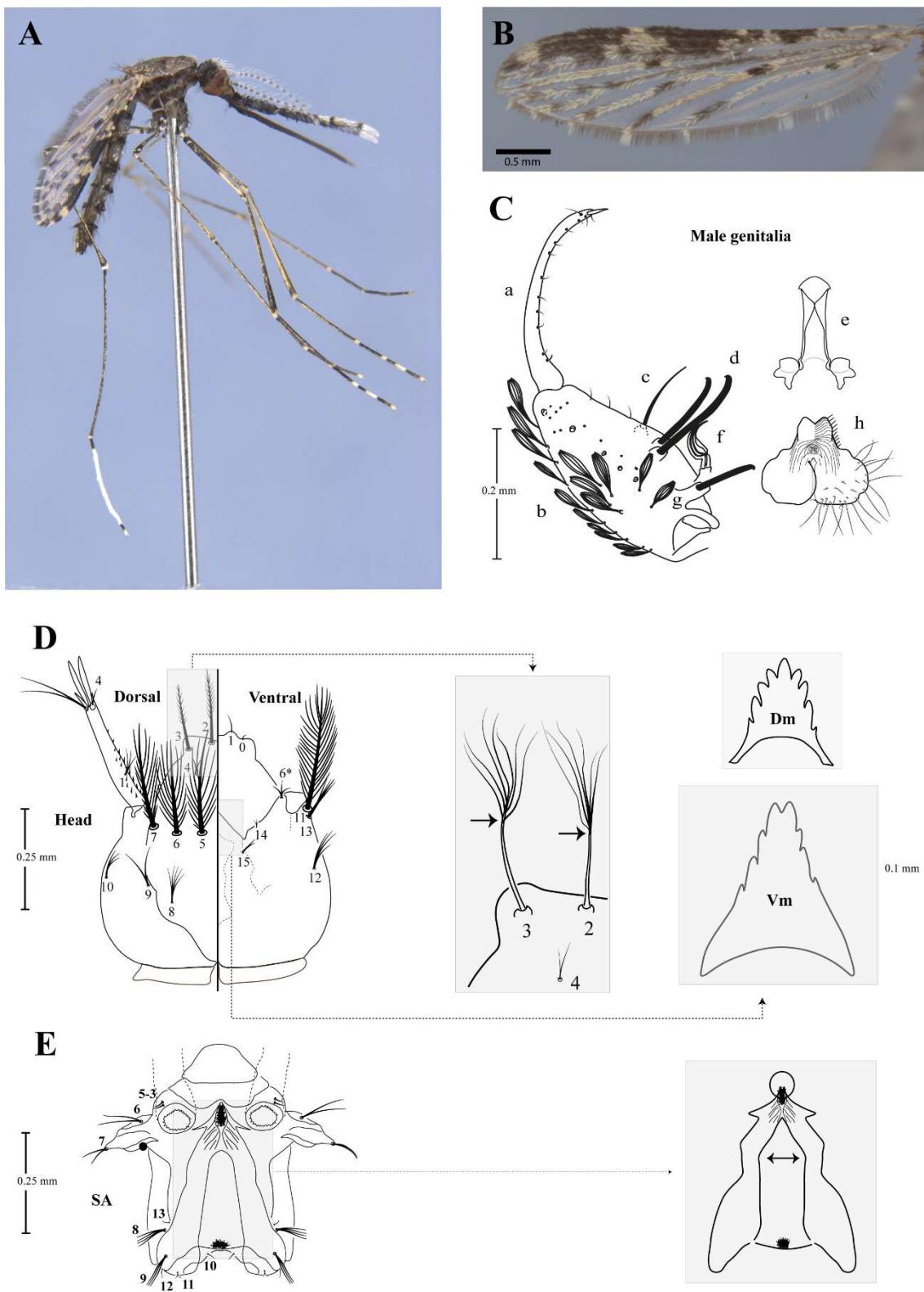


Figure 5. Diagnostic characters for *Anopheles* sp. nr. *konderi*. A) Adult female habitus, B) Female left wing, C) Male genitalia, **a**; gonostylus, **b**; gonocoxite, **c**; internal seta, **d**; accessory setae, **e**; aedeagus, **f**; dorsal claspete, **g**; tubercle of parabasal spine, **h**; ventral claspete, D) 4th larva head, details for diagnosis, clypeal setae and **Vm**; ventromentum and **Dm**; dorsomentum, E) SA; spiracular apparatus, diagnostic details on the contour of the spiracular plate.

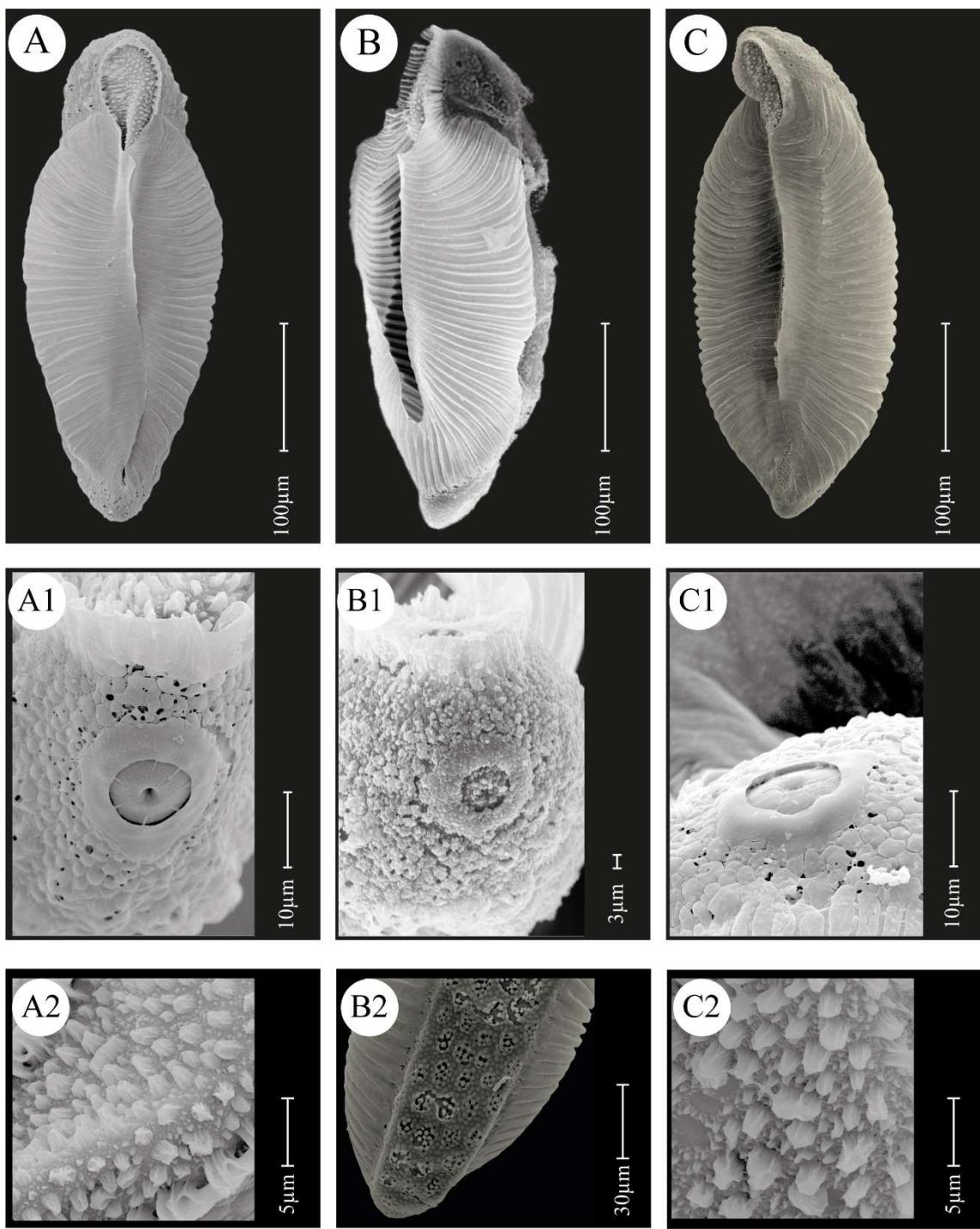


Figure 6. Ultrastructure of eggs for three species of the Oswaldoi-Konderi complex. *Anopheles oswaldoi* A (A, A1 and A2), *An. oswaldoi* B (B, B1 and B2) and *An. konderi*. s.s. (C, C1 and C2). A, B and C. Habitus eggs; A1, B1 and C1. details of the micropyle; A2 and C2. detail anterior deck tubercles; B2. detail of chorionic cells, dorsal plastron. Scales in micrometers (µm).

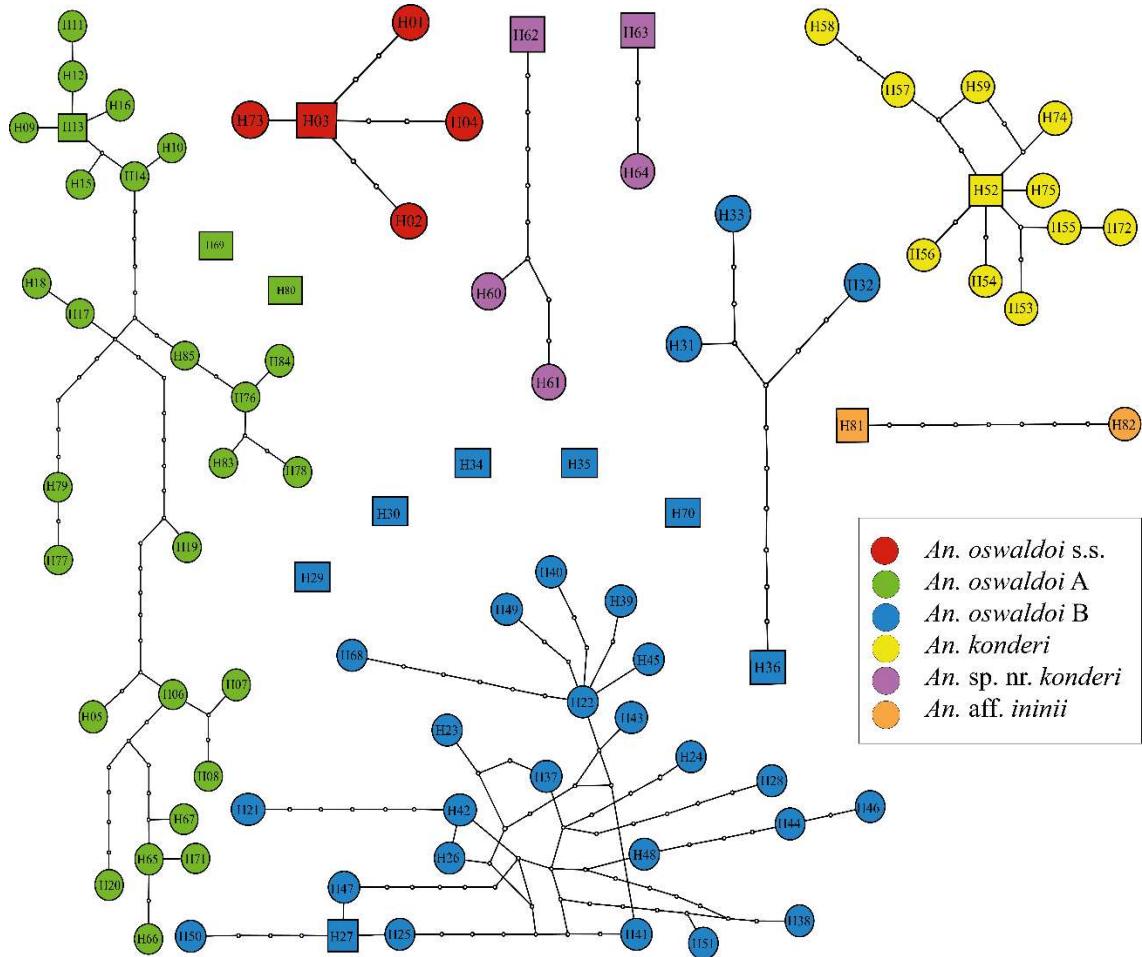


Figure 7. Haplotypes network based on parsimony and 95% confidence limit of connectivity. The species are represented by different colors for better visualization of the groups. The square polygons represent the simulation of the ancestral haplotype, the colored circles represent the recovered haplotypes.

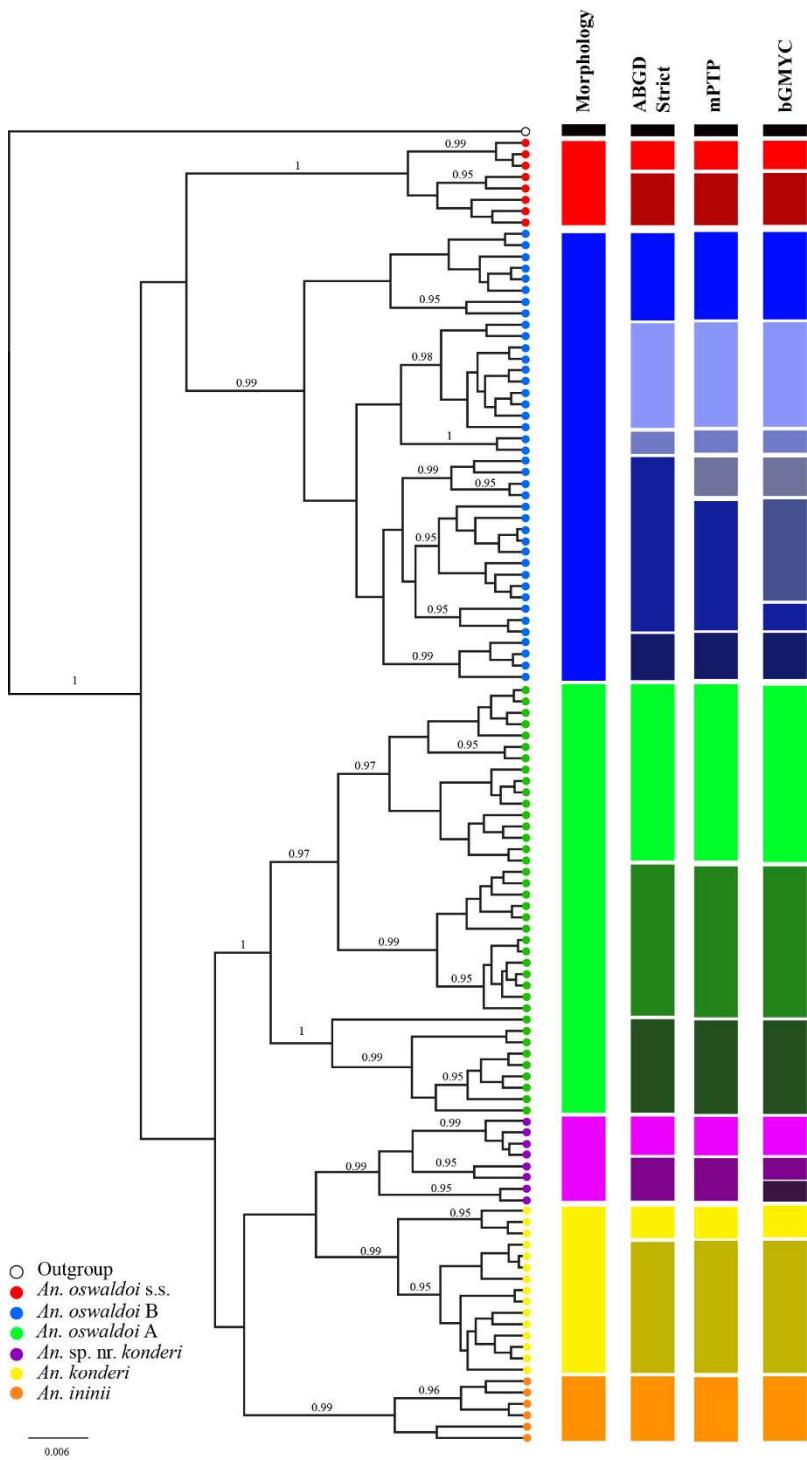


Figure 8. Topology of the Bayesian Inference tree and species delimitation analysis. The consensus tree (HKY + I + G) of the *COI* gene. The support of the branches represents the posterior probability ($\geq 70\%$). The terminal symbols in the tree are the 116 haplotypes recovered, including *An. ininii*. As an external group, *An. marajoara* was included. The first colored vertical bar shows the delineation based on characters of the male genitalia (ventral claspete and apex of the aedeagus). The next three bars are species delimitations based on genetic data using the approaches: genetic distance, evolutionary models, and Bayesian inference, with ABGD strict, mPTP and bGMYC algorithms, respectively.

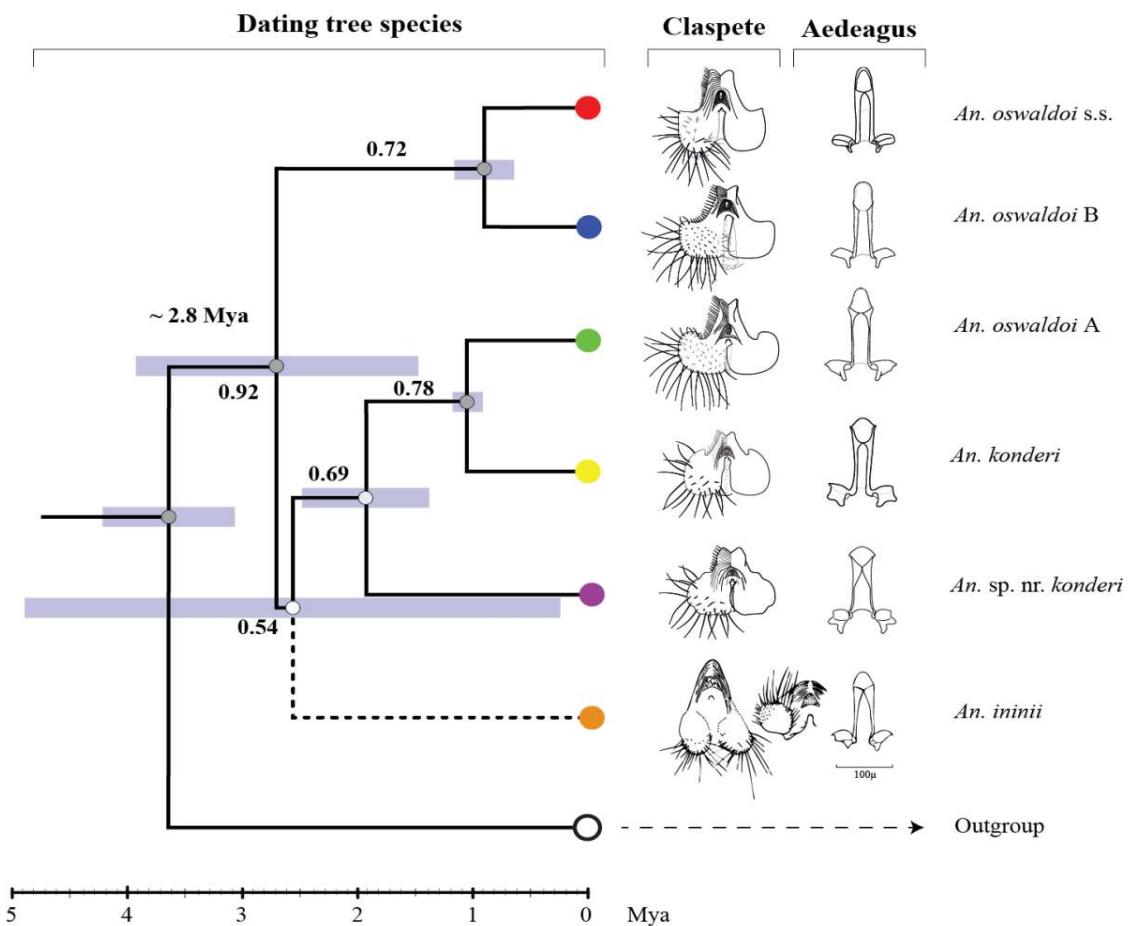


Figure 9. Tree of species, generated with maximum credibility, using multispecies coalescence model. Analysis performed with concatenated data (*COI* + *ITS2*). Using molecular dating for the *COI* gene, assuming a relaxed clock and a mutation rate of 2.3% every million years (Mya). Estimates of the average node divergence time are reported at 95% HPD intervals (higher posterior density) and are represented as bars in the nodes. The supports in the branches are indicated by a gray circle from 0.95BPP to 0.70BPP. Male genitalia (claspete and aedeagus) were illustrated for each species. The dotted branch of *An. ininii* was included only based on *COI* data.

SUPPLEMENTARY FILES

Supplementary table 1. List of haplotypes (*COI*), species and localities sampled for the Oswaldoi-Konderi complex in the Brazilian Amazon basin.

Hap.	nº	Species	Samples
H01	1	<i>An. oswaldoi</i> s.s.	Transacreana
H02	3	<i>An. oswaldoi</i> s.s.	Coari
H03	2	<i>An. oswaldoi</i> s.s.	Coari
H04	1	<i>An. oswaldoi</i> s.s.	Rio Branco
H05	1	<i>An. oswaldoi</i> s.s.	Rio Branco
H06	2	<i>An. oswaldoi</i> A	Pitinga
H07	2	<i>An. oswaldoi</i> A	Pitinga (1); Calçoene (1)
H08	1	<i>An. oswaldoi</i> A	Pitinga
H09	1	<i>An. oswaldoi</i> A	Serra do Cachorro
H10	1	<i>An. oswaldoi</i> A	Serra do Cachorro
H11	2	<i>An. oswaldoi</i> A	Lábrea
H12	1	<i>An. oswaldoi</i> A	Lábrea
H13	1	<i>An. oswaldoi</i> A	Lábrea
H14	1	<i>An. oswaldoi</i> A	Lábrea
H15	1	<i>An. oswaldoi</i> A	Lábrea
H16	2	<i>An. oswaldoi</i> A	Lábrea
H17	1	<i>An. oswaldoi</i> A	Lábrea
H18	1	<i>An. oswaldoi</i> A	Lábrea
H19	1	<i>An. oswaldoi</i> A	Lábrea
H20	2	<i>An. oswaldoi</i> A	Lábrea (1); Coari (1)
H21	1	<i>An. oswaldoi</i> A	Coari
H22	1	<i>An. oswaldoi</i> A	Coari
H23	2	<i>An. oswaldoi</i> A	Mata Fome (1); Anajás (1)
H24	1	<i>An. oswaldoi</i> A	Santa Barbara
H25	1	<i>An. oswaldoi</i> A	Serra do Cachorro
H26	1	<i>An. oswaldoi</i> A	Serra da Mocidade
H27	3	<i>An. konderi</i> s.s.	Viruá
H28	1	<i>An. konderi</i> s.s.	Viruá
H29	1	<i>An. konderi</i> s.s.	Viruá
H30	7	<i>An. konderi</i> s.s.	Viruá (2); Autazes (3); Borba (2)
H31	1	<i>An. konderi</i> s.s.	Viruá
H32	2	<i>An. konderi</i> s.s.	Viruá
H33	2	<i>An. oswaldoi</i> A	Novo Progresso
H34	1	<i>An. oswaldoi</i> A	Novo Progresso
H35	1	<i>An. oswaldoi</i> A	Novo Progresso
H36	2	<i>An. oswaldoi</i> A	Novo Progresso
H37	1	<i>An. oswaldoi</i> A	Novo Progresso
H38	1	<i>An. oswaldoi</i> A	Novo Progresso
H39	1	<i>An. oswaldoi</i> A	Novo Progresso
H40	1	<i>An. oswaldoi</i> A	Novo Progresso

H41	1	<i>An. oswaldoi</i> A	Novo Progresso
H42	1	<i>An. oswaldoi</i> A	Novo Progresso
H43	2	<i>An. oswaldoi</i> A	Novo Progresso
H44	1	<i>An. oswaldoi</i> A	Novo Progresso
H45	1	<i>An. oswaldoi</i> A	Novo Progresso
H46	2	<i>An. oswaldoi</i> A	Novo Progresso
H47	2	<i>An. oswaldoi</i> A	Novo Progresso
H48	2	<i>An. oswaldoi</i> B	ESEC Maracá
H49	5	<i>An. oswaldoi</i> B	ESEC Maracá
H50	2	<i>An. oswaldoi</i> B	ESEC Maracá
H51	1	<i>An. oswaldoi</i> B	ESEC Maracá
H52	4	<i>An. oswaldoi</i> B	ESEC Maracá
H53	1	<i>An. oswaldoi</i> B	ESEC Maracá
H54	3	<i>An. oswaldoi</i> B	ESEC Maracá
H55	1	<i>An. oswaldoi</i> B	ESEC Maracá
H56	1	<i>An. oswaldoi</i> B	ESEC Maracá
H57	1	<i>An. oswaldoi</i> B	ESEC Maracá
H58	2	<i>An. oswaldoi</i> B	ESEC Maracá
H59	1	<i>An. oswaldoi</i> B	ESEC Maracá
H60	1	<i>An. oswaldoi</i> B	ESEC Maracá
H61	1	<i>An. oswaldoi</i> B	ESEC Maracá
H62	1	<i>An. oswaldoi</i> B	ESEC Maracá
H63	1	<i>An. oswaldoi</i> B	ESEC Maracá
H64	1	<i>An. oswaldoi</i> B	ESEC Maracá
H65	1	<i>An. oswaldoi</i> B	ESEC Maracá
H66	1	<i>An. oswaldoi</i> B	ESEC Maracá
H67	1	<i>An. oswaldoi</i> B	ESEC Maracá
H68	1	<i>An. oswaldoi</i> B	ESEC Maracá
H69	1	<i>An. oswaldoi</i> B	ESEC Maracá
H70	1	<i>An. oswaldoi</i> B	ESEC Maracá
H71	1	<i>An. oswaldoi</i> B	ESEC Maracá
H72	1	<i>An. oswaldoi</i> B	ESEC Maracá
H73	1	<i>An. oswaldoi</i> B	ESEC Maracá
H74	1	<i>An. oswaldoi</i> B	ESEC Maracá
H75	1	<i>An. oswaldoi</i> B	ESEC Maracá
H76	1	<i>An. oswaldoi</i> B	ESEC Maracá
H77	1	<i>An. oswaldoi</i> B	ESEC Maracá
H78	1	<i>An. oswaldoi</i> B	ESEC Maracá
H79	1	<i>An. oswaldoi</i> B	ESEC Maracá
H80	1	<i>An. oswaldoi</i> B	ESEC Maracá
H81	1	<i>An. oswaldoi</i> B	Cantá
H82	5	<i>An. oswaldoi</i> B	Ferreira Gomes (1); Tartarugalzinho (1); Serra do Navio (3)
H83	1	<i>An. oswaldoi</i> B	Ferreira Gomes
H84	1	<i>An. oswaldoi</i> B	Serra do Navio
H85	1	<i>An. oswaldoi</i> B	Ferreira Gomes

H86	1	<i>An. oswaldoi</i> B	Viruá
H87	1	<i>An. oswaldoi</i> B	Viruá
H88	1	<i>An. oswaldoi</i> B	Tartarugalzinho
H89	10	<i>An. konderi</i> s.s.	Santa Barbara (1); Ilha de Santana (3) Mata Fome (3) Carvão (2); Manacapuru (1)
H90	3	<i>An. konderi</i> s.s.	Autazes
H91	3	<i>An. konderi</i> s.s.	Autazes
H92	2	<i>An. konderi</i> s.s.	Autazes
H93	17	<i>An. konderi</i> s.s.	São Miguel (14); Serra do Cachorro (3)
H94	2	<i>An. konderi</i> s.s.	São Miguel (1); Porto velho (1)
H95	1	<i>An. konderi</i> s.s.	Serra do Cachorro
H96	1	<i>An. konderi</i> s.s.	Carvão
H97	1	<i>An. konderi</i> s.s.	Carvão
H98	1	<i>An. konderi</i> s.s.	Carvão
H99	1	<i>An. konderi</i> s.s.	Carvão
H100	1	<i>An. konderi</i> s.s.	Novo Progresso
H101	2	<i>An. sp. nr. konderi</i>	Rio Branco
H102	1	<i>An. sp. nr. konderi</i>	Porto Velho
H103	6	<i>An. sp. nr. konderi</i>	Nova Olinda do Norte (1); Novo Remanso (3); Coari (1); Viruá (1)
H104	1	<i>An. sp. nr. konderi</i>	Transacreana
H105	2	<i>An. sp. nr. konderi</i>	Sena Madureira (1); Rio Branco (1)
H106	1	<i>An. sp. nr. konderi</i>	Coari
H107	1	<i>An. sp. nr. konderi</i>	Novo Remanso
H108	1	<i>An. sp. nr. konderi</i>	Novo Remanso
H109	1	<i>An. oswaldoi</i> s.l.	Viruá
H110	1	<i>An. oswaldoi</i> s.l.	Coari
H111	1	<i>An. oswaldoi</i> s.l.	Coari
H112	1	<i>An. ininii</i>	Novo Progresso
H113	1	<i>An. ininii</i>	Novo Progresso
H114	1	<i>An. ininii</i>	Novo Progresso
H115	1	<i>An. ininii</i>	Novo Progresso
H116	1	<i>An. ininii</i>	Novo Progresso

Supplementary table 2. Parsimony informational sites for MOTUs (Molecular Operational Taxonomic Units) obtained with molecular data from the *COI* gene barcode (mitochondrial DNA), detected for a database of 193 mosquito sequences of the Oswaldoi-Konderi complex, of the Brazilian Amazon.

[11111111222222222223333333333333344]
[4455567899913477899011222457779223345556677889900]
[28178687069124179281034258384695473681470328140358]
An. oswaldoi s.s.	AAAATTTAATTAAACAACTTATTATAACTTTAATTATTTT
An. oswaldoi A [H05]	...TC.CAC.....T..T..C.C.....CT..C.....C
An. oswaldoi A [H69]	...TC.CG.....T..T..C.C..C.GCG..C.....C
An. oswaldoi A [H80]	..GTCC..C.....T..T.G.CC.....C..T.C.....G.C..C
An. oswaldoi B [H21]C.G.T.C.....T....G.C.....T....CC.C....
An. oswaldoi B [H29]G.T.C....GG.T..T..C.C..G..TC....CCG....
An. oswaldoi B [H30]	G.....G.G.C....G.T.....C.C..G..T.....CC....
An. oswaldoi B [H34]G.C.C.....T.....C.C..G..T..C..CC..G....
An. oswaldoi B [H35]	..G.....C.T.....GGT.TT.G.C..CC.A.G.C..T.A.GA...C
An. oswaldoi B [H70]	.G.....G.....G.T.....C.C.....T.....CC.....
An. oswaldoi B [H36]G.T.C.....G.T.....C.C..G.....C..C.....
An. sp. nr. konderi [H60]CC.....T..T..CC..CCG..T.....T....C
An. sp. nr. konderi [H63]C.....G..T..T..CC..G.CG..T.....T.....T....
An. konderi s.s. [H52]G...CC..C.....TG.T...C..G.C....G.C.....
An. ininii [H81]	.T.....C..TC.CAA.G.TGTT..CC..C.G.....TG.C..ACCC
[44444444444444455555555555555566666]
[1223445577889900113344566677788901122]
[1036143917063878061747015703626795814]
An. oswaldoi s.s.	CAATATTTGTATTTATTTTTGTATTCTATTC
An. oswaldoi A [H05]	T.G..C..CA.....A.GC..T.....
An. oswaldoi A [H69]	TGG..C..CA.....A.GC..T.....T
An. oswaldoi A [H80]	T.G..C..A.....A.G..T.....T
An. oswaldoi B [H21]	T.G.....A.GC.A.....C..A.....TC.T
An. oswaldoi B [H30]	T.G.G....A.GC.A....C..C..A.....TC.T
An. oswaldoi B [H34]	T.G.....GC.A.....C.CA.....TC.T
An. oswaldoi B [H35]	TTG...C..A..C..A..C..A.AA.A.CT.T.CT
An. oswaldoi B [H70]	T.G.....A.GC.A.....C.CA.G.....TC.T
An. oswaldoi B [H36]	T.G.....GC.A.....C.CA.G.....TC..
An. sp. nr. konderi [H60]	T..C.....A....A.G..C.....A..A.T...CT
An. sp. nr. konderi [H63]	T..C....CCA....A.G..C....A.G.A.T...CT
An. konderi s.s. [H52]	T.....A..C.....C.....A..CT.....
An. ininii [H81]C...AG.....C...AAGAC.T.....

Supplementary table 3. Parsimony informational sites for MOTUs (Molecular Operational Taxonomic Units) obtained with molecular data from the ITS 2 marker (Second Transcribed Internal Spacer - ribosomal DNA), detected for a database of 33 mosquito sequences of the Oswaldoi-Konderi complex, in the Amazon Brazilian.

[122222333333344444444]
[506889113556900112346]
[790282349260539465420]
<i>An. oswaldoi s.s.</i> [H01]	ACTC-AACACCATCGTGGAC-
<i>An. oswaldoi s.s.</i> [H02]	CTC-A.CAC.ATC.CG.---
<i>An. oswaldoi A</i> [H03]	GC.....A.C.A...GA.
<i>An. oswaldoi A</i> [H04]	G..A.....A.C.A...GA.
<i>An. oswaldoi B</i> [H05]	.T..A..-AGCC.A.CA..A
<i>An. konderi</i> [H06]	.TAAAG....A.CTA...GAA
<i>An. konderi</i> [H07]	.T..A.....A.CTA...GAG
<i>An. sp. nr. konderi</i> [H08]	.TAAAGG...A.CTA...GAA
<i>An. sp. nr. konderi</i> [H09]	.T.AAG....A.C.A...GA.
<i>An. sp. nr. konderi</i> [H10]	.TAAAGG....A.C.A.....A
<i>An. sp. nr. konderi</i> [H11]	.TAAGG..T.A.C.A.....

Capítulo 2

***Anopheles (Nyssorhynchus) tadei*: A New Species of the Oswaldoi-konderi complex (Diptera, Culicidae, Anophelinae), morphological and molecular comparisons with *An. konderi* sensu stricto**

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***Anopheles tadei* não é um nome válido nesta publicação.**

Abstract

Currently, the Oswaldoi-Konderi complex (*Anopheles*, *Nyssorhynchus*) is composed of five species, distinguished, and delimited by molecular markers. Of these, two species have been formally described, *Anopheles oswaldoi* s.s. and *An. konderi* s.s.; however, *An. konderi* was considered a synonymy of *An. oswaldoi* for many years and in 2004 it was resurrected from *An. oswaldoi*. However, the true identity of *An. konderi* s.s. remains unclear, because in the resurrection study of *An. konderi* s.s. was included a mixture of the two morphotypes from Coari, state of Amazonas, Brazil (its type locality). Subsequently, molecular studies indicated that more species could exist within *An. konderi*. This study aimed to establish the identity of *An. konderi* s.s. and to describe the new species near *An. konderi* based on the morphological and molecular data. For this, six wild-caught females were collected in the municipality of Coari and separated individually to lay eggs. The progenies (F₁) were reared to obtain all stages of development and then the eggs, larva, pupa, and adult (male and female) were used for morphological characterization. In addition, the *An. konderi* s.s. neotype material was re-examined and compared with the material of new species. One 4th instar larvae of each progeny were also used in the molecular analyses with three markers: mtDNA *COI* barcode region, *CAD* gene and ITS2 rDNA nuclear region. The findings of the morphological and molecular analyses clearly showed two distinct groups. The first group was identical to the material of the neotype *An. konderi* s.s. and the second belonged to specimens that informally is named of the *An.* sp. near *konderi* or *An. konderi* B, which it is described in the present study as *Anopheles tadei* n. sp.

Key words: Brazilian Amazon region, Cryptic species, Molecular markers, Speciation.

Introduction

Anopheles konderi Galvão & Damasceno (1942) was originally described based on the unique form of the aedeagus male specimens collected from the south bank of Solimões river, municipality of Coari, State of Amazonas, Brazil (Galvão & Damasceno 1942). This species is morphologically very similar to *An. oswaldoi* (Peryassú 1922), which can only be differentiated by the shape of the apex of the aedeagus (Causey *et al.* 1946; Coutinho *et al.* 1946; Deane *et al.* 1946; Flores-Mendoza *et al.* 2004). The apex of the aedeagus in *An. konderi* is round and broad, with a small pointed lateral leaflet (small protuberance) on both sides, whereas in *An. oswaldoi* s.s. the apex of the aedeagus is elongate, much longer than wider and without leaflets (Galvão & Damasceno 1942). Due their high external morphological similarities, *An. konderi* was treated as a synonym of *An. oswaldoi* for long time (Lane 1953; Faran 1980; Faran & Linthicum 1981). Studies carried out in the 1990s proposed that *An. konderi* was distinct from *An. oswaldoi*, which most likely comprised a species complex (Klein & Lima 1990; Lounibos *et al.* 1997; Marrelli *et al.* 1999). This hypothesis was confirmed with molecular markers (Marrelli *et al.* 1999) and subsequent studies concluded that *An. oswaldoi* s.l. comprised three species - *An. oswaldoi* s.s., *An. oswaldoi* A and *An. oswaldoi* B (Scarpassa & Conn 2006; Ruiz-Lopez *et al.* 2013). *Anopheles konderi* was also shown to comprise at least two species – *An. konderi* s.s. and *An. sp. nr. konderi* [or *An. konderi* B] (Scarpassa & Conn 2006; Sallum *et al.* 2008; Motoki *et al.* 2011; Ruiz-Lopez *et al.* 2013; Saraiva *et al.* 2018). Phylogenetic analyses indicated that these two complexes are paraphyletic; therefore, they are collectively referred here to as the Oswaldoi-Konderi Complex (Ruiz-Lopez *et al.* 2013; Saraiva *et al.* 2018).

Given the apparent complexity of the Oswaldoi-Konderi Complex, it is necessary to revisit the true identity of *An. konderi* s.s. for conclusively fix the identity of the species in

the strict sense and; thus, to allow the formal description of other closely associated taxa. Although Flores-Mendoza *et al.* (2004) redescribed *An. konderi* s. s. and assigned a neotype, in the study of the description, these authors mixed the two forms of *An. konderi* from a broad area of their distribution, including samples from Peru, Bolivia and Brazil (Costa Marques and Coari, type locality of *An. konderi*). Moreover, in the redescription study there are illustrations of the two forms occurring in sympatry in Coari (AM), which was confirmed with molecular data in the present study. In fact, at least three species of the Oswaldoi-Kondesi Complex co-occur at the type locality of *An. konderi* s.s. in Coari, Brazil, further adding to the confusion over the identity of *An. konderi*.

The aim of the present study was to fix the morphological (re-examine of neotype material) and molecular identity of *An. konderi* s.s. by associating with the molecular data from three markers (*COI*, *CAD* and *ITS2*) and the description of the second species near *An. konderi*, informally named from *An. sp. nr. konderi* (Ruiz-Lopez *et al.*, 2013) or *An. konderi* B (Foster *et al.*, 2013).

Materials and methods

Mosquito collection

Mosquitoes were collected with a Shannon model (Shannon 1939) light trap. The trap was installed inside a fragment of the preserved forest, Chácara do Tarcísio, municipality of Coari, State of Amazonas, Brazil ($04^{\circ} 07' 11.41''$ S, $63^{\circ} 4' 56.88''$ W) during July 2018, in the southern bank of the Solimões River, Coari municipality, the type locality from *An. konderi* s.s. (Galvão & Damasceno 1942). For comparison, in this study, we also included descendants from progenies of two females collected in the municipality of Borba, State of Amazonas, Brazil ($04^{\circ} 25' 39.16''$ S, $59^{\circ} 55' 08.83''$ W), which were morphologically

and molecularly identified as *An. konderi* s.l. and compared with material from Coari (Table 1).

After collections in the field, the mosquitoes were maintained alive in paraffin cups closed with nets. In each cup, a sugary solution (10% glucose) was offered to the mosquitoes. The cups were packed in a sealed Styrofoam box and transported to the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, Amazonas, Brazil. The mosquitoes (females) were morphologically identified using the key of Faran & Linthicum (1981) and those morphologically like *An. konderi*/*An. oswaldoi* s.l. were placed individually into oviposition cups. After oviposition, 20 eggs from each progeny were preserved in 4% glutaraldehyde solution for scanning electron microscopy (SEM) analysis. The remaining eggs were placed in trays containing distilled water for hatching. The larvae were fed with fish food (*Tetramin®*) until they became 4th instar. Fourth-instar larvae from each progeny were separated and stored at freezer (-80°C) for molecular analysis. The remaining larvae were maintained until the pupal stage, when they were transferred to screened cups for emergence of adults. Associated larval and pupal exuviae were mounted on microscope slides with Canada balsam. Male genitalia were dissected, transferred to 10% KOH solution for 12 hours, stained with acid fuchsine and slide-mounted with Canada balsam. Adult mosquitoes were mounted on entomological pins and examined. A single voucher specimen of each developmental stage was deposited at the INPA entomological collection.

Morphological characterization

Morphological characters of adult males and females and male genitalia were examined and measured. The morphological terminology used herein is as follows: for adults (Harbach & Knight 1980), for wing spots (Wilkerson & Peyton 1990) and for wing veins

(Belkin 1962). The pupal and larval setae were also examined, and the branches were counted for the description of *An. tadei* n. sp. Abbreviations used in the life stages are as follows: F - adult female; M - adult male; MG - male genitalia; L - larvae; Le - larval exuviae; Pe - pupal exuviae. The larvae, pupae and adults were examined under fluorescent light in a Leica MZ 12.5 stereomicroscope. Photographs were taken using a Leica M205A stereomicroscope, with a Leica DMC4500 digital camera in Leica Application Suite v.4.10 (Leica Microsystems Wetzlar, Germany). A series of partially focused digital images was stacked using the Helicon Focus, v. 6.8, to produce final images with high quality.

DNA Extraction, amplification, and sequencing

Genomic DNA was isolated from fourth-instar larvae using the phenol-chloroform extraction protocol (Green & Sambrook 2012) and the DNA pellet was suspended in 30 µL of sterile water. A small aliquot of this DNA was stored at -20°C and then used as template for polymerase chain reaction (PCR) amplification of the DNA barcoding region of the mtDNA *COI* gene, *CAD* gene and rDNA ITS2 (Internal Transcribed Spacer 2) region. The remaining aliquots of the extracted DNA are kept frozen at -80°C as voucher DNA in Laboratory of Populations Genetics and Evolution of Mosquitoes at INPA, Manaus, Brazil.

The *COI* gene (DNA barcoding region) was amplified using the universal LCO-1490 (59 - GGT CAA CAA ATC ATA AAG ATA TTG G - 39) and HCO-2198 (59 - TAA ACT TCA GGG TGA CCA AAA ATC A - 39) primers (Folmer *et al.* 1994). Each PCR reaction had a total volume of 25 µL, containing 1 µL of DNA and 24 µL of Mix comprising 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 2.5 pmole of each primer, 200 µM each dNTPs, 2.5 U of Taq polymerase (Platinum High Fidelity Taq DNA Polymerase, Invitrogen Inc., Carlsbad, CA) and distilled water. The amplification program consisted of

3 min of denaturation at 94° C, 35 cycles at 94° C, 55° C and 72° C for 1 min each, followed by 7 min of extension at 72° C.

The ITS2 region was amplified using the primer pair 5.8SF (ATC ACT CGG CTC GTG GAT CG) and 28SR (ATG CTT AAA TTT AGG GGG TAG TC) as described by Collins *et al.* (1987). The PCR reactions had a final volume of 25 µL, containing 1 µg of DNA, 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 5 pmol of each primer, 200 µM of each deoxynucleoside triphosphate (dNTP) and 2.5 U of Taq polymerase (Platinum High Fidelity Taq DNA Polymerase; Invitrogen Inc., Carlsbad, CA). PCR amplifications consisted of 2 min of denaturation at 94° C and 34 cycles of 94° C, 57° C, and 72° C for 30 second each, followed by 10 min of extension at 72° C.

For the *CAD* nuclear gene, two pairs of primers were used, the pair first was for the PCR reaction CAD338F (ATG AAR TAY GGY AAT CGT GGH CAY AA) and CAD680R (AAN GCR TCN CGN ACM ACY TCR TAY TC) and the pair second for the sequencing reaction CAD_F (CCM RSC GST GCT ACA TGA C) and CAD_R (GAT GAT GAG CTG RGY CGA GTG) only (Foster *et al.* 2013). Each PCR reaction contained 3 µL of template DNA (about 1/50 to 1/100 of the amount extracted from a single sample); 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 to 2.4 mM MgCl₂; 2.5 mL DMSO; 100 picomoles of each initiator; 200 mM each dNTPs; Taq 2.5 U polymerase; and the remaining volume of ultra-pure water to 25 µL. The amplification protocol consisted of a 2 min denaturation at 94°C, 35 cycles at 94°C for 30s, 57°C for 30s and 72°C for 1 min each, followed by a 7 min extension at 72°C.

After the PCR reaction, the amplified products were checked on a 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, USA), observed under UV light to verify the size and quality of fragments. The amplified products were purified with PEG precipitate (20% polyethylene glycol 8000/2.5M NaCl). Sequencing reactions were performed in both directions using the ABI Big Dye Terminator Kit, and ABI

automated sequencer 3130x1 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses

The DNA sequences obtained for each marker were automatically aligned with ClustalW (Higgins & Sharp 1988) and edited in BioEdit v.7.2.5 (Hall 2011) with the help of Chromas Lite v 2.6.5 (<http://technelysium.com.au/wp/chromas/>). The consensus sequences of the *COI* barcode region were checked for the presence of stop codons, pseudogenes or Numts. The genetic distances were assessed using the Kimura 2-Parameter (*COI* gene) and uncorrected-*p* distance (*CAD* gene and ITS2) in MEGA v. 7 (Kumar *et al.* 2016). Bayesian inference (BI) analyses (separated and combined datasets) were inferred in MrBayes v. 2 (Huelsenbeck & Ronquist 2001) using evolutionary models previously selected in the program JModelTest v. 2.1.10 (Posada 2008). BI analysis was performed with two simultaneous and independent runs of the Markov Chain and Monte Carlo (MCMC), with a 25% burning and 10 million generations, while sampling every 1,000 generations. Bayesian posterior probabilities (BPP) were used to evaluate the nodal support of the clades. In these analyses, *Anopheles oswaldoi* s.s. (Peryassú) was used as the outgroup. To delimit the geographic distribution of *An. konderi* s.l., the sequences from previous studies were downloaded and then compared with the sequences obtained in the present study (Table 1).

Results

The six progenies obtained from Coari were morphologically separated into two morphotypes. Of these, four progenies had the characters of the male genitalia and the 4th instar larva identical to the *An. konderi* (Neotype) (Galvão & Damasceno 1942; Flores-

Mendoza *et al.* 2004). The two remaining progenies of Coari were distinct in these characters, which were also present in the series of paratypes deposited by Flores-Mendoza et al. (2004). Thus, these traits raised for male genitalia and 4th instar larva, which were observed in the neotype, were established to separate *An. konderi* from *An. tadei* n. sp. and corroborated with molecular data. Specimens of the paratype series that were morphologically identical to *An. tadei* n. sp. were put as their synonym.

DIAGNOSE. *Anopheles tadei* n. sp. can be distinguished from *An. konderi* by a few characters present in the larva and male genitalia (Figure 1A, B). In the adult and pupal stages, the two species are identical. In the male genitalia, the distinction is given by the sclerotization of the apex of the aedeagus. In *An. tadei* n. sp., basal sclerotization is V-shaped, with a short apex ($37,71 \pm 0,01\mu\text{m}$) (Figure 1A-4), whereas in *An. konderi* it is U-shaped, giving a longer aspect to the apex of the aedeagus ($51,18 \pm 0,01\mu\text{m}$) (Figure 1B-4). In the ventral claspete of *An. tadei* n. sp. the basal lobe is round, with no spare lobe (Figure 1A-3), whereas in *An. konderi* the basal lobe has a very noticeable overhang (Figure 1A-3). Still in the ventral claspete, the length is shorter in *An. tadei* n. sp. ($123.56 \pm 0.05\mu\text{m}$) than in *An. konderi* ($131.30 \pm 0.05\mu\text{m}$). The space between the pre-apical plate and the median sulcus was significantly larger in *An. tadei* n. sp. ($24.58 \pm 0.02\mu\text{m}$) than in *An. konderi* ($18.52 \pm 0.02\mu\text{m}$).

In the fourth instar larva, the clypeal setae (Setae 2,3-C) of *An. tadei* n. sp. are bifurcate from the middle portion, these branches are long and exceptionally smooth (Figure 1B-1). In *An. konderi* the clypeal setae are also bifurcate from the middle portion; however, the secondary branches arise from the primary branches (Figure 1A-1). The spiracular apparatus of *An. tadei* n. sp. presents a wider conformation of this structure (Figure 1B-2), whereas in *An. konderi* s.s. the spiracular apparatus presents concave entrances giving a narrow appearance to this structure (Figure 1A-2).

Morphological description

Anopheles (Nyssorhynchus) tadei Saraiva & Scarpassa new species

FEMALE - Integument dark brown with light contrasting areas (Figure 2A). *Head* (Figure 2B, C): Interocular space with frontal tuft of long white setae and curved white spatulate scales, curved scales along ocular margin, apex immediately posterior to frontal tuft with erect white spatulate scales and few long pale yellow setae; remainder of vertex and occiput with brownish spatulate scales; postgena with a tuft of dark brown spatulate scales and a few white and semi-erect spatulate scales at junction of eyes; clypeus bare. Antenna dark brown; pedicel with white, decumbent spatulate scales on dorsal surface; flagellomere 1 with semi-erect white scales on medial and lateral surfaces and white decumbent scales at base of dorsal surface (Figure 2C). Proboscis with dark decumbent scales and short setae, length 1.99–2.32 mm (mean 2.14 ± 0.15 mm) ($n = 10$); length of maxillary palpus 1.92–2.22 mm (mean 2.10 ± 0.11 mm) ($n = 10$). Maxillary palpomere (MPlp) 1 - 3 predominantly with semi-erect dark scales, palpomere 1 dark, with erect scales on dorsal surface, MPlp 2 dark, with narrow ring of pale scales at apex of dorsal surface, MPlp 3 dark, with apical white band and spot of sparse pale scales on dorsal surface, MPlp 4 with dark scales in ventral region and predominantly colourless on dorsal surface with small dark rings on proximal and distal ends, MPlp 5 with white scales and setae on dorsal surface, and dark scales at base (Figure 2B). *Thorax* (Figure 2D, E): Anterior promontory with long pale set form scales, usually not extending too far onto acrostichal area. Foretarsomeres (Ta-I) Ta-I1–Ta-I3 with pale scales at apices, length of pale apices of Ta-I1: 0.04–0.05 mm (mean 0.04 mm) ($n = 10$), Ta-I2 0.04–0.12 mm (mean 0.07 ± 0.02 mm) ($n = 10$), Ta-I3: 0.07–0.19 mm (mean 0.12 ± 0.02 mm), Ta-I4 totally dark ($n = 10$), Ta-I5 with

apical band of yellowish scales, length 0.04–0.07 mm (mean 0.06 ± 0.01 mm) ($n = 10$). Midtarsus (Ta-II) predominantly dark, with narrow yellow ring at apices of Ta-II1–Ta-II3, Ta-II4 dark and Ta-II5 with pale apical scales. Hindtarsomere (Ta-III), Ta-III2 with proximal 0.14–0.15 mm (mean 0.15 mm) ($n = 10$) dark-scaled, Ta-III3 and predominantly white-scaled, Ta-III5 with a proportion of basal 0.5 dark-scaled and remainder pale-scaled (Figure 1H). *Wing*: Length 2.89–3.52 mm (mean 3.23 ± 0.1 mm) ($n = 30$); wing spot measurements in Table 2; veins with scales white and yellowish pale spots; prehumeral pale, humeral pale, presector pale, sector pale, accessory sector pale, subcostal pale and preapical pale spots present in 100% of wings examined ($n = 30$). Radial vein with dark spot in distal third; R_s predominantly dark, with narrow pale scales at fork of R_{2+3} and R_{4+5} and few pale scales at base; R_{2+3} with pale and dark scales mixed, dark spot more evident at base and dark and pale scales mixed at apex before furcation of R_2 and R_3 ; R_{4+5} mainly pale-scaled with small spot of dark scales at proximal and distal ends; vein M variable, mainly dark-scaled, with pale scales from base to mid region or mostly pale with dark spots at middle and distal end; M_{1+2} and M_{3+4} with 0.5 of length; Cu mainly pale, with small dark spot on distal 0.2 before furcation of CuA and CuP; CuA and CuP mainly pale, CuA with 2 small dark spots distal to base and small preapical dark spot, CuP also mainly pale, with preapical dark spot; wing fringe with pale spots at apices of all veins; details of wing veins in Figure 2F. *Abdomen* (Figure 2G): Dark brown integument; terga II–VII each with pale scales in sub-triangular pattern, pale and yellow scales uniformly distributed on tergum VIII; posterolateral tufts of dark scales present on segments II–VII; sterna with a few moderately long setae.

MALE - Like female except for sexual differences. Maxillary palpus (MPlp) with dark and pale yellow markings; semi-erect scales on basal 0.5 half of MPlp-2, decumbent on rest of MPlp-2 and MPlp-3–5; MPlp-2 with pale scales at apex, dorsal surface with a pale spot on basal 0.5; MPlp-3 with pale scales at base, long setae at apex; MPlp-4 with pale

scales on dorsal surface, dark scales at apex and base and long setae at base; MPlp-5 with dark setae ventrally surface and pale scales on dorsal surface. *Genitalia* (Figure 3C): Segment VIII with tergum and narrow sternum with spatulate scales and long setae, scales slightly wider on sternum than on tergum. Gonocoxite elongate; tergal surface with 4 or 5 setae and subapical surface with 1 or 2 setae; tubercle of the parabasal spine large, 0.4 length of the spine; dorsomedian rim 0.11–0.14 (mean 0.12 ± 0.01) length gonocoxite; length of gonostylus 0.32–0.38 (mean 0.37 ± 0.01); gonostylar claw spiniform, long. *Dorsal claspette*: Pedicel narrow, base rounded, leaflets broad, expanded in median area. *Ventral claspette* (Figure 3D): Spiculate, extending to a narrow apex, laterally rounded basal lobule with long setae at basal margin. Aedeagus (Figure 3C) with fine lateral leaflets at apex, apex wider than long imparting a rounded to triangular shape, leaflets of variable, evident under light microscope at 20x magnification. Two morphotypes were observed. The first showed the apex of the flattened aedeagus with evident lateral leaflets. The second morphotype showed the apex of the triangular aedeagus with more extended lateral leaflets. In all forms the basal sclerotization of the apex of the aedeagus had V-conformation, giving this structure a flattened aspect.

PUPA - (Figure 3). Positions and development of setae in Figure 2A, B; range and modal number of branches in Table 3. Measurements based on 20 specimens. *Cephalothorax*: Light brown integument with dark spots on legs, and dorsal area without a defined pattern; trumpet length 0.4–0.42 mm (mean 0.41 ± 0.01 mm), truncate and widened apically; distinct tracheoid area; meatus length 0.19–0.20 mm (mean 0.20 ± 0.01 mm); pinna moderately pigmented, length 0.28–0.29 mm (mean = 0.28 ± 0.01). *Abdomen*: yellowish tegument with some dark ventral areas, segment II length 2.38–2.50 mm (mean 2.44 ± 0.02 mm); seta 1-II,III with lateral branches; seta 1-IV–VII single and long. *Paddle*: Pale, slightly

paler than abdomen, oval, slightly longer than broad; margins with spicules, more developed distally.

LARVA (fourth instar) - Positions and development of setae in Figure 4; range and modal number of branches in Table 4. Measurements based on 20 specimens unless indicated otherwise. *Head*: Length 0.53–0.54 mm (mean 0.53 ± 0.01 mm); width 0.54–0.55 mm (mean 0.54 ± 0.01 mm); integument dark brown, dark spots on areas and posterolateral regions of dorsal apotome along posterior parts of frontal ecdysis lines as shown in Figure 4C, ventral areas of lateralia, labiogula and along hypocranial ecdysial line; dorsomentum strongly sclerotized, blackened, with 4 lateral teeth on either side of median apical tooth (Figure 4D); ventromentum slightly sclerotized, with 4 teeth on each side (Figure 4D'); collar strongly pigmented and sclerotized. Setae 2,3-C bifurcate, branches thin, distance between bases of 2-C = 0.02 mm ($n = 10$) and 3-C = 0.04 mm ($n = 10$); clypeal index (distance between 2-C and 3-C bases on one side / distance between 2-C bases) 1.10–1.43 (mean 1.33 ± 0.1). Seta 4-C with 2–6 short branches. Antennal length 0.26–0.28 mm (mean 0.27 ± 0.1 mm), with numerous spicules, seta 1-A with 6–9 branches, inserted at 0.05–0.08 mm (mean 0.07 ± 0.01 mm) from base. *Thorax*: Integument pale, Setae 1,2-P borne on separate tubercles, 1-P, with 9–18 narrow, smooth, and pointed leaflets, 2-P with 12–19 branches. *Abdomen*: Integument pale. Seta 0-II, III large; 1-I–VII palmate with smooth leaflets, 1-I with 9–14 leaflets, poorly pigmented; 1-II and 1-VII, with smooth, darker leaflets; seta 1-X inserted outside saddle, which is incomplete. Pecten plate with 4 long spines and 14–16 short spines. Spiracular apparatus: Lateral arms of medial plate developed, elongate, projecting to spiracular process. Posterior margin of segment X with numerous short spike-like spicules. Segment X: covered with fine spicules; seta 1-X if saddle, inserted on ventral margin of saddle. Anal papillae hyaline, length 0.43–0.46 mm (mean 0.44 ± 0.1 mm); length of seta 4-X 0.65–0.77 mm (mean 0.68 ± 0.19 mm).

TYPE OF MATERIAL - designated male holotype, with association larval and pupal exuvia and male genitalia, from the progeny litter of the Shannon trapped female progeny: south bank of Solimões River, Coari ($3^{\circ} 57'S$, $63^{\circ} 12'W$), State of Amazonas, Brazil. *COI* voucher sequence XX-XX, *CAD* gene XX-XX and ITS2 XX-XX, specimen, col. J. F. Saraiva, in 23-VII-2018, deposited at the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil.

ETYMOLOGY. The name of this species is a tribute to Dr. Wanderli Pedro Tadei, in recognition of his significant contribution to anopheline studies and other mosquitoes in the Brazilian Amazon region. Currently, he is a senior researcher at the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil, where he works since 1979. Dr. Tadei started to study anophelines analysing inversion chromosome polymorphism in *Anopheles darlingi* populations. Later, he expanded his studies for ecological and behavioural patterns and malaria transmission dynamic as well as control methods in species of the *Anopheles*, *Aedes* and *Culex* genera, with emphasis in the vector species. Dr. Tadei published approximately two-hundred scientific articles and contributed enormously for the training of human resources, which he trained over 70 masters and doctoral students. Many of these students are currently scientists and teachers in distinct Brazilian institutions.

BIONOMICS. *An. tadei* n. sp. was collected inside the rain forest, along with *An. kondneri* s.s. in the municipality of Coari, state of Amazonas, Brazil. The specimens were captured between 19:00 and 20:00 hours. Although these species bite humans, they are considered zoophiles. However, further investigations will be needed to determine ecological and behavioural patterns between the two species.

MEDICAL IMPORTANCE. The role of *An. tadei* n. sp. in the transmission of malaria is amply unknown. Infection experiments showed that *An. oswaldoi* s.l. is more

susceptible to *Plasmodium* spp. infection than *An. konderi* s.l. (Marrelli *et al.*, 1999). However, more studies need to be done to assess the vectoral competence of these species.

Molecular characterization

MtDNA *COI* fragment consisted of 644 bp, ITS2 fragment comprised 474 bp and *CAD* gene 793 pb. The A+T content of the *COI* fragment was 67.5% in all sequences. The mean base composition of the eight sequences was 29.1% A, 38.4% T, 15.6% C and 16.9% G. The percentage of A+T in the ITS2 fragment was 47.6%, and the composition of nucleotides was 27.3% A, 20.3% T, 27.5% C and 24.9% G; this finding corresponded to those observed in the previous studies of the Oswaldoi-Konderi Complex (Ruiz-Lopez *et al.* 2013; Saraiva *et al.* 2018). The *CAD* gene presented 38 variable sites and 30 informative sites for parsimony. ITS2 region presented 20 variable sites and 18 informative sites. The composition of variable sites is shown in the supplementary files (4-6).

The voucher sequences from *An. konderi* s.s. and *An. tadei* n. sp. have been deposited under the following accession numbers: *An. konderi* s.s. [*COI* gene (XX_XX), ITS2 (XX_XX) and *CAD* gene (XX_XX)] and *An. tadei* n. sp. [*COI* gene (XX_XX), ITS2 (XX_XX) and *CAD* gene (XX-XX)].

The DNA sequences for the *COI* gene separated the eight sequences (six from Coari and two from municipality of Borba) into two groups. The first group corresponded to *An. tadei* n. sp. ($BPP = 0.98$), while the other groups corresponded to *An. konderi* s.s. ($BPP = 1$), each subgroup within *An. konderi* s.s. were moderately supported ($BPP = 0.83$ and 0.89) (Supplementary Figure 1). The two subgroups from *An. konderi* s.s. were not recovered by morphological analysis, being identical to the neotype material deposited by Flores-Mendoza *et al.* (2004). The interspecific genetic distance (K2P) for *COI* between *An.*

konderis s.s. and *An. tadei* n. sp. was 4.0%, whereas the intraspecific distances were 0.5% in *An. konderi* s.s. and 0.2% in *An. tadei* n. sp. (Table 5). On the other hand, the BI tree generated with nuclear markers recovered only two strongly supported groups (BPP = 1). BI tree from the ITS2 database (Supplementary Figure 2) recovered the species (*An. konderi* s.s. and *An. tadei* n. sp.) with an interspecific distance of 3.8% (p-distance uncorrected). In the BI tree for the *CAD* gene, the distance p was 4.2% between two species (Table 5). BI tree with combined data (*COI* + *CAD* + ITS2) recovered two groups, *An. tadei* n. sp. and *An. konderi*, with high support (BPP = 1), and two subgroups in clade of *An. konderi* s.s. heavily supported (BPP = 0.90 and 0.95) (Figure 6). Although the subgroups within *An. konderi* were maintained, the genetic distances between them were very low (0.1-0.4%) for all markers used (Table 5), likely reflecting intraspecific variability. The variable positions of the three markers are shown in the supplementary tables (3-5).

GEOGRAPHIC DISTRIBUTION. According to our data and sequence records of the previous studies (Table 1), *An. tadei* n. sp. occurs in Peru (Madre de Dios and Loreto Province), Ecuador (Orellana Province), Colombia (Caquetá Department) and Brazil (State of Amazonas: Coari, Nova Olinda do Norte and Novo Remanso; State of Acre: Senador Guiomar, Transacreana Highway and Rio Branco; State of Rondônia: São Miguel, Porto Velho). Of these, at least in three locations (Coari, São Miguel and Porto Velho) *An. tadei* n. sp. is sympatric to *An. konderi* s.s. (Scarpassa & Conn 2006; Sallum *et al.* 2008; Saraiva *et al.* 2018) (Figure 7).

MATERIAL EXAMINED: HOLOTYPE: slide with dissected male genitalia associated with the larva and pupa (1MG, 1Le, 1Pe), BRAZIL, Amazonas, Coari, JF Saraiva coll., 23-VII-2018. PARATYPES: two blades with; 2MG, 2Le, 2Pe, and more 2F, 2M mounted on entomological pins, State of Amazonas, Coari, JF Saraiva coll. They are deposited in the Entomological Collection of the Instituto Nacional de Pesquisas da

Amazônia, Manaus, Amazonas, Brazil. Par. Types of *An. konderi* s.s., synonymised with *An. tadei* n. sp.: Slide nº. 1630 - 3MG, 2MPeLe, 1FPeLe, Senador Guiomar, Ramal Oco do Mundo, State of Acre, R. Santos coll. Slide nº. 1631 - 1MG, 2Le, 2Pe, Candeias do Jamari, State of Rondônia, C. Flores-Mendoza and MT Marrelli colls.

Discussion

Flores-Mendoza et al. (2004) also found *An. konderi* s.s. and *An. tadei* n. sp. in municipality of Coari, but in the redescription study, the authors considered all material examined as *An. konderi* s.s. and the morphological differences observed likely were considered as variations intraspecific. Besides, they did not carry out molecular analysis that could show to the existence of more than one species. Molecular data obtained from previous studies (Scarpassa and Conn, 2006; Sallum et al., 2008; Motoki et al., 2011; Saraiva et al., 2018), the suspicions that it was not a single species can be clearly shown. However, molecular data were not obtained from the *An. konderi* s.s. neotype, however, the morphological differences observed were congruent with the molecular differences of the progenies of our study, which made it possible to fix the name *An. konderi* s.s. and synonymize the paratypes (nº1630 and nº1631) deposited by Flores-Mendoza et al. (2004) with *An. tadei* n. sp.

Coari, now the type locality of *An. konderi* and *An. tadei* n. sp., also has presence of *An. oswaldoi* species A, recorded by Scarpassa and Conn (2006) and Saraiva et al. (2018). *Anopheles oswaldoi* A was captured in the Igarapé do Isidoro, further east of the Sítio do Tarcísio. Although, *An. oswaldoi* A was not collected together with the two species of the present study, it has already been reported in sympatric with *An. konderi* in Serra do Cachorro, Oriximiná (PA) and Mata fome, Macapá (AP) by Saraiva et al. (2018). In addition, in Pará, Tadei et al. (1998) reported a single specimen infected with *P. falciparum* identified

as *An. oswaldoi* s.l. that based on our data, it can refer to both *An. oswaldoi* A and *An. konderi*. Thus, additional studies will clarify the vectoral importance of these species.

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LIST OF FIGURES

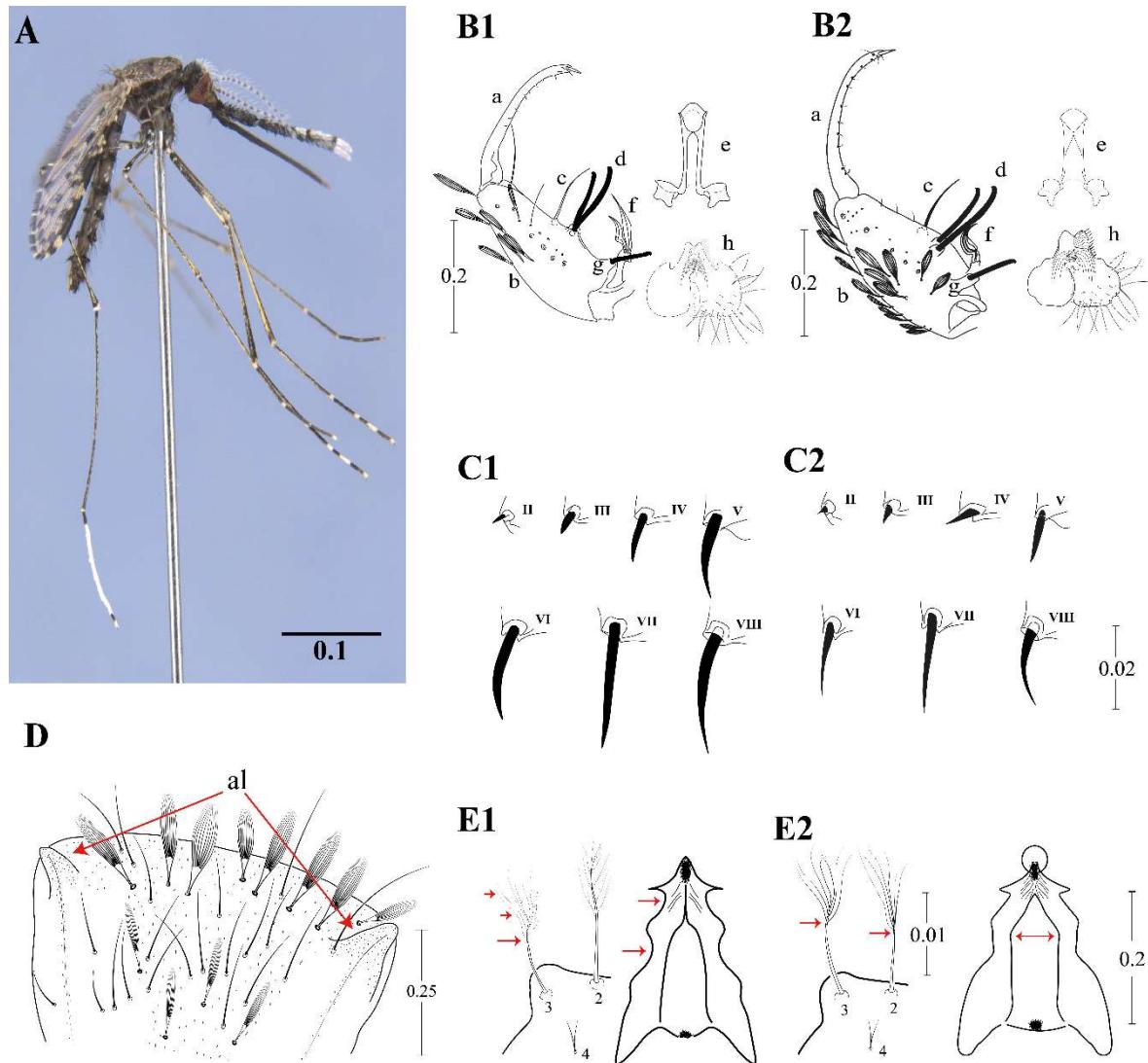


FIGURE 1. Morphological differences between (1) *An. konderi* s.s. and (2) *An. tadei* n. sp. (A) Habitus of *An. tadei* n. sp. (B) male genitalia, with details of the sclerotization of the aedeagus in V and U, and lateral lobes of the ventral claspete, (C) spines of the abdominal segments (II-VIII) of the pupa (D) 8th abdominal segment of the male (Neotype of *An. konderi* s.s.), with details of the presence of lateral lobes (al), absent in *An. tadei* n. sp., (E) differences in the fourth instar larvae, details of the branching pattern of the clypeal setae and the shape of the spiracular plate. Scale in mm.

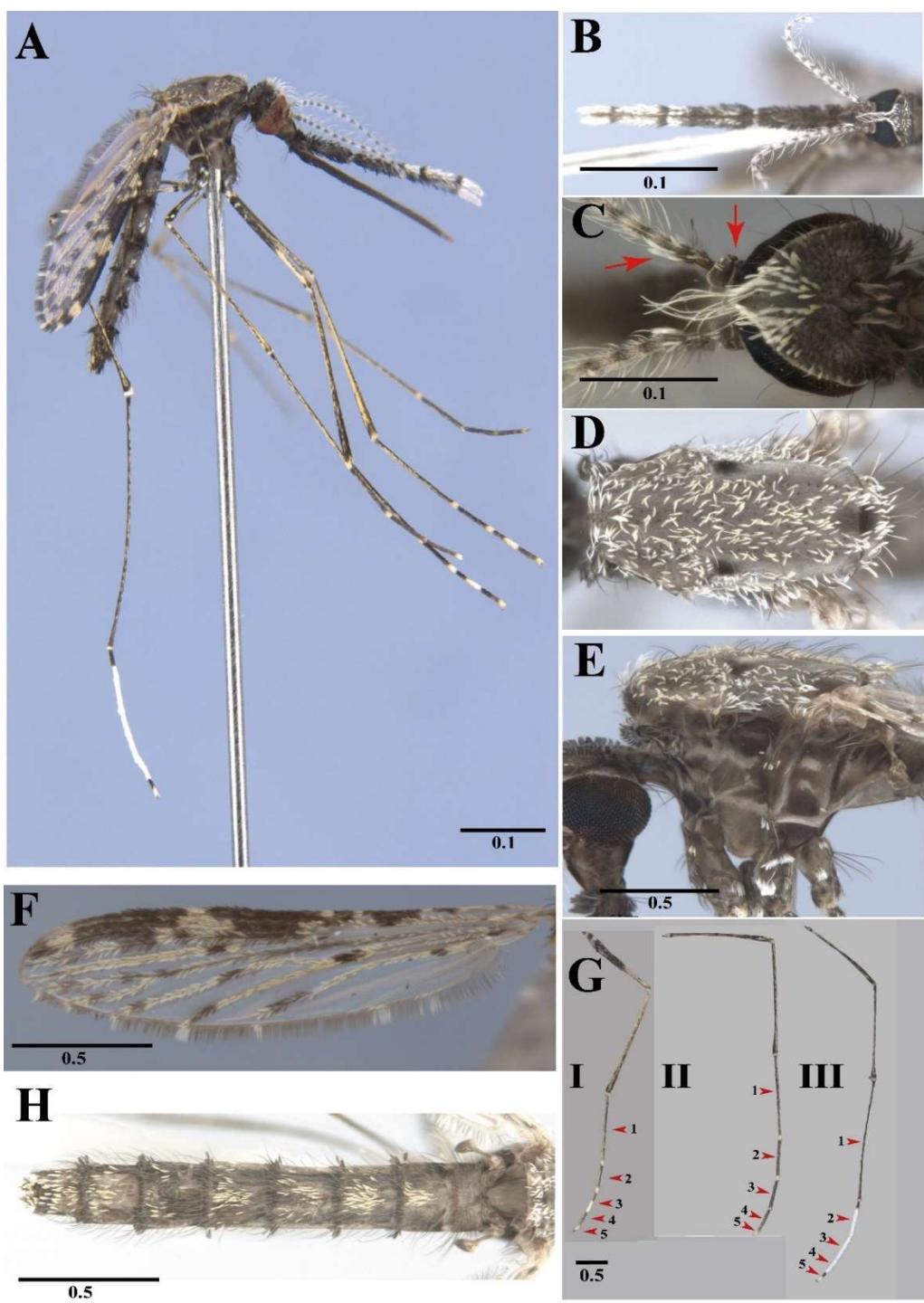


FIGURE 2. External morphology of the adult female of *Anopheles tadei* n. sp. A, Habitus; B, full dorsal view of the head (palpus and antennae); C, dorsal view of the head, details of the pedicel and 1st flagellomere with scales; D, dorsal view of mesonotum and scutellum; E, lateral view of the thorax; F, wing; G, legs – foreleg (I), midleg (II) and hindleg (III) with ornamentation of tarsomeres; H, abdomen (dorsal view).

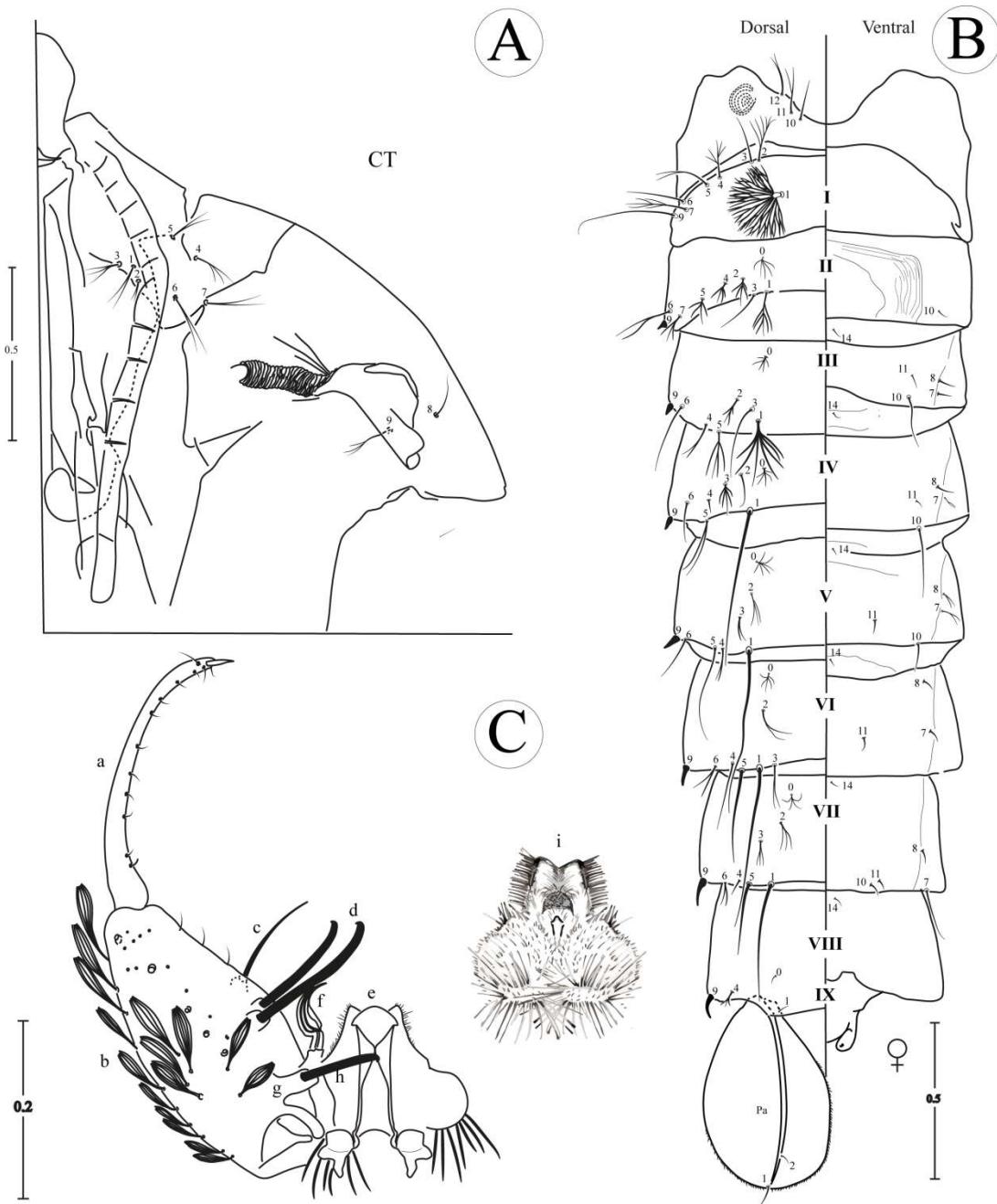


FIGURE 3. Pupa and male genitalia of *Anopheles tadei* n. sp. A - B, Pupa: C, male genitalia. a, gonostylus; b, gonocoxite; c, internal seta; CT, Cephalothorax; d, accessory setae; e, aedeagus; f, dorsal claspette; g, tubercle of parabasal spine; h i, ventral claspette; P, paddle; I-IX, abdominal segments; 1-14, setae of specified areas.

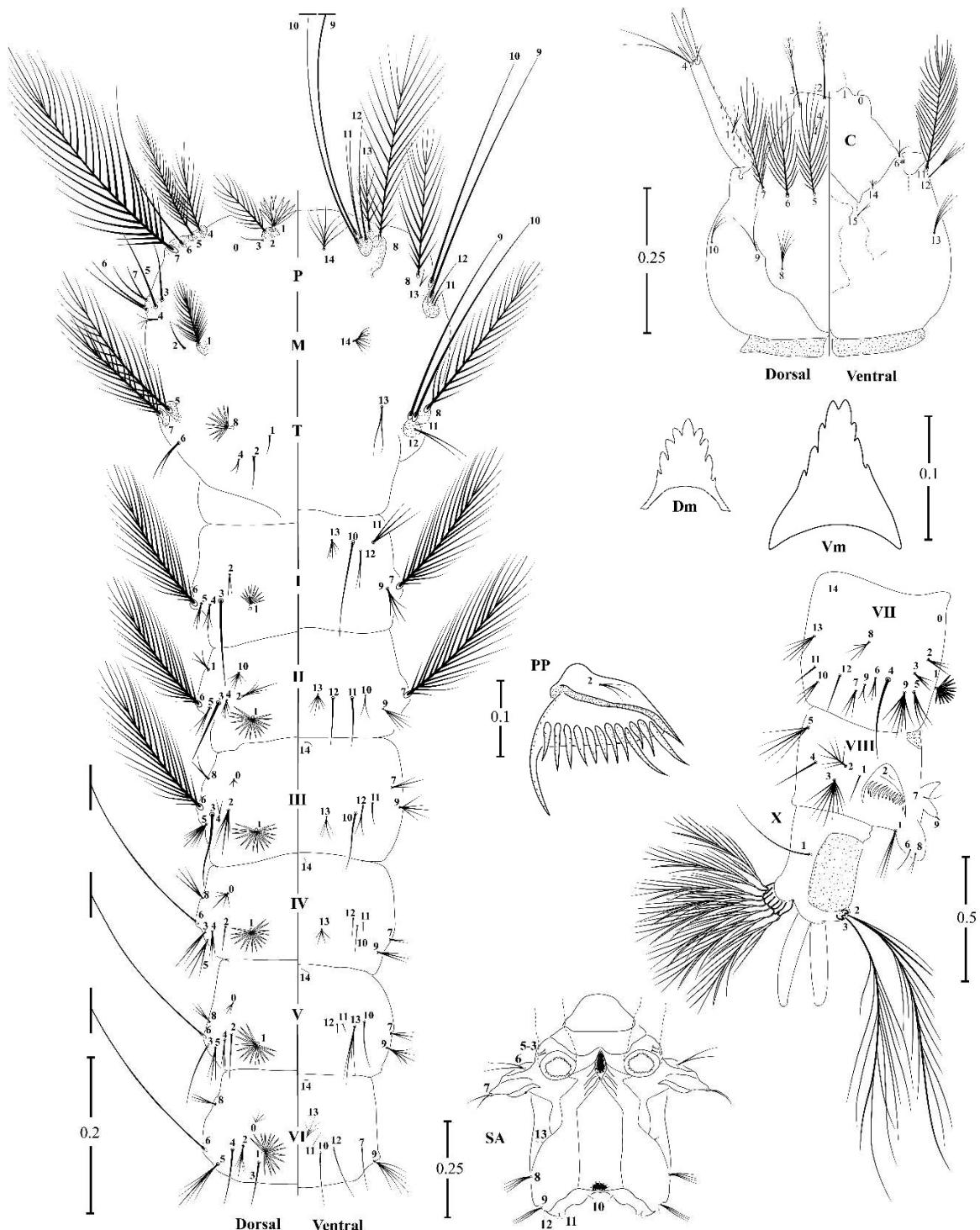


Figure 4. Fourth-instar larva of *Anopheles tadei* n. sp. A; antenna, C; cranium, Dm; dorsomentum, M; mesothorax, P; prothorax, PP; pecten plate, SA; spiracular apparatus, T; metathorax, Vm; ventromentum, I-VIII; abdominal segments, X; anal lobe. Scales in mm.

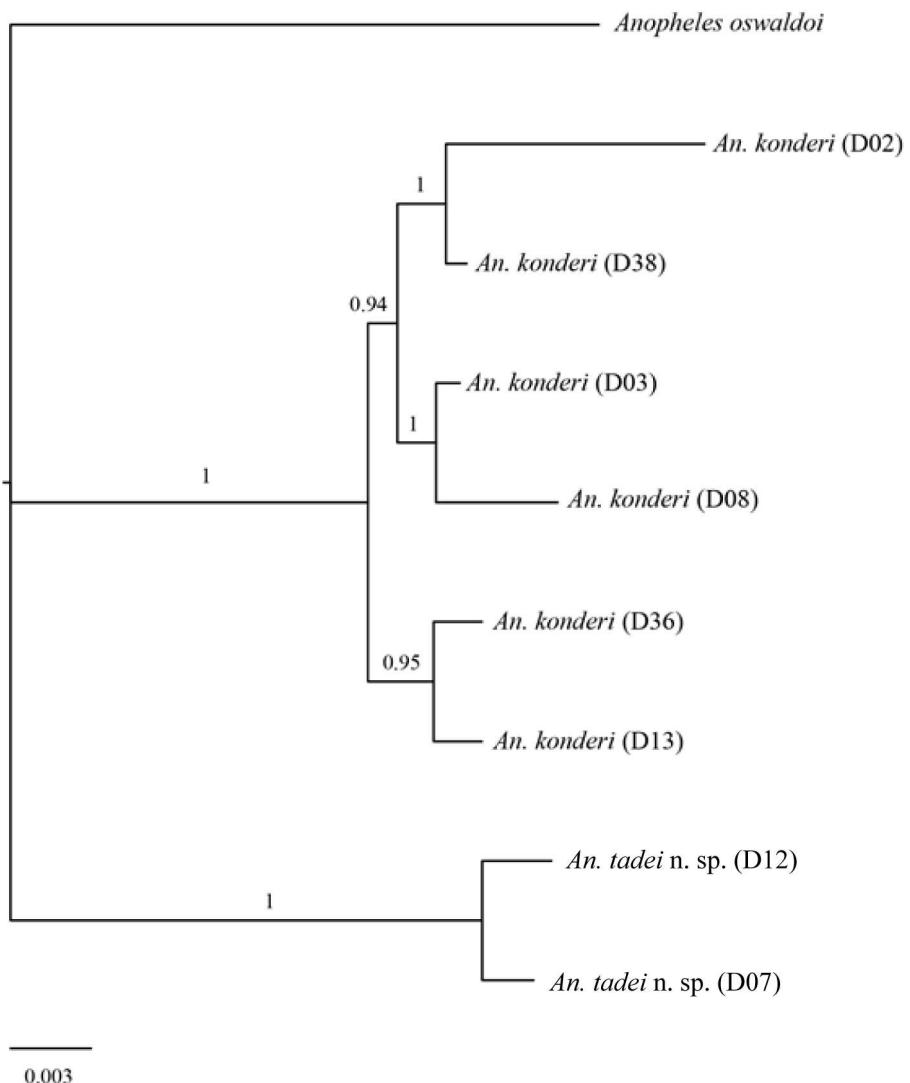


FIGURE 6. Bayesian Inference tree generated with the combined data (*COI*, *ITS2* and *CAD*). Numbers on the branches indicate Bayesian posterior probability (≥ 0.70). *Anopheles oswaldoi* s.s. was included as outgroup.

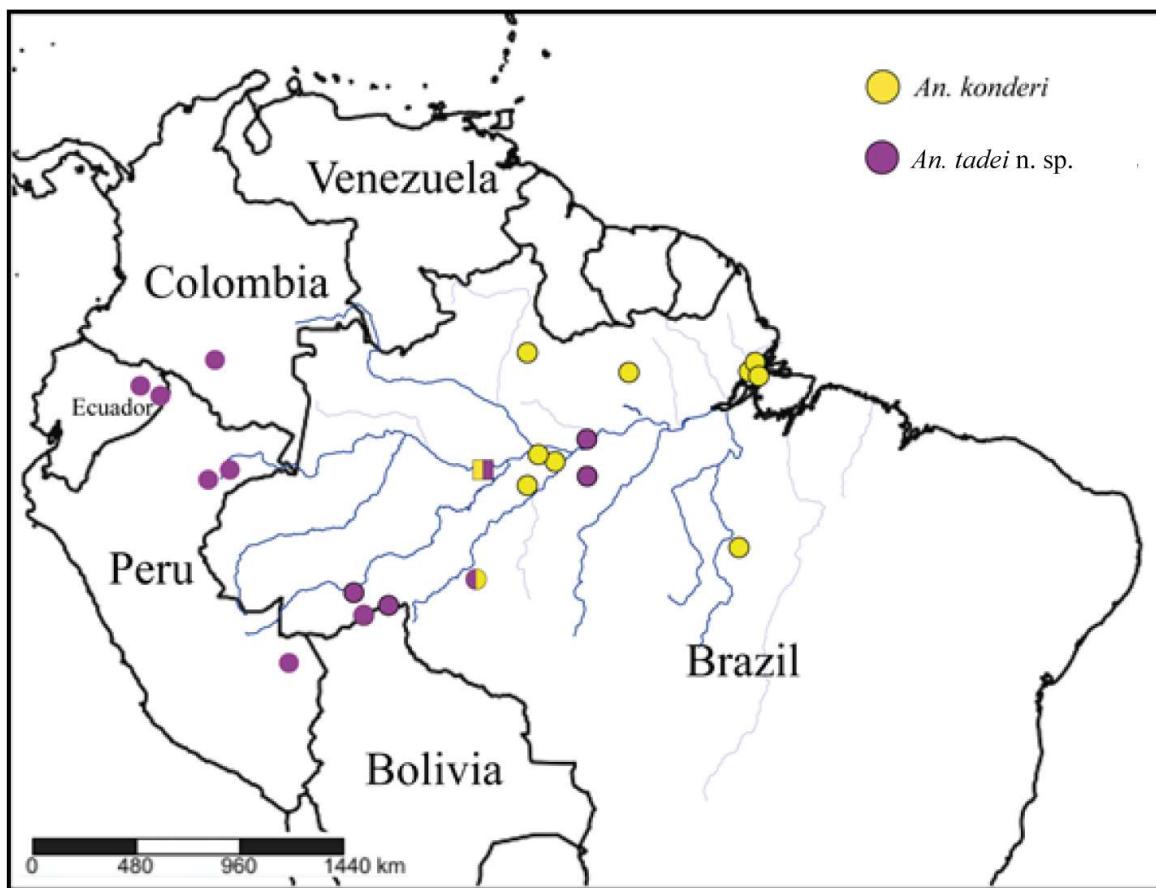


FIGURE 7. Geographic distribution of *An. tadei* n. sp. and *An. konderi* s.s. Geographic distribution range inferred based on molecular data (*COI*, *CAD* and *ITS2*) of this study and from previous studies.

TABLE 1. Information for specimens and references of the sequences (*COI*, *CAD* and *ITS2*).

GenBank accession n°. or field specimen ID	Species	COI	CAD	ITS2	Municipality, State	References
MG241939	<i>An. tadei</i> n. sp.	X			Nova Olinda do Norte, Amazonas	Saraiva et al. (2018)
COD02_779	<i>An. konderi</i>	X			Coari, Amazonas	This Study
COD02_707	<i>An. konderi</i>		X		Coari, Amazonas	This Study
COD02_791	<i>An. konderi</i>			X	Coari, Amazonas	This Study
COD03_708	<i>An. konderi</i>	X			Coari, Amazonas	This Study
COD03_756	<i>An. konderi</i>		X		Coari, Amazonas	This Study
COD03_787	<i>An. konderi</i>			X	Coari, Amazonas	This Study
COD07_782(1)	<i>An. tadei</i> n. sp.	X			Coari, Amazonas	This Study
COD07_782(2)	<i>An. tadei</i> n. sp.		X		Coari, Amazonas	This Study
COD07_782(3)	<i>An. tadei</i> n. sp.			X	Coari, Amazonas	This Study
COD08_709	<i>An. konderi</i>		X		Coari, Amazonas	This Study
COD08_790(1)	<i>An. konderi</i>	X			Coari, Amazonas	This Study
COD08_790(2)	<i>An. konderi</i>			X	Coari, Amazonas	This Study
COD12_781	<i>An. tadei</i> n. sp.	X			Coari, Amazonas	This Study
COD12_710	<i>An. tadei</i> n. sp.		X		Coari, Amazonas	This Study
COD12_788	<i>An. tadei</i> n. sp.			X	Coari, Amazonas	This Study
COD13_711	<i>An. konderi</i>		X		Coari, Amazonas	This Study
COD13_794(1)	<i>An. konderi</i>	X			Coari, Amazonas	This Study
COD13_794(2)	<i>An. konderi</i>			X	Coari, Amazonas	This Study
BOD38_713	<i>An. konderi</i>		X		Borba, Amazonas	This Study
BOD38_800(1)	<i>An. konderi</i>	X			Borba, Amazonas	This Study
BOD38_800(2)	<i>An. konderi</i>			X	Borba, Amazonas	This Study
BOD36_712	<i>An. konderi</i>		X		Borba, Amazonas	This Study
BOD36_797(1)	<i>An. konderi</i>	X			Borba, Amazonas	This Study
BOD36_797(2)	<i>An. konderi</i>			X	Borba, Amazonas	This Study
MG241930	<i>An. konderi</i> s.l.	X			Autazes, Amazonas	Saraiva et al. (2018)
MG241931	<i>An. konderi</i> s.l.	X			Autazes, Amazonas	Saraiva et al. (2018)
MG241932	<i>An. konderi</i> s.l.	X			Autazes, Amazonas	Saraiva et al. (2018)
MG241934	<i>An. konderi</i> s.l.	X			São Miguel, Rondônia	Saraiva et al. (2018)
MG241935	<i>An. konderi</i> s.l.	X			São Miguel, Rondônia	Saraiva et al. (2018)
MG241936	<i>An. konderi</i> s.l.	X			Oriximiná, Pará	Saraiva et al. (2018)
JF437965	<i>An. konderi</i> s.l.	X			Plácido de Castro, Acre	Motoki et al. (2011)
JF437974	<i>An. konderi</i> s.l.	X			Monte Negro, Rondônia	Motoki et al. (2011)
MH844298	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)
MH844246	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)
MH844215	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)
MH844125	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)
MH844124	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)
MH844123	<i>An. konderi</i> s.l.	X			Acrelândia, Acre	Bourke et al. (2018)

MH844286	<i>An. konderi</i> s.l.	X	Acrelândia, Acre	Bourke et al. (2018)
MH844246	<i>An. konderi</i> s.l.	X	Acrelândia, Acre	Bourke et al. (2018)
KF809033	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Ruiz-Lopez et al. (2013)
KF809032	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Ruiz-Lopez et al. (2013)
KF809031	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Ruiz-Lopez et al. (2013)
KF809030	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Ruiz-Lopez et al. (2013)
JF437968	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437967	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437966	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF923716	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Foster et al. (2013)
JF437962	<i>An. konderi</i> s.l.	X	Monte Negro, Rondônia	Motoki et al. (2011)
EU636801	<i>An. konderi</i> s.l.	X	Plácido de Castro, Acre	Motoki et al. (2011)
JF437964	<i>An. konderi</i> s.l.	X	Monte Negro, Rondônia	Motoki et al. (2011)
JF437963	<i>An. konderi</i> s.l.	X	Monte Negro, Rondônia	Motoki et al. (2011)
JF437961	<i>An. konderi</i> s.l.	X	Monte Negro, Rondônia	Motoki et al. (2011)
JF437960	<i>An. konderi</i> s.l.	X	Monte Negro, Rondônia	Motoki et al. (2011)
MG263756	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Saraiva et al. (2018)
JF437928	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
MG263755	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Saraiva et al. (2018)
JF437938	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437937	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437932	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437933	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437931	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437930	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437929	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437927	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437936	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437935	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437934	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)
JF437926	<i>An. konderi</i> s.l.	X	Macapá, Amapá	Motoki et al. (2011)

TABLE 2. Wing spot measurements (in mm) for males and females of *An. tadei* n. sp. collected in the municipality of Coari, state of Amazonas, Brazil ($n = 20$).

Wing spot	Female			Male		
	Range	Mean	SD (\pm)	Range	Mean	SD (\pm)
Basal pale + prehumeral pale	0.19–0.22	0.20	0.01	0.22–0.23	0.23	0.01
Prehumeral dark	0.06–0.07	0.07	0.01	0.04–0.05	0.05	0.02
Humeral pale	0.16–0.20	0.18	0.02	0.19–0.21	0.20	0.01
Humeral dark	0.15–0.18	0.17	0.01	0.15–0.16	0.16	0.03
Presector pale	0.07–0.08	0.07	0.01	0.06–0.07	0.07	0.01
Presector dark	0.34–0.35	0.35	0.01	0.38–0.39	0.39	0.02
Sector pale	0.19–0.22	0.21	0.01	0.22–0.25	0.24	0.00
Proximal sector pale	0.05–0.06	0.06	0.01	0.05–0.06	0.05	0.02
Accessory sector pale	0.02–0.04	0.03	0.01	0.08–0.09	0.08	0.02
Distal sector dark	0.67–0.73	0.71	0.02	0.60–0.61	0.61	0.03
Subcostal pale	0.13–0.15	0.14	0.01	0.19–0.23	0.21	0.01
Preapical dark	0.71–0.76	0.73	0.03	0.55–0.56	0.56	0.03
Preapical pale	0.06–0.14	0.11	0.03	0.12–0.14	0.13	0.00
Apical dark	0.11–0.13	0.12	0.01	0.11–0.12	0.11	0.03

TABLE 3. Number, range and (mode) of branches of setae of the pupa of *An. tadei* n. sp. collected in the municipality of Coari, state of Amazonas, Brazil ($n = 20$).

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

n.c. - not counted.

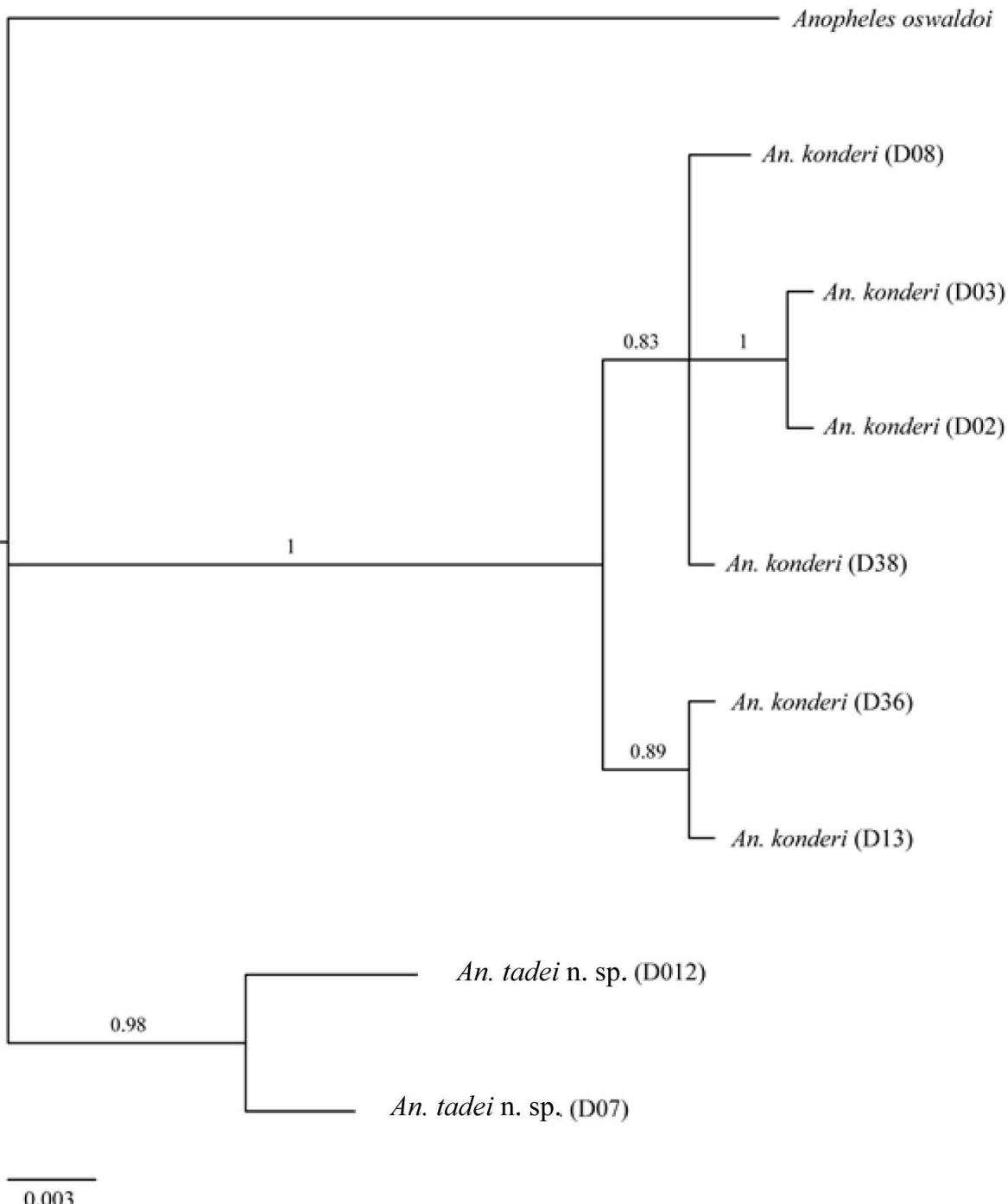
TABLE 4. Number, range and (mode) of branches of setae of the fourth-instar larva of *An. tadei* n. sp. collected in the municipality of Coari, state of Amazonas, Brazil ($n = 20$).

Nº	Head C	Thorax				Abdominal segments								
		P	M	T	I	II	III	IV	V	VI	VII	VIII	X	
0	1	n.c.	-	-	-	5-10 (7)	5-6 (5)	3-4 (4)	5-6 (6)	4-6 (4)	4-5 (4)	2-3 (3)	-	
1	1	11-14 (14)	24-2 (25)	1	9-11 (11)	19-22 (20)	20-26 (22)	24-26 (24)	17-19 (18)	20-26 (24)	17-19 (18)	1	1	
2	12-18 (18)	9-14 (12)	1-2 (2)	1-2 (1)	1-2 (2)	5-6 (5)	4-5 (4)	1	1-3 (3)	3-4 (4)	4-6 (6)	5-8 (5)	16-18 (17)	
3	15-18 (18)	1	1	9-12 (12)	1-2 (1)	1	4-5 (4)	2-3 (2)	1-2 (2)	1	2-3 (3)	12-13 (12)	11-14 (10)	
4	2-4 (4)	16-18 (18)	2-3 (3)	1-3 (3)	2-4 (4)	5-9 (9)	1-2 (1)	1	1-2 (2)	1	1	1	8*	
5	12-16 (12)	26-32 (29)	1	37-51 (38)	3-4 (3)	4-7 (6)	6-8 (6)	3-4 (4)	5-6 (6)	5-6 (6)	8-9 (8)	4-9 (9)	-	
6	15-16 (15)	1	2-3 (2)	1-2 (2)	26-34 (31)	26-39 (34)	23-25 (24)	1	1	1	2-4 (3)	1-S	4-8 (6)	
7	19-23 (19)	23-35 (31)	2-4 (3)	38-47 (45)	23-33 (28)	24-35 (28)	1-2 (2)	5-7 (6)	2-3 (3)	2-3 (2)	3-6 (5)	1-S	3-8 (6)	
8	4-7 (4)	22-32 (30)	23-24 (23)	35-45 (40)	-	n.c.	n.c.	2-4 (2)	3-4 (3)	2-3 (2)	n.c.	4-S	1-2 (1)	
9	5-6 (6)	1	1	1	3-4 (4)	2-4 (3)	5-8 (6)	4-6 (5)	4-6 (6)	9-11 (11)	3-5 (4)	3-S	1	
10	1-3 (3)	1	1	1	1	3-4 (3)	1-2 (1)	1-2 (2)	1	1-2 (2)	1-3 (3)	5-S	2-4 (4)	
11	n.c.	1-4 (2)	1-2 (1)	1	2-3 (3)	1-2 (1)	2-3 (2)	1-3 (3)	1-3 (3)	1-2 (2)	1-2 (2)	1-S	2-4 (4)	
12	1-3 (3)	1	1	1-2 (2)	1-2 (2)	1	2-3 (2)	1-4 (3)	2-3 (2)	1-2 (2)	1	-	-	
13	6-8 (8)	3-5 (5)	4-5 (4)	n.c.	6-8 (6)	n.c.	3-4 (4)	4-6 (6)	4-5 (4)	11-12 (12)	3-5 (4)	-	-	
14	1-4 (2)	7-8 (8)	7-11 (9)	-	-	-	1	1	1	1	1	1	-	
15	3-5 (4)	-	-	-	-	-	-	-	-	-	-	-	-	

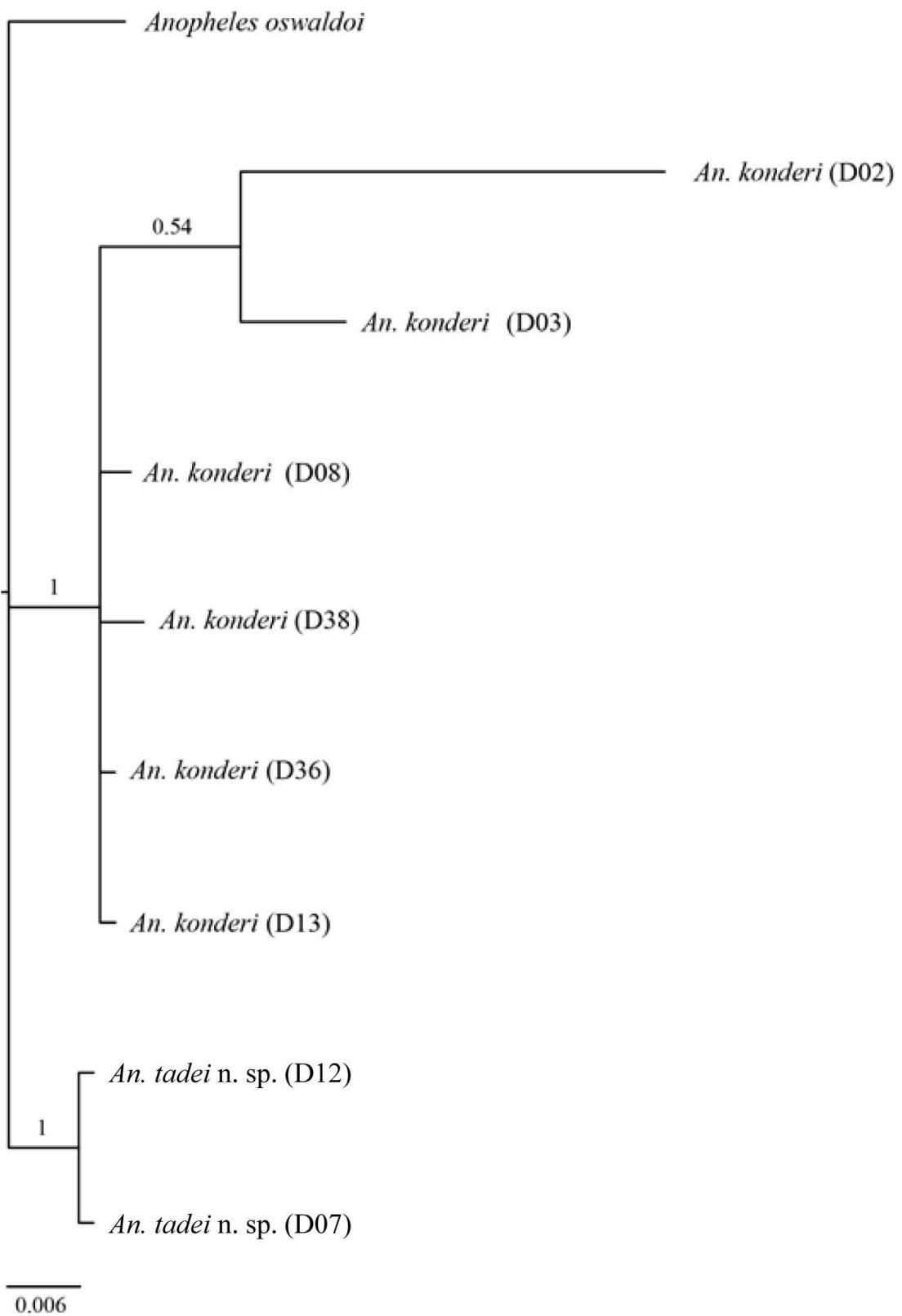
n.c. – not counted, * – 8 pairs.

TABLE 5. Mean genetic distance values based on the Kimura 2-Parameters model obtained for the *COI* gene and uncorrected p-distance model obtained for the *CAD* gene and ITS2 region.

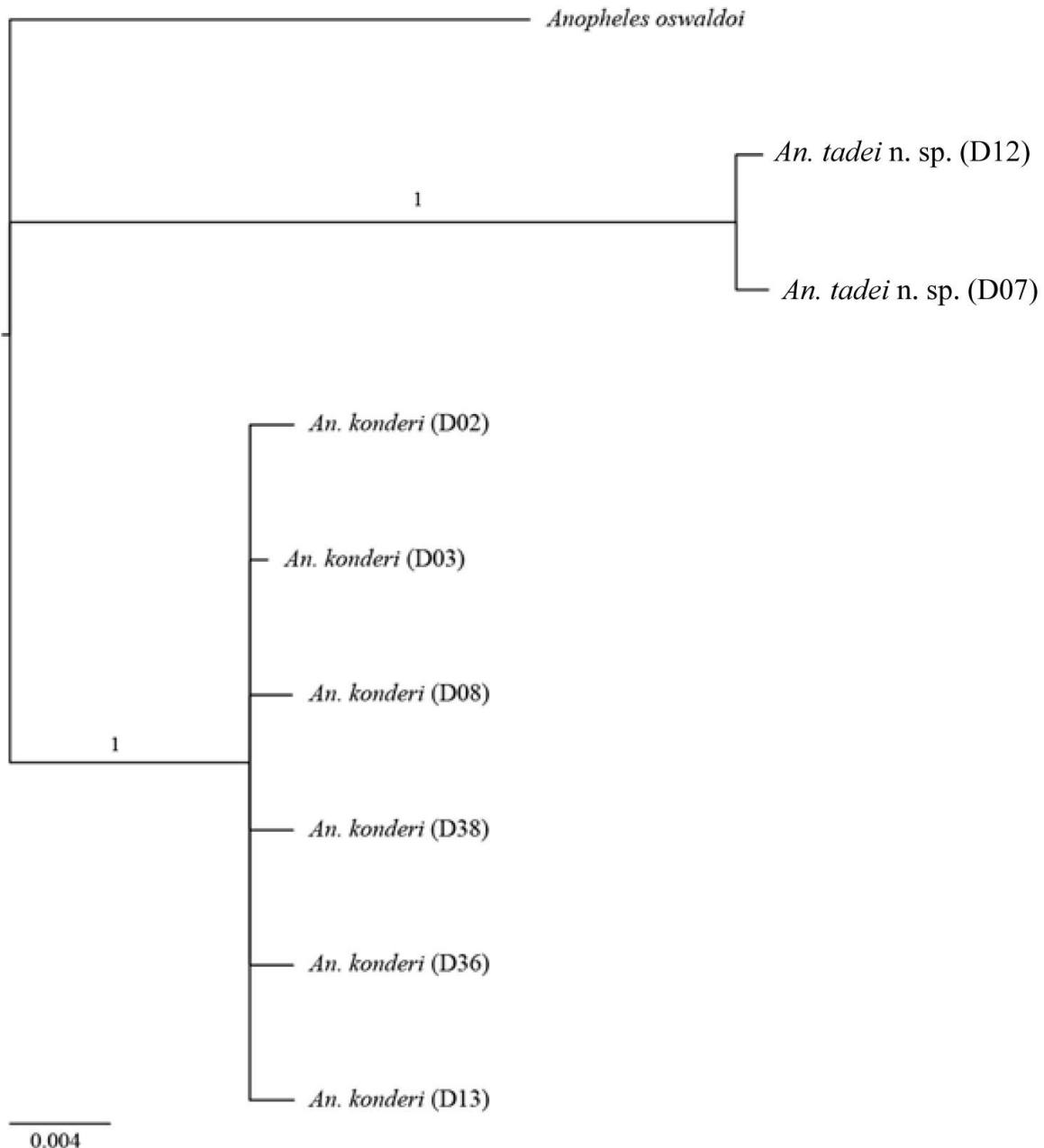
Species	<i>COI</i>	<i>CAD</i>	ITS2
<i>An. konderi</i> s.s. (intraspecific values)	0.005 ± 0.002	0.002 ± 0.001	0.001 ± 0.001
<i>An. tadei</i> n. sp. (intraspecific values)	0.002 ± 0.002	0.001 ± 0.001	0.002 ± 0.002
<i>An. konderi</i> x <i>An. tadei</i> n. sp.	0.040 ± 0.008	0.042 ± 0.007	0.038 ± 0.009



Supplemental Figure 1. Bayesian Inference tree generated for the COI data of *An. tadei* n. sp. (GRT + G model). Numbers at branches indicate Bayesian posterior probability (≥ 0.70). *Anopheles oswaldoi* s.s. were included as outgroup.



Supplemental Figure 2. Bayesian phylogenetic analysis of the Internal Transcribed Spacer 2 (ITS2) of *An. tadei* n. sp. (F81 + I model). Numbers on the branches indicate Bayesian posterior probability (≥ 0.70). *Anopheles oswaldoi* s.s. was included as outgroup.



Supplemental Figure 3. Bayesian phylogenetic analysis of the nuclear *CAD* gene of *An. tadei* n. sp. (GTR + I + G model). Numbers on the branches indicate Bayesian posterior probability (≥ 0.70). *Anopheles oswaldoi* s.s. was included as outgroup.

Supplemental table 4. Variable sites for the *COI* gene.

		1	2	2	2	2	2	3	3	3	3	3	3	4	4	4	5	5	5	5	6	6	6	6					
Sequences		4	5	8	9	3	1	2	5	7	7	2	3	4	4	5	8	0	1	9	0	1	8	8	9	2	2	3	4
		2	7	7	6	2	0	8	8	6	9	4	6	5	8	1	1	8	4	3	7	0	2	6	1	1	4	9	2
<i>An. konderi</i> Coari D02		A	G	C	A	C	G	C	T	G	T	A	G	T	C	C	A	T	T	C	T	A	T	C	A	C			
<i>An. konderi</i> Coari D03		G	.	.	G	.	.	T	T	T	.	.	.	T	T			
<i>An. konderi</i> Coari D08		G	.	.	G	.	.	T	G	.	
<i>An. konderi</i> Coari D13		T	G	.	
<i>An. konderi</i> Borba D36		T	
<i>An. konderi</i> Borba D38		T	
<i>An. tadei</i> n. sp. Coari D07		.	A	T	.	T	A	.	C	A	C	G	A	A	T	T	T	C	C	T	A	G	A	T	G	C	T	.	
<i>An. tadei</i> n. sp. Coari D12		.	A	T	.	T	A	.	C	A	C	G	A	A	T	T	T	C	C	T	A	G	A	T	G	C	T	.	T

Fragment size = 644 bp.

Supplemental table 5. Variable sites for the *CAD* gene.

	1	1	1	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	5	5	5	6	6	6	6	7	7	7	7					
Sequences data	9	1	1	2	4	5	6	8	1	2	2	3	6	0	1	2	4	4	7	0	7	9	4	4	6	6	6	7	8	9	0	4	6	9
	7	5	9	7	9	9	9	7	4	0	9	0	3	7	0	5	6	9	0	2	2	0	4	7	0	1	4	3	8	4	9	2	6	0
<i>An. konderi</i> Coari D02	T	T	G	T	T	G	T	A	T	A	A	C	G	T	A	C	G	G	C	G	C	A	G	C	G	A	T	T	G	A	T	C	T	
<i>An. konderi</i> Coari D03	G
<i>An. konderi</i> Coari D08	G	G	.	.	.
<i>An. konderi</i> Coari D13	G	A	
<i>An. konderi</i> Borba D36	G	.	A	
<i>An. konderi</i> Borba D38	G	A	A	.	.	
<i>An. tadei</i> n. sp. Coari D07	C	G	C	C	C	A	A	C	T	C	G	G	A	A	C	G	G	.	C	T	C	A	G	C	.	C	C	C	A	.	C	G	C	
<i>An. tadei</i> n. sp. Coari D12	C	G	C	C	C	A	A	C	T	C	G	G	A	A	C	G	G	.	C	T	C	A	G	C	.	C	C	C	A	.	G	C	G	C

Fragment size = 793 bp.

Supplemental table 6. Variable sites for the ITS2 region.

	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	
Sequences data	1	1	4	9	1	2	4	5	9	0	0	0	0	1	3	3	4	4	6	6
	7	8	3	4	6	4	5	4	9	2	3	5	8	9	1	7	3	5	0	8
<i>An. konderi</i> Coari D02	-	-	T	A	C	A	A	T	C	G	A	G	A	C	T	A	C	C	G	A
<i>An. konderi</i> Coari D03	-	-	A	.	.	
<i>An. konderi</i> Coari D08	G	C	A	.	.		
<i>An. konderi</i> Coari D13	-	-	
<i>An. konderi</i> Borba D36	-	-	
<i>An. konderi</i> Borba D38	T	C	
<i>An. tadei</i> n. sp. Coari D07	G	A	C	T	.	G	G	C	A	C	G	A	C	T	C	G	T	A	A	C
<i>An. tadei</i> n. sp. Coari D12	G	A	C	T	A	G	G	C	A	C	G	A	C	T	C	G	T	A	A	C

Fragment size = 474 bp.

Capítulo 3

**Filogeografia de *Anopheles (Nyssorhynchus) oswaldoi* A (Diptera:
Anophelinae) da Amazônia brasileira: uma espécie do Complexo
Oswaldoi-Konderi**

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Resumo

Anopheles oswaldoi A pertence ao complexo Oswaldoi-Konderi juntamente com outras quatro espécies (*An. oswaldoi* s.s., *An. oswaldoi* B, *An. konderi* e *An. sp. nr. konderi*). Esta espécie até o momento foi reportada somente na bacia amazônica (Brasil e Colômbia), sendo que no Brasil é amplamente distribuída. Estudos anteriores apontaram que *An. oswaldoi* A pode ser um vetor secundário de malária na região amazônica. Além disso, o mais recente estudo molecular revelou que *An. oswaldoi* A pode consistir de pelo menos três linhagens genéticas. No presente estudo amostras de *An. oswaldoi* A da Amazônia brasileira foram analisadas para a região variável do gene *COI* (DNAm_t) para testar a hipótese se estas linhagens genéticas representam espécies incipiente dentro *An. oswaldoi* A. Quarenta e dois indivíduos procedentes de três localidades (Novo Progresso - PA, Parna do Viruá - RR e Lábrea - AM) foram sequenciados. Elevada diversidade genética foi observada, com 28 haplótipos. As análises populacionais indicaram cinco grupos na rede de haplótipos e no BAPS. Forte estruturação genética foi observada para a população de Lábrea (AM). A datação molecular estimou uma divergência muito recente (~1,02 milhões de anos) para a população de Lábrea, no período Pleistoceno. Estudos subsequentes devem ser realizados para investigar o papel de cada grupo como vetor de malária humana na Amazônia.

Palavras-chave: *Anopheles oswaldoi*, Complexo Oswaldoi-Konderi, Genética de populações, Malária.

Introdução

A região Neotropical concentra uma rica biodiversidade que vem sendo extensamente estudada para compreender os processos de diversificação biológica (Rull, 2008). Diversas hipóteses foram propostas para explicar os processos geradores de biodiversidade na Amazônia, por exemplo, a hipótese dos rios (Wallace, 1852), hipótese dos refúgios (Haffer, 1969; Vanzolini e Williams, 1970) e hipótese de gradientes ecológicos (Blackburn e Gaston, 1996; Hawkins, 2001). Entretanto, recentemente estas hipóteses estão sendo mais rigorosamente testadas, graças aos avanços analíticos e teóricos das últimas décadas (Moritz *et al.* 2000). Em especial, a filogeografia, que têm como objetivo central entender os princípios e processos que governam a distribuição geográfica das linhagens intraespecíficas ou de espécies relacionadas, baseada na distribuição espacial de genealogias gênicas (Avise, 2000). Além disso, estes avanços também têm proporcionado *insight* para novas hipóteses de diversificação (Hoorn *et al.*, 2010; Antonelli e Sanmartín, 2011).

Os anofelinos da região Neotropical mostram uma extraordinária diversidade e complexidade, em parte, devido à capacidade desses insetos dípteros de se adaptarem à uma ampla variedade de nichos ecológicos (Grimaldi e Engel, 2005). Somente dentro do subgênero *Nyssorhynchus* há uma extensa variação morfológica e genética, que podem estar relacionadas à processos de divergência recente de linhagens (Conn e Mirabello, 2007). Estes processos de divergência muitas vezes são atribuídos às mudanças climáticas ocorridas em diferentes épocas, que teriam provocado modificações nos habitats originais (Conn e Mirabello, 2007). Entretanto, devido à escassez de dados sobre a distribuição dos vetores e até mesmo a ausência de um padrão de distribuição geográfica dos mosquitos,

dificultam a compreensão de quais processos estarão envolvidos na origem da diferenciação populacional ou ainda no processo de especiação (Saraiva *et al.*, 2018).

Outro fator complicador para entender os padrões filogeográficos, se deve à uma grande quantidade de complexos de espécies dentro do gênero *Anopheles* Meigen (Harbach, 2004), incluindo várias espécies de vetores. Os complexos de espécies são grupos de espécies morfologicamente idênticas ou quase idênticas e que são nomeadas como uma única espécie (Mayr, 1963). No grupo Oswaldoi (*Anopheles*, Subgênero *Nyssorhynchus*), sete das 15 espécies consistem de complexos de espécies crípticas (Marrelli *et al.*, 2006; Motoki *et al.*, 2009; Rosa-Freitas *et al.*, 1998; Silva-do-Nascimento *et al.*, 2007; Sallum *et al.*, 2008; Scarpassa e Conn, 2006) e algumas destas estão implicadas na transmissão de malária (Quiñones *et al.*, 2006).

Recentemente, estudos confirmaram que o complexo Oswaldoi-Konderi, comprehende cinco espécies (*An. oswaldoi* s.s., *An. oswaldoi* B, *An. oswaldoi* A, *An. konderi* e *An. sp. nr. konderi*), com ampla distribuição geográfica na Amazônica brasileira (Saraiva *et al.*, 2018). Estudos retrospectivos, de infecção natural e a distribuição das espécies deste complexo permitiram inferir que *An. oswaldoi* A, *An. oswaldoi* B e *An. sp. nr. konderi* podem ser vetores de malária na região amazônica (Ruiz-Lopes *et al.*, 2013). Entretanto, estudos subsequentes mostraram que muitas dessas espécies ocorrem em simpatria nas localidades em que foram detectados mosquitos infectados, tornando difícil conhecer qual destas espécies estaria envolvida na transmissão do parasita da malária sem testes de infecção e identificação molecular subsequente (Saraiva *et al.*, 2018). Além disso, *An. oswaldoi* A mostrou uma ampla distribuição na bacia amazônica brasileira, com pelo menos três linhagens genética, provavelmente de divergência recente, apontadas por Saraiva e colaboradores (2018).

O presente estudo teve como objetivo estudar os processos de divergência de *An. oswaldoi* A utilizando dados genéticos, gene *COI*, para entender quais processos históricos se deu a diversificação do complexo na região amazônica.

Materiais e Métodos

Captura dos mosquitos

As amostras de *An. oswaldoi* A foram coletadas em três localidades da Amazônia brasileira: Mina Palito, município de Novo progresso (MP), Estado do Pará (06°26'4.17"S; 55°57'3.60"W), Parque Nacional do Viruá (Parna do Viruá), município de Caracaraí (PV), Estado de Roraima (01°29'1.29"N; 60°59'7.58"W) e Palmari, município de Lábrea (LB), Estado do Amazonas (7°39'52.3"S; 65°04'11.1"W). As amostras foram obtidas entre setembro de 2016 e janeiro de 2018, com armadilha luminosa, modelo Shannon (Shannon, 1939). As amostras de LB foram gentilmente cedidas pelo Dr. Ronildo B. Alencar e foram coletadas com armadilha luminosa tipo CDC. Os mosquitos capturados foram armazenados em tubos de 1,5mL, contendo etanol 95%. As amostras foram preservadas no freezer -20°C até a extração do DNA.

Extração de DNA, amplificação por PCR, purificação e reação de Sequenciamento

O DNA genômico foi extraído individualmente das pernas dos mosquitos adultos coletados na natureza. Para a extração de DNA foi utilizado o protocolo Fenol-Clorofórmio, descrito em Green e Sambrook (2012). Um fragmento de ~1.200 bp do gene *COI* (Citocromo Oxidase Subunidade I) do DNA mitocondrial, foi amplificado com os primers UEA3 e UEA10, com as condições da reação da PCR descritas em Zhang e Hewitt (1997). Os produtos de PCR foram checados em gel de agarose à 1%, analisado e

fotodocumentado. O produto da reação da PCR foi purificado com PEG 8,000 e posteriormente sequenciado utilizando o Kit Big Dye Terminator Cycle. Todas as amostras foram sequenciadas em ambas as direções (Forward e Reverse) utilizando o analisador automático ABI 3130 xl (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) no Laboratório Temático do Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus.

Alinhamento, edição das sequências e seleção do modelo evolutivo

As sequências foram alinhadas separadamente (Forward e Reverse) utilizando o alinhamento automático do ClustalW e editadas manualmente no BioEdit v.7.2.5 (Hall 2011) com o exame visual dos eletroferogramas no Chromas Lite v.2.6.5 (<http://technelysium.com.au/wp/chromas/>). Posteriormente, as sequências consenso foram geradas a partir das fitas separadas (Forward e Reverse) e checadas novamente no Chromas Lite. As sequências consenso foram comparadas com aquelas depositadas previamente no GenBank® (<http://ncbi.nlm.nih.gov>) com a ferramenta BLAST® - Basic Local Alignment Search Tool (Altschul *et al.* 1997). Esta etapa teve como objetivo verificar as possíveis contaminações, tais como amplificação de sequências que não corresponde à *Anopheles* spp. Posteriormente, o banco de dados foi analisado para a seleção do melhor modelo evolutivo de substituição nucleotídica, utilizando o jModelTest v.2.1.10 (Darriba *et al.* 2012) e da aplicação da correção com o Critério de Informação Akaike (AIC).

Inferências bayesianas e estimativa do tempo de diversificação

A árvore de gene foi gerada no programa BEAST v.1.10.4 (Drummond *et al.*, 2012), utilizando o modelo evolutivo previamente selecionado no jModelTest. Para esta

análise foi considerada a taxa de mutação de 2,3% a cada milhão de anos (ou seja, 0.0115 substituições/sítios por linhagem), previamente proposta para o gene *COI* de insetos (Brower, 1994), com o relógio molecular relaxado (*uncorrelated lognormal*), utilizando o modelo de especiação "*Yule process*". Quatro corridas independentes foram realizadas, com 200 milhões de gerações cada e com amostragens à cada 10 mil passos. As corridas eram checadas se haviam atingido o valor de convergência esperado (ESS > 200) no programa Tracer v.1.4 (Rambaut e Drummond, 2014). Em seguida, as quatro corridas foram combinadas utilizando o LogCombiner (Drummond *et al.*, 2012). As árvores foram summarizadas no programa TreeAnnotator v.1.10.4 (Drummond *et al.*, 2012) e computada a árvore de máxima credibilidade (MCC). A árvore final gerada foi visualizada e editada no programa FigTree v.1.4.4 (Rambaut, 2018). Como grupo externo foi utilizado duas sequências de *An. konderi* s.s. de Coari, Estado do Amazonas.

Diversidade genética, demográfica e estrutura populacional

As populações estudadas foram agrupadas por localidade de origem e, em seguida, foram calculados os valores de distância genética intra e interpopulacional no programa MEGA v.7.0.26 (Kumar *et al.* 2018). Para avaliar a diversidade genética, foram estimados os valores de diversidade haplotípica (H) e nucleotídica (π). No Arlequim v.3.5 (Excoffier e Lischer, 2010) foi testado a hipótese nula de panmixia entre as populações, realizando uma Análise de Variância Molecular (AMOVA), que avalia a existência de populações geneticamente divergentes. Os testes de neutralidade, D de Tajima (Tajima, 1989) e Fs de Fu (Fu, 1997), foram empregados para verificar cenários de expansão populacional, considerando sítios segregantes, frequência de mutações, permitindo detectar desvios da neutralidade e flutuações demográficas históricas (expansão ou retração). Estas análises foram realizadas no programa DnaSP v.6.0.73 (Rozas *et al.*, 2017), baseadas em 10.000

réplicas (simulações de coalescência) assumindo neutralidade e equilíbrio nas populações estudadas.

Para inferir as relações entre os haplótipos recuperados, uma rede de haplótipos foi gerada no programa PopArt v.1.7 (French *et al.*, 2014), utilizando o método *Median-Joining* (MJ) (Bandelt *et al.*, 1999). Para identificar estruturações mais profundas dentro de *An. oswaldoi* A, foi realizada uma análise da estrutura genético-populacional utilizando o programa BAPS v.6 (*Bayesian Analysis of Population Structure*) (Corander *et al.*, 2008). A primeira foi uma análise *mixture* dos indivíduos definindo o número máximo de grupos (K) para em seguida ser realizada uma análise de *admixture*.

Por fim, uma reconstrução filogeográfica espaço-temporal foi inferida utilizando os mesmos parâmetros de corrida da árvore bayesiana no BEAST e o mesmo modelo selecionado no jModelTest. O *Bayesian of Relaxed Random Walk* (RRW) foi utilizado para inferir preliminarmente a localização geográfica do nó ancestral, assim como, a difusão contínua das linhagens ao longo do espaço e do tempo, levando em consideração as incertezas na topologia recuperada (Lemey *et al.*, 2010). A árvore de máxima credibilidade (MCC) foi utilizada para gerar uma representação visual da expansão das linhagens ao longo do tempo, utilizando o *Continous Tree* implementado no programa SPREAD v.1.0.7 (Bielejec *et al.*, 2011), o arquivo kml (*Keyhole Markup Language*) gerado foi visualizado sobre o mapa do Google Earth (<http://earth.google.com>).

Resultados

Quarenta e dois espécimes de *An. oswaldoi* A foram analisados de três localidades da Amazônia brasileira. Após o alinhamento, a sequência consenso do gene *COI* apresentou 1.211pb de comprimento. Vinte e oito haplótipos foram identificados (Tabela

suplementar 1) e não houve compartilhamento de haplótipos entre as três localidades estudadas. As amostras da localidade MP apresentaram o maior número de haplótipos (15), sendo a maioria únicos ($n=14$); apenas o haplótipo H2 foi compartilhado por três indivíduos, mas da mesma localidade. A amostra de PV gerou 10 haplótipos. quatro haplótipos tiveram o maior número sequências. H17 teve três sequências e os haplótipos H18, H20 e H21 apresentaram duas sequências cada. A amostra de LB apresentou o menor número de haplótipos ($n=3$) denominados de H26, H27 e H28. A figura 1 apresenta a distribuição dos haplótipos por localidade estudada.

A composição média de nucleotídeos, excluindo o grupo externo, foi de 29,5% (A), 39,4% (T), 16,2% (C) e 14,9 (G), com o conteúdo de A+T de 68,9%. O banco de dados apresentou ainda 70 sítios filogeneticamente informativos para o critério de parcimônia de um total de 147 sítios variáveis. A distância genética calculada foi a menor entre MP e LB (2,2%), enquanto a maior distância p encontrada foi entre PV e as populações de LB e MP (3,0%). Os valores de distância genética intra-populacional foram 1,5% e 1,4% para MP e PV, respectivamente, e de 0,5% para LB (Tabela 1).

As três populações estudadas foram separadas em cinco grupos na rede de haplótipos *Median-joining* (Figura 2). A população MP apresentou quatro subgrupos que se subdividiram de maneira fortemente reticulada e com homoplasias no centro da rede, conectando as três populações. O haplótipo H10 de MP foi separado por 63 eventos mutacionais, estimados pelo PopArt. A amostra de PV apresentou três grupos. O haplótipo H25 de PV originou de um nó distinto dos demais indivíduos da população. Na população de LB, os haplótipos foram conectados em um único grupo e originaram do mesmo nó, próximo à população de MP. Poucos eventos mutacionais separam os indivíduos dentro desta população, com exceção do H27, que apresentou oito eventos mutacionais (Figura 2).

Os índices de diversidade genética (Tabela 2) mostram altos valores de diversidade haplotípica para as populações de MP ($0,978 \pm 0,031$) e PV ($0,943 \pm 0,040$), enquanto em LB esta estimativa apresentou valor menor ($0,711 \pm 0,086$). Os valores apresentados refletiram claramente no número de haplótipos recuperados em cada população, em que MP foram obtidos 15 haplótipos, enquanto LB somente três haplótipos recuperados. Com relação à diversidade nucleotídica (π) os valores foram baixos, variando de 0,005 a 0,015.

Os testes de neutralidade D de Tajima para as populações MP e PV tiveram valores negativos, mas não foram significantes, enquanto o teste Fs de Fu foi negativo apenas para MP e não foi significante (Tabela 2). Portanto, com base neste resultado, o modelo neutro não deve ser rejeitado.

Na análise do BAPS, os mesmos cinco grupos foram recuperados. Nesta análise, a amostra de MP apresentou três grupos (sem o grupo 5), enquanto na rede de haplótipos foram observados quatro grupos. O Log ótimo estimado para os cinco grupos pelo BAPS, foi de $Ln -1614.6374$ (Máxima Verossimilhança) indicando estruturação dentro de *An. oswaldoi* A (Figura 2).

A inferência bayesiana (Figura 3) também separou as três populações analisadas em quatro grupos. A MP foi a amostra que apresentou maior número de haplótipos que estiveram presente em outros grupos, seus haplótipos foram recuperados dentro de três grupos (G-01, G-02 e G-03). O haplótipo 10 de MP (G-01), foi o mais divergente, recuperado como um ramo fora do grande clado que forma os outros quatro grupos restantes. O grupo G-04 reuniu apenas haplótipos de PV. Nenhuma população recuperada, apresentou monofilia recíproca. Por exemplo, apesar do grupo 4 reunir maioria de haplótipos de PV, o H25 (PV), foi recuperado em G-02. LB foi restrita ao G-

03, mas ainda é um grupo parafilético por apresentar um haplótipo (H08) da população de MP (Figura 4).

A análise hierárquica de variância molecular (AMOVA), incluindo as três populações estudadas (não agrupadas), revelou elevada e significante estruturação genética entre as populações ($F_{ST} = 0.552, p < 0.001$) (Tabela 3). A diferenciação genética par-a-par usando a estimativa F_{ST} apresentou valores muito elevados e significantes (0.506-0.671) (Tabela 4), consequentemente os valores de Nm foram menores que 1 indicando isolamento geográfico entre elas. As populações PV e LB foram as mais diferentes (0.671), e tiveram o menor Nm (0.245), número de migrantes por geração. O nível de significância nominal ($P < 0.05$) ajustado pelo procedimento sequencial de Bonferroni (=0,02), mostrou valores que indicam forte diferenciação genética entre as populações (Tabela 4).

O modelo de difusão RRW estimou a origem geográfica das linhagens de *An. oswaldoi* A para o interflúvio dos rios Tapajós e Madeira, no Estado do Pará, próximo à calha do rio Amazonas (Latitude: 95.87; Longitude: 42.01, 80% HPD). A estimativa de divergência desta espécie é de ~1.8 milhões de anos atrás (m.a.a.) para a primeira linhagem (MP). Em seguida, a população MP apresentou uma expansão geográfica de ~1.49 m.a.a. Em seguida à ~1.18 m.a.a. há expansão para população de PV, no lado oposto do rio Amazonas. Por fim, a população LB surge da expansão de MP à ~1.02 m.a.a. (Figura 3). As estimativas da difusão espacial para *An. oswaldoi* A foram datadas entre o final do Plioceno (~1.8 m.a.a.) e início do Pleistoceno (a partir de ~1.6 m.a.a.), indicando uma divergência muito recente.

Discussão

Anopheles oswaldoi A é uma das espécies não descrita do complexo Oswaldoi-Konderi. Para a sua descrição taxonômica será necessário o exame do material tipo de *An. aquacaelestis* Curry, que atualmente é o único sinônimo de *An. oswaldoi* s.s. (Saraiva *et al.* 2018) registrado no Panamá por Curry (1932); entretanto, nossos dados indicam que *An. oswaldoi* A têm distribuição restrita à Amazônia brasileira e colombiana (Ruiz-Lopez *et al.* 2013; Saraiva *et al.* 2018) e apenas *An. oswaldoi* B coincide com a distribuição de *An. aquacaelestis*. De qualquer forma, será prudente reexaminar o material tipo para fornecer estabilidade taxonômica a estas duas espécies, que morfologicamente, também podem ser separadas por meio do exame da genitália masculina, em que o claspete ventral e a forma do ápice do edeago são claramente distintos das demais espécies do complexo Oswaldoi-Konderi. Por outro lado, os grupos ou linhagens encontradas neste estudo e nas análises anteriores (Saraiva *et al.*, 2018), não apresentaram diferenças morfológicas claras que pudessem separá-las.

Nossos resultados reforçam as observações do estudo anterior (Saraiva *et al.* 2018), em que três linhagens genéticas foram identificadas, utilizando uma região menor do gene *COI* (DNA Barcode) para *An. oswaldoi* A. Além disso, ao utilizarmos as sequências de Moju, Pará, do estudo de Scarpassa e Conn (2006), as três sequências de Moju agruparam com as nossas sequências de Mina Palito, reforçando a hipótese de três grupos fortemente suportados. Desta forma, embora no presente estudo tenham sido amostradas apenas três localidades, devido à baixa densidade desta espécie na mesma localidade, inferior à quatro indivíduos, optamos em utilizar apenas as localidades que tiveram número igual ou superior à 10 indivíduos, tornando as análises populacionais

mais robustas. Com uma análise mais ampla provavelmente estes três grupos permaneceriam.

Como revelado neste estudo, as três populações estudadas foram fortemente apoiadas para formar quatro grupos na árvore de inferência bayesiana. Com exceção do haplótipo H10 que foi altamente divergente. H10 foi recuperado como grupo mais basal, e pode representar sub-amostragens, podendo indicar um novo grupo dentro de *An. oswaldoi* A não recuperado aqui, ou ainda, se novas amostragens forem realizadas, esta diferença pode ser diluída, e em futuras análises H10 pode ser recuperado dentro um clado maior, e mais diversos. Desta forma, novas amostragens gerarão informações que esclarecerão esta problemática.

Os testes de análise hierárquica e de distância par-a-par F_{ST} para as populações estudadas também mostraram elevada estruturação genética. Tendo em vista a recente divergência entre estes grupos, hipotetizamos que em uma escala macro, a distância geográfica e os grandes cursos dos rios da Amazônia podem atuar como barreira ao fluxo gênico. E na escala micro, a disponibilidade de criadouros viáveis e a cobertura florestal podem atuar também como fator limitante à dispersão de *An. oswaldoi* A. Estudos mostraram que as barreiras geográficas são um dos principais determinantes da estrutura genética dos mosquitos em comparação com a distância geográfica (Braginets *et al.* 2003; Markianos *et al.*, 2016). No entanto, a distância geográfica e as barreiras ao fluxo gênico podem operar em combinação para gerar estrutura genética (Feng *et al.*, 2017). Por outro lado, é questionável se os rios também atuam como barreira efetiva para os mosquitos, pois para a atuação de uma barreira física proporcionada pelo rio, deve-se levar em consideração aspectos da biologia e ecologia de cada organismo analisado. Por exemplo, para 14 grupos taxonômicos (incluindo algumas ordens de insetos: Hymenoptera [Apidae e Formicidae], Coleoptera, Lepidoptera, Isoptera e

Orthoptera) o rio Madeira não foi uma barreira efetiva para delimitar as áreas de endemismo (Santorelli *et al.*, 2018). Além disso, com o tamanho corporal reduzido dos mosquitos e o fluxo de vento, pode ser um fator que pode proporcionar que indivíduos ultrapassem barreiras físicas e colonizem de novos ambientes (Gilliest e Wilkes, 1974; Lehmann *et al.*, 2014, Endo *et al.*, 2018). Isto pode estar ocorrendo para mosquitos que apresentaram haplótipos recuperados em margens opostas dos grandes rios na bacia amazônica.

Anopheles oswaldoi s.l. é caracterizado como zoofílico, e, portanto, restrito à ambientes com algum grau de preservação da cobertura florestal que possibilite a manutenção tanto de animais mamíferos para o repasto sanguíneo quanto de criadouros sombreados e calmos para a permanência e desenvolvimento das formas imaturas (ovos, larvas e pupas) (Forattini, 2002). As comparações pareadas (valores *Fst*) e a análise de variância molecular (AMOVA) mostraram que há forte estruturação genética entre as três localidades analisadas para *An. oswaldoi* A. Considerando-se que *An. oswaldoi* s.l. apresenta restrições quanto aos criadouros, que se caracteriza em estás sempre em áreas florestadas, sombreada, com vegetação emergente ou flutuante (Faran e Lithicum, 1981; Forattini, 2002), propomos também que a existência de haplótipos altamente divergentes, podem indicar sub-amostragens, tendo em vista a sensibilidade ecológica desta espécie sofrer à alterações nos seus criadouros naturais. Portanto, há de se considerar que mudanças na paisagem também podem provocar isolamento entre populações que anteriormente eram coesas e com livre fluxo gênico. Medeiros-Sousa e colaboradores (2019) ao conduzir estudo sobre os efeitos das mudanças na paisagem e a distribuição de *An. cruzii*, concluíram que mudanças no ambiente devido a atividades humanas seriam os responsáveis pela redução de *An. cruzii* na região de Mata Atlântica no Brasil. Originalmente, *An. cruzii* possuía ampla distribuição no Brasil, estendendo-

se das regiões sul ao nordeste do país (Zavortink, 1973). No entanto, com o aumento do desmatamento na Mata Atlântica, sua distribuição diminuiu consideravelmente (Forattini, 2002; Laporta *et al.* 2011).

Em nossos dados, os grupos revelados indicaram que a estruturação das populações iniciou entre ~1.8 ma.a. e ~1.02 m.a.a. Nesse período, mudanças na paisagem devido a alterações climáticas podem ter sido os responsáveis pela reconfiguração na distribuição dos organismos, e, além disso, isolado populações que demonstram estruturação genética atualmente. Tendo em vista a estruturação revelada neste estudo, são necessários mais estudos para elucidar o papel de cada grupo como vetor da malária e assim propor maneiras eficientes de controle.

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Lista de Figuras:

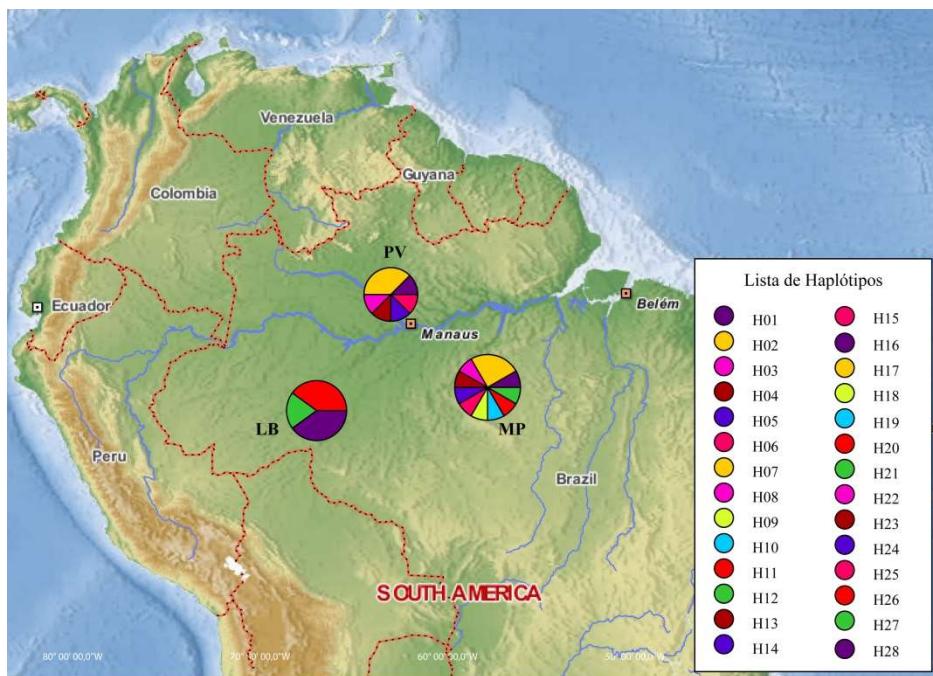


Figura 1. Mapa de distribuição dos haplótipos nos pontos de amostragens de *An. oswaldoi* A da região amazônica. Os haplótipos foram coloridos e representados segundo a proporção encontrada em cada localidade. MP - Mina Palito/PA, PV - Parna do Viruá/RR e LB - Lábrea/AM.

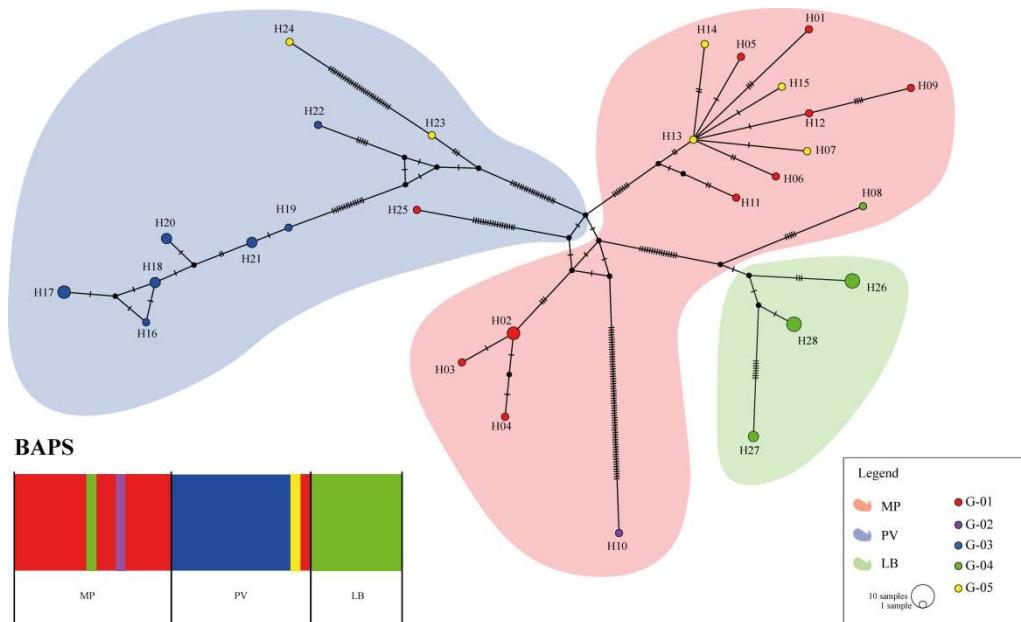


Figura 2. Rede de haplótipos *Median-Joining* e BAPS inferidos a partir dos 28 haplótipos observados nas três populações de *An. oswaldoi* A. Cada grupo recuperado está colorido conforme o BAPS. MP - Mina Palito/PA, PV - Parna do Viruá/RR e LB - Lábrea/AM, coloridos em; vermelho, azul e verde, respectivamente.

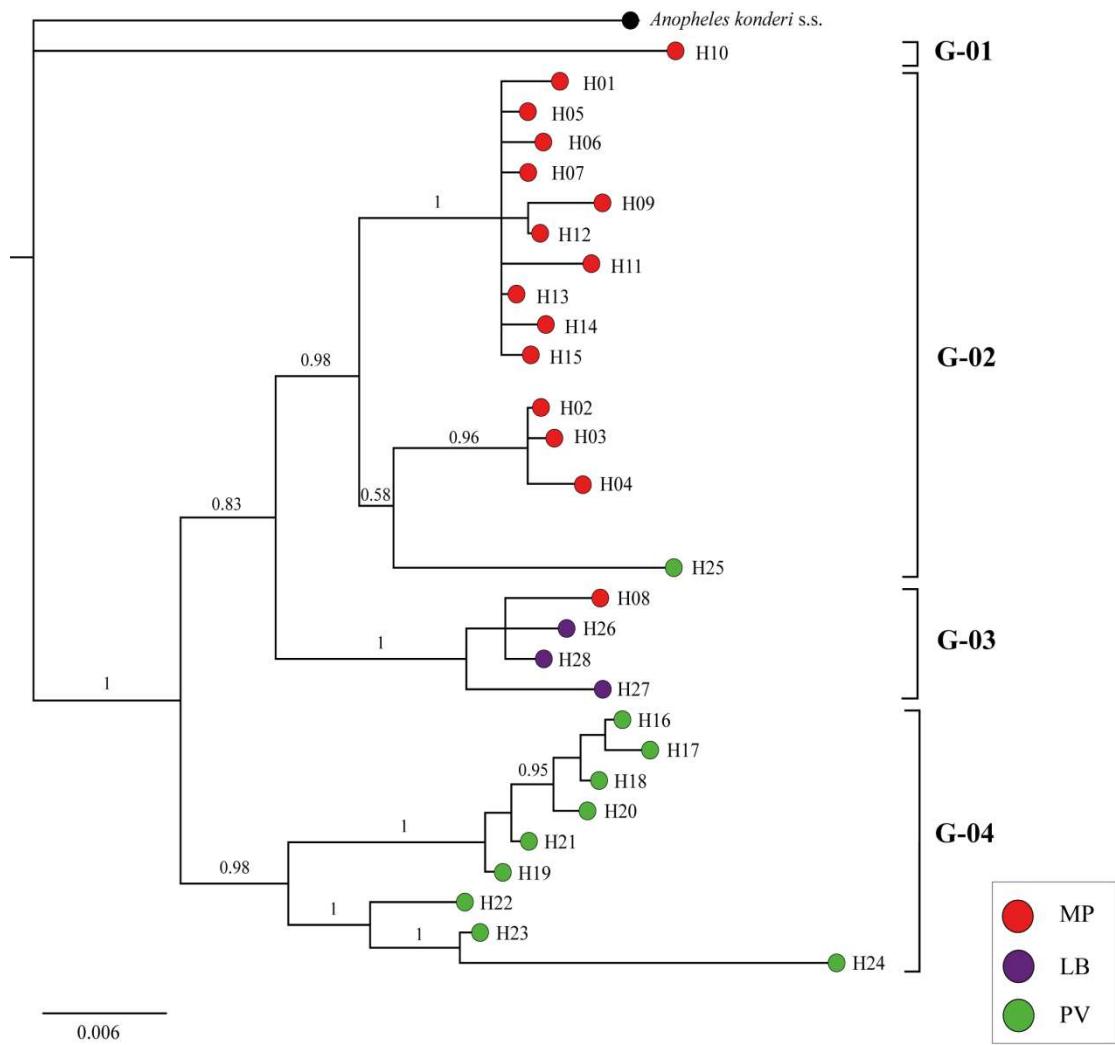


Figura 3. Inferência bayesiana com o gene *COI* de *An. oswaldoi* A. Os terminais da árvore representam os haplótipos ($n = 28$) recuperados para as três localidades estudadas (MP - Mina Palito/PA, LB - Lábrea/AM e PV - Parna do Viruá/RR). O suporte sobre os ramos é o valor de probabilidade posterior (BPP). Grupos foram delimitados e nomeados em ordem: G-01 a G-04. *Anopheles konderi* s.s. (Coari/AM) foi utilizado como grupo externo.

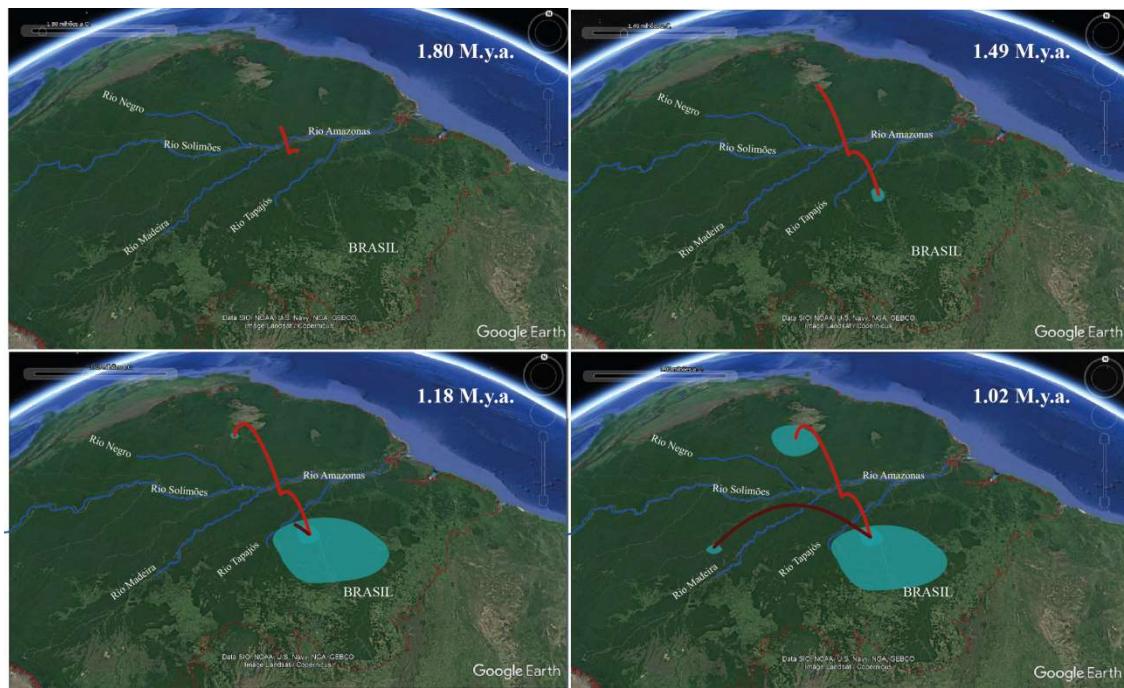


Figura 4. Difusão espaço-temporal bayesiana de três populações de *An. oswaldoi* A, espécie do complexo Oswaldoi-Konderi, em quatro intervalos de tempo. As reconstruções são baseadas na árvore de credibilidade máxima do clade estimada com uma abordagem filogeográfica bayesiana heterogênea e relaxada ao acaso (RRW). O sombreamento representa uma incerteza de 80% - HPD na localização dos ramos ancestrais, com tons mais escuros e claros representam eventos de difusão mais antigos e recentes, respectivamente.

LISTA DE TABELAS

Tabela 1. Distância genética (uncorrected-p) média (intra e interespecífica) para três populações de *Anopheles oswaldoi* A na Amazônia brasileira, obtido com 2.000 réplicas de bootstrap.

Populações	MP	PV	LB	<i>An. konderi</i>
MP	0.015 ± 0.002	0.004	0.003	0.005
PV	0.030	0.014 ± 0.002	0.004	0.006
LB	0.022	0.030	0.005 ± 0.001	0.006
<i>An. konderi</i>	0.053	0.057	0.052	0.002 ± 0.001

MP - Mina Palito, Novo Progresso (PA); PV - Parna do Viruá (RR); LB - Lábrea (AM). Os valores de distância intraespecífica estão destacados em negrito e o desvio padrão em azul.

Tabela 2. Índices de diversidade genética inferida para três populações de *Anopheles oswaldoi* A.

Populações	nº	NH	NS	Hd ± SD	π ± SD	Tajima's D	Fu's Fs
MP	17	15	93	0,978 ± 0,031	0,015 ± 0,005	-1,644	-2,167
PV	15	10	62	0,943 ± 0,040	0,014 ± 0,004	-1,164	1,472
LB	10	3	13	0,711 ± 0,086	0,005 ± 0,001	0,907	5,269
Total	43	31	133	0,978 ± 0,010	0,022 ± 0,002	-1,040	-2,025

nº: número de sequências, NH: número de haplótipos, NS: número de sítios segregantes, Hd: diversidade haplotípica, π: diversidade nucleotídica, SD: desvio padrão.

Tabela 3. Análise hierárquica (AMOVA) da variação genética em três populações de *An. oswaldoi* A da Amazônia brasileira.

Fonte da variação	d.f.	Soma dos quadrados	Componentes da variância	Porcentagem da variância
Todas as populações	2	275.355	9.51119 Va	56.03
Dentro das populações	39	297.122	7.46466 Vb	43.97
Total	41	566.476	16.97584	

Índice de Fixação FST: 0.56028

d.f.: graus de liberdade, teste de significância com 10.000 permutações.

Tabela 4. Distância genética par-a-par FST e matriz com o número de migrantes por geração (Nm), destacado em negrito na diagonal superior.

	MP	PV	LB
MP	-	0.486	0.472
PV	0.506	-	0.245
LB	0.514	0.671	-

Valor de significância p=0,05 (FST). Correção de *bonferroni* = 0,02.

Tabela suplementar 1. Lista de haplótipos e número de indivíduos por localidades.

Haplótipos	nº	Grupo	Localidade, Município e Estado
H01	1	G-02	Mina Palito, Novo Progresso, Pará.
H02	3	G-02	Mina Palito, Novo Progresso, Pará.
H03	1	G-02	Mina Palito, Novo Progresso, Pará.
H04	1	G-02	Mina Palito, Novo Progresso, Pará.
H05	1	G-02	Mina Palito, Novo Progresso, Pará.
H06	1	G-02	Mina Palito, Novo Progresso, Pará.
H07	1	G-02	Mina Palito, Novo Progresso, Pará.
H08	1	G-03	Mina Palito, Novo Progresso, Pará.
H09	1	G-02	Mina Palito, Novo Progresso, Pará.
H10	1	G-01	Mina Palito, Novo Progresso, Pará.
H11	1	G-02	Mina Palito, Novo Progresso, Pará.
H12	1	G-02	Mina Palito, Novo Progresso, Pará.
H13	1	G-02	Mina Palito, Novo Progresso, Pará.
H14	1	G-02	Mina Palito, Novo Progresso, Pará.
H15	1	G-02	Mina Palito, Novo Progresso, Pará.
H16	1	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H17	3	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H18	2	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H19	1	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H20	2	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H21	2	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H22	1	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H23	1	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H24	1	G-04	Parque Nacional do Viruá, Caracaraí, Roraima.
H25	1	G-02	Parque Nacional do Viruá, Caracaraí, Roraima.
H26	4	G-03	Palmari, Lábrea, Amazonas.
H27	2	G-03	Palmari, Lábrea, Amazonas.
H28	4	G-03	Palmari, Lábrea, Amazonas.

H=Haplótipos; G-01 à G-04=Grupos. Os grupos foram delimitados na árvore de inferência bayesiana.

3. CONSIDERAÇÕES FINAIS

- O presente estudo confirmou a existência de cinco espécies no complexo Oswaldoi-Konderi, apoiada em dados morfológicos, especialmente nos caracteres da genitália masculina (ápice do edeago e claspete ventral) e dados moleculares. Os dados moleculares superestimaram o número de unidades taxonômicas operacionais moleculares significativas (MOTU's), entre 14 e 18, com o gene *COI*.
- *Anopheles ininii*, registrado pela segunda vez no Brasil, foi recuperado como grupo irmão de *An. konderi*, *An. oswaldoi* A e *An. tadei* n. sp. Estudos futuros serão necessários para indicar se esta espécie pertence de fato ao complexo Oswaldoi-Konderi. Para isso, será necessário incluir nas análises sua espécie irmã, *An. sanctieli* cuja distribuição é restrita, até o momento, reportada na Guiana Francesa.
- *Anopheles tadei* n. sp. foi descrita, apoiada em dados moleculares e morfológicos. Esta espécie era denominada informalmente de *An. sp. nr. konderi* por Ruiz-Lopez *et al.* (2013) e *An. konderi* B por Foster *et al.* (2013). Os parátipos de *An. konderi* s.s. designados por Flores-Mendoza *et al.* (2004) a partir de material obtido nos Estados do Acre e Rondônia foram sinonimizados neste estudo com a nova espécie *An. tadei*, baseando-se nos caracteres da genitália masculina e no estágio larval. **O nome *Anopheles tadei* não é válido nesta publicação.**
- As duas localidades Anajás e Porto Velho analisadas para verificar infecção por *Plasmodium* spp. foram as mesmas onde *An. oswaldoi* s.l. havia sido reportado infectado em estudos anteriores. Entretanto, nenhum espécime coletado neste estudo foi positivo para a infecção por *Plasmodium* spp. Este resultado se deve ao baixo número de indivíduos do complexo Oswaldoi-Konderi capturados.
- O estudo filogeográfico de *An. oswaldoi* A mostrou expansão populacional e evidente estruturação genética para esta espécie. Os estudos anteriores, baseado em distribuição geográfica de *An. oswaldoi* A e os dados de infecção por *Plasmodium* spp. sugerem que *An. oswaldoi* A é um vetor potencial de malária. Embora não se observou mosquitos infectados, os resultados das análises filogeográficas indicaram três grupos genéticos muito divergentes que podem apresentar capacidade vetorial distintas.

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