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Research Article

Keywords: Culicoides, Biodiversity, Amazon, blood meal analysis, Oropouche virus

Posted Date: August 11th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10025629/v1

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Diversity, Host-Feeding Patterns, and Oropouche Virus Surveillance in *Culicoides* (Diptera: Ceratopogonidae) from the Western Brazilian Amazon

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Abstract

Culicoides Latreille biting midges (Diptera: Ceratopogonidae) are hematophagous insects of medical and veterinary importance due to their role in arbovirus transmission. In the neotropical region, species diversity remains incompletely characterized, particularly in the Amazon Basin. This study assessed the diversity and composition of *Culicoides* in Rondônia, Brazil, compared assemblages between forest fragments and peridomiciliary environments, identified vertebrate blood meal sources, and evaluated the presence of Oropouche virus (OROV) RNA. Specimens were collected using light traps and protected human attraction and identified based on morphological characters. Blood meals were analyzed using mitochondrial DNA markers, and RT-qPCR performed OROV detection. A total of 1,058 specimens were examined, representing 76 taxa, including 46 identified species and 30 morphotypes. The most abundant species were *Culicoides leopoldoi* Ortíz, *Culicoides glabellus* Wirth & Blanton, and *Culicoides pseudodiabolicus* Fox. Nine species were recorded for the first time in Rondônia, including four new records for Brazil. Forest fragments exhibited higher species richness and diversity than peridomiciliary areas, which harbored fewer

species but included taxa of epidemiological relevance. Blood meal analysis revealed a predominance of avian hosts, particularly *Gallus gallus* Linnaeus, as well as mammals, including humans, indicating opportunistic feeding behavior. No OROV RNA was detected in the 414 pools analyzed. These findings demonstrate high *Culicoides* diversity in western Amazonia and highlight the concentration of potential vector species at forest–human interfaces. Although no evidence of OROV circulation was detected during the sampling period, the presence of anthropophilic and generalist species underscores the importance of continued surveillance.

Keywords *Culicoides,* Biodiversity, Amazon, blood meal analysis, Oropouche virus

Introduction

Biting midges of the family Ceratopogonidae (Diptera) are a diverse group of small insects of ecological and sanitary importance, particularly due to hematophagous species capable of transmitting pathogens. These insects typically measure 2–5 mm in length and are characterized by short wings, absence of ocelli, and marked sexual dimorphism (Szadziewski 2011). Taxonomic identification relies primarily on morphological characters of the thorax, wings, and genitalia (Ferreira-Keppler et al. 2014). Immature stages develop in aquatic, semi-aquatic, or organic-rich terrestrial habitats, and many species act as vectors of pathogens of medical and veterinary relevance (Forattini 1957; Costa et al. 2013; Ronderos et al. 2018).

Within this family, the genus *Culicoides* Latreille stands out for its wide geographic distribution and epidemiological importance. Approximately 1,413 species are currently recognized worldwide, with 303 occurring in the Neotropical region. In Brazil, 153 species have been recorded, including 123 in the Amazon region (Borkent et al. 2025; Santarém and Felippe-Bauer 2024). Adult identification is mainly based on wing patterns and genital structures (Ronderos and Díaz 2015), although cryptic diversity remains a major challenge, particularly in highly biodiverse regions where integrative taxonomic approaches are still limited.

Culicoides species occupy a wide range of habitats, enabling persistence in both natural and anthropogenic environments (Mellor et al. 2000). Females are obligate blood feeders and often exhibit opportunistic host use, feeding on birds, mammals, reptiles, and amphibians (Borkent and Spinelli 2007; Carvalho 2021). However, host preference varies among species and environmental contexts, directly influencing vector–host–pathogen interactions and shaping transmission dynamics. In this context, molecular tools based on mitochondrial markers, particularly cytochrome *b*, have become essential for identifying vertebrate blood meal sources and elucidating feeding ecology (Townzen et al. 2008; Kent 2009).

Several *Culicoides* species are recognized vectors of arboviruses of medical and veterinary importance, including bluetongue virus, African horse sickness virus, Schmallenberg virus, and Oropouche virus (OROV) (Shelley and Coscarón 2001; Borkent and Spinelli 2007). OROV represents a growing public health concern in the Americas, with more than 29,000 reported cases between 2024 and 2025 across at least 11 countries, including Brazil, Bolivia, Colombia, Cuba, Ecuador, Guyana, and Peru (PAHO 2024). The Amazon region remains endemic, with transmission affecting both rural and urban populations and reinforcing the need for continuous entomological surveillance.

Oropouche fever is an acute febrile illness characterized by nonspecific clinical symptoms such as headache, myalgia, and arthralgia, which complicates clinical diagnosis and may lead to underreporting (Travassos da Rosa et al. 2017). Although OROV primarily circulates in sylvatic and urban cycles, *Culicoides paraensis* Goeldi, is recognized as its principais urban vector (Pinheiro et al. 1981). Recent molecular evidence has also suggested the involvement of additional potential vector species during outbreaks in the Brazilian Amazon, highlighting the complexity of transmission systems in anthropogenic environments (Feitoza et al. 2025).

Despite its epidemiological relevance, knowledge of *Culicoides* diversity, feeding behavior, and arbovirus circulation in the western Amazon remains limited. Most studies have been concentrated in eastern Amazonia, leaving substantial gaps in understanding species composition, ecological interactions,

and pathogen dynamics in regions such as Rondônia (Aitken et al. 1975). Ongoing environmental changes, including deforestation, agricultural expansion, and urbanization, may further reshape vector communities and increase opportunities for arbovirus emergence and spillover (Weaver and Reisen 2010; Lourenço-de-Oliveira and Failloux 2017).

In this context, a multi-approach strategy integrating taxonomy, ecological analysis of species assemblages, molecular identification of blood meal sources, and molecular screening for arboviruses is essential to improve understanding of vector dynamics in changing landscapes.

Therefore, this study aimed to (i) characterize the diversity and composition of *Culicoides* species in multiple municipalities of Rondônia state; (ii) compare assemblages between forest fragments and peridomiciliary environments; (iii) identify vertebrate blood meal sources using moleculares tools; and (iv) investigate the occurrence of Oropouche virus RNA in field-collected specimens.

Materials and Methods

Study Area

The study was conducted in the state of Rondônia, located in the western Brazilian Amazon, northern Brazil, encompassing an area of 237,754.17 km² and comprising 52 municipalities. The state capital is Porto Velho. Rondônia borders the states of Amazonas to the north, Acre to the west, Mato Grosso to the east, and Bolivia to the south and west. According to the latest population estimates, the state has approximately 1.58 million inhabitants (IBGE 2023).

The regional climate is classified as humid tropical, characterized by two distinct seasonal periods: a dry season from June to August and a rainy season from October to April (SEDAM 2003). Mean annual temperature is approximately 25.5 °C, with maximum temperatures reaching up to 34.4 °C (Aragão et al. 2018).

Rondônia is situated within a transition zone between the Central Brazilian and Amazonian geomorphological domains, comprising predominantly Amazon rainforest formations, interspersed with Cerrado vegetation and transitional ecosystems (SEDAM 2003). The state's hydrographic network is extensive and includes major river systems such as the Madeira, Ji-Paraná, Mamoré, Guaporé, and Jamari rivers.

Field collections were conducted in ten municipalities representing: Alta Floresta d'Oeste, Ariquemes, Cabixi, Colorado do Oeste, Cerejeiras, Guajará-Mirim, Jaru, Ouro Preto do Oeste, and Vilhena, as well as the district of Abunã (Porto Velho municipality) (Fig. 1).

Sampling Design

Municipalities were selected based on: (i) previous records of human Oropouche fever cases and (ii) logistical feasibility for field activities.

In each municipality, two environments were sampled: peridomicile areas and forest fragments. Five modified HP light traps were installed per site (one in peridomicile and four in forest), totaling 10 traps per municipality. Traps operated from 18:00 to 06:00 for three consecutive nights. Collected specimens were transferred to 50-mL Falcon tubes containing 80% ethanol.

Field collections were carried out over two consecutive years (2023 and 2024) at the same sampling sites and habitat types, with surveys conducted in alternate months.

For virus detection, *Culicoides* were collected using protected human attraction with manual aspirators in peridomicile areas from 15:00 to 18:30 for two consecutive days per site. Sampling was conducted during 2023 and 2024.

The total sampling effort comprised 200 HP light-trap deployments (720 trap-hours) and 60 hours of protected human attraction collections.

Specimen Processing and Identification

Specimens were transported to the Medical Entomology Laboratory of Fiocruz Rondônia, sorted, and preserved in 92% ethanol. Specimens collected for viral analysis were stored in RNAlater® and preserved in liquid nitrogen.

For identification, specimens were slide-mounted in Berlese medium (Cerqueira 1943) and identified using standard morphological keys (Wirth et al. 1988; Spinelli et al. 1993, 2005; Veras 2001; Felippe-Bauer et al. 2009, 2013). Identification was based on wing patterns, antennal morphology, palpal structures, spermathecae, and male genitalia. Wing images were obtained using a Nikon Eclipse Ni-E microscope with a DS-Ri2 camera and analyzed in NIS-Elements v. 5.30.

Blood Meal Identification

Females presenting visible blood meals were dissected, and abdominal remnants were stored at −20 °C to preserve host DNA. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following an adapted protocol. Abdomens were individually macerated in 180 µL of ATL buffer, supplemented with 2.5 µL of proteinase K, and incubated overnight at 56 °C. Subsequent steps followed the manufacturer's protocol, with final DNA elution performed in 30–100 µL of AE buffer.

PCR amplification targeted a 358 bp fragment of the vertebrate mitochondrial *cytochrome b* gene using primers cytb1 and cytb2 (Boakye et al., 1999). Reactions were performed in a final volume of 20 µL using GoTaq® Colorless Master Mix (Promega). Thermal cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 75 °C for 5 min. PCR products were visualized by electrophoresis on 2% agarose gels stained with GelRed®, using a 100 bp molecular weight marker, and documented with an ImageQuant LAS 4000 system.

RNA Extraction and Oropouche Virus Detection

Specimens collected by human attraction were pooled (≤10 individuals per pool). RNA extraction followed the Trizol® LS protocol (Naveca et al. 2017), with MS2 bacteriophage as an exogenous control.

Phase separation was achieved using chloroform, followed by isopropanol precipitation and ethanol washing. All extraction steps were performed using a temperature-stabilized rack at −20 °C to ensure RNA integrity. RNA pellets were resuspended in nuclease-free DEPC-treated water.

RNA concentration was quantified using a NanoDrop™ One spectrophotometer (Thermo Scientific®). Oropouche virus RNA detection was performed by RT-qPCR using the TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems), following an adapted protocol by Naveca et al. (2017). Reactions were conducted in a final volume of 10 µL on a QuantStudio™ 7 Pro system. A positive human OROV RNA sample was used as a control in all assays.

Statistical and Spatial Analyses

Data were analyzed using descriptive statistics (relative frequency and abundance). Diversity was assessed using Simpson's index expressed as effective number of species (q = 2) and species richness (q = 0) (Chao et al. 2014; Hsieh et al. 2016). Diversity profiles were constructed using Rényi indices. Rarefaction curves were used to assess sampling sufficiency. Analyses were conducted in RStudio, PAST, and Excel. DNA sequences were edited in MEGA 11 and compared using BLASTn. Associations between *Culicoides* species and host sources were visualized using Circos (Krzywinski et al. 2009).

Results

Species Composition, Abundance, and Diversity of *Culicoides* in Rondônia

A total of 1,058 *Culicoides* specimens were examined from the state of Rondônia, comprising 831 females (78.54%) and 227 males (21.46%). The material was assigned to 76 taxa, including 46 valid species and 30 morphotypes (Table 1; Figs. 2–3). These taxa belong to four subgenera (*Anilomyia*, *Avaritia*, *Haematomyidium*, and *Hoffmania*) and are distributed across nine informal species groups: *carpenteri*, *dasyophrus*, *debilipalpis*, *fluvialis*, *guttatus*, *leoni*, *limai*, *paraensis*, and *reticulatus*.

Five species are recorded for the first time in Rondônia: *Culicoides aldomari* Felippe-Bauer & Spinelli, *C. crucifer* Clastrier, *C. franklini* Spinelli, *C. parauapebensis* Trindade and Felippe-Bauer, and *C. peruvianus* Felippe-Bauer. Additionally, four species represent new records for Brazil: *C. austroparaensis* Spinelli, *C. ferreyrai* Cazorla & Spinelli, *C. trifidus* Spinelli & Wirth, and *C. willinki* Spinelli & Wirth.

The most abundant species were *C. leopoldoi* Ortíz (153 specimens; 14.46%), *C. glabellus* Wirth & Blanton (92; 8.70%), and *C. pseudodiabolicus* Fox (73; 6.90%), while all remaining species exhibited relative abundances below 5%. Species richness per municipality ranged from 2 to 46 taxa, and abundance varied from 4 to 400 specimens.

The highest species richness was recorded in Ouro Preto do Oeste (46 taxa), Ariquemes (42), and Jaru (31), followed by Porto Velho (21), Guajará-Mirim (19), and Cerejeiras (9). *Culicoides leopoldoi* showed the widest distribution, occurring in nine municipalities, followed by *C. glabellus* (six municipalities) and *C. insignis* Lutz (five municipalities) (Table).

Shannon–Wiener diversity values (H′) ranged from 0.96 in Cerejeiras to 3.17 in Ouro Preto do Oeste, whereas Pielou's evenness (J′) varied from 0.43 to 0.94 (Table 1). All diversity analyses were conducted at the municipal level using pooled data from forest fragments and peridomiciliary areas. Rarefaction curves did not reach an asymptote, indicating that additional sampling would likely increase the observed species richness (Fig. 4). Rényi diversity profiles revealed marked differences in diversity patterns among municipalities (Fig. 5), which were statistically significant (Dunn's test; $p < 0.0001$).

Comparison between environments

A total of 963 specimens representing 76 taxa were collected in forest fragments, whereas 95 specimens representing 21 taxa were obtained from peridomiciliary areas. Forest fragments exhibited higher diversity ($H' = 3.53$) and evenness ($J' = 0.81$) compared with peridomiciliary areas ($H' = 2.34$; $J' = 0.76$) (Table 1).

Rarefaction curves indicated greater potential species richness in forest fragments (Fig. 6). Rényi diversity profiles also demonstrated higher diversity and lower dominance in forest fragments compared with peridomiciliary environments (Fig. 7). A statistically significant difference between environments was detected (Mann–Whitney U test; $p < 0.0001$).

Detection of Oropouche Virus in *Culicoides*

Specimens analyzed for Oropouche virus detection were obtained exclusively through protected human attraction collections and were analyzed independently from those collected using light traps. A total

of 3,975 specimens belonging to the *Culicoides paraensis* species group were collected using this method. Due to the presence of cryptic species within this complex, specimens were identified and treated at the species-group level.

For Oropouche virus (OROV) RNA detection, 414 *Culicoides* pools were screened using RT-qPCR. No pool showed detectable amplification for OROV RNA (Fig. 8).

Identification of blood meal sources

A total of 54 engorged or partially engorged female *Culicoides* were analyzed, of which 70.4% were collected in peridomiciliary areas. Molecular analyses identified 10 vertebrate host species, including birds and mammals, with no evidence of mixed blood meals.

Birds represented the primary blood meal source, with *Gallus gallus* Linnaeus, accounting for 59.3% of identified meals. Among mammals, *Coendou prehensilis* Lacépède (13%), *Ateles chamek* Humboldt (7.4%), *Sus scrofa* Linnaeus (5.6%), and *Homo sapiens* Linnaeuswere identified; the latter was associated with specimens of the *Culicoides paraensis* species group.

Culicoides leopoldoi and *C. forattinii* Ortíz exhibited distinct feeding patterns. *C. leopoldoi* was associated with five vertebrate host species, whereas *C. forattinii* fed predominantly on birds (Fig. 9).

Discussion

This study documents a high diversity of *Culicoides* in Rondônia, with 76 taxa recorded, representing approximately 58% of the species known from the Brazilian Amazon. These findings reinforce the region as a major biodiversity hotspot for Ceratopogonidae and are consistent with previous surveys conducted in Amazonian environments (Farias et al. 2015; Carvalho et al. 2017; Farias et al. 2020; Carvalho et al. 2021a).

The predominance of females reflects their hematophagous behavior and the greater efficiency of light traps in capturing host-seeking females (Farias et al. 2020). Males, which typically remain near breeding sites and exhibit reduced dispersal, are frequently underrepresented in light-trap collections, a pattern widely reported in *Culicoides* surveys (Farias et al. 2020), and probably this trap is not very effective of males of *Culicoides* (Farias et al. 2024). This bias highlights the importance of complementary sampling methods for a more accurate characterization of population structure.

The detection of nine species newly recorded for Rondônia, namely *C. aldomari*, *C. austroparaensis* Spinelli, *C. crucifer* Clastrier, *C. ferreyrai* Ronderos, *C. franklini*, *C. parauapebensis*, *C. peruvianus*, *C. trifidus* Spinelli, and *C. willinki* Spinelli, including four new records for Brazil (*C. austroparaensis, C. ferreyrai, C. trifidus and C. willinki*), significantly expands current knowledge of the geographic distribution of the genus and highlights persistent taxonomic gaps in the Amazon region. Before this study *C. aldomari*, *C. crucifer* and *C. franklini* had been recorded in Brazil only from the states of Amazonas and Pará, whereas *C. parauapebensis* was known exclusively from Pará and *C. peruvianus* had been reported only from Rio de Janeiro and São Paulo (Santarém & Felippe-Bauer 2024).

Furthermore, *C. austroparaensis*, *C. ferreyrai*, and *C. willinki* were previously known only from Argentina, whereas *C. trifidus* had been reported from both Argentina and Costa Rica (Borkent & Dominiak

2025). The records presented here considerably expand the known distribution of these species and reinforce the importance of the western Amazon as an understudied region for *Culicoides* diversity. These findings corroborate the results of Carvalho et al. (2021), who reported 12 new records of *Culicoides* species for Rondônia in the Jamari National Forest, demonstrating that the diversity and distribution of the genus in the southwestern Amazon remain incompletely documented. Together, these studies emphasize the need for continued faunistic surveys and taxonomic efforts to improve our understanding of *Culicoides* biodiversity in the region.

Forest fragments exhibited higher richness and diversity of *Culicoides* than peridomiciliary environments. This pattern may be associated with greater habitat heterogeneity and the availability of suitable breeding and resting sites in forested areas. Similar trends have been reported in Amazonian studies, which consistently show higher *Culicoides* diversity in preserved habitats, underscoring the importance of forest environments for maintaining ceratopogonid biodiversity (Carvalho et al. 2017; Farias et al. 2020; Carvalho et al. 2021a).

Peridomiciliary areas, although less diverse, concentrated species of recognized epidemiological importance, such as *C. leopoldoi*, *C. insignis*, and *C. paraensis*. Their presence in anthropogenic settings highlights the adaptability of certain *Culicoides* species to human-modified environments and underscores the epidemiological relevance of forest–urban interfaces. The dominance of a limited number of species in these habitats may reflect the effects of environmental disturbance on community composition, favoring species with broader ecological tolerances (Carvalho et al. 2021; Silva et al. 2026).

Several species recorded in this study are confirmed or suspected vectors of pathogens of medical and veterinary importance. *Culicoides insignis* and *C. pusillus* Lutz, the main vectors of Bluetongue virus in South America, and *C. paraensis*, the primary urban vector of Oropouche virus, were widely distributed across municipalities and environments, increasing the potential for arbovirus transmission at forest–human interfaces (Pinheiro *et al*. 1976; Borkent & Spinelli, 2007).

Additionally, numerous species documented here have been described as anthropophilic or frequently associated with human environments, including *C. batesi* Wirth and Blanton, *C. benarrochi* Ortíz & Mirsa, *C. debilipalpis* Lutz, *C. diabolicus* Hoffman, *C. foxi* Ortíz, *C. gabaldoni* Ortíz, *C. glabellus*, *C. guttatus* Coquillett, *C. ignacioi* Forattini, *C. leopoldoi*, *C. limai* Barretto, *C. lutzi* Costa Lima, *C. ocumarensis* Ortíz, *C. paraignacioi* Spinelli, *C. peruvianus* Felippe-Bauer, *C. pseudodiabolicus* and *C. pusillus* (Wirth & Blanton 1973; Aitken et al. 1975; Castellón et al. 1990; Castellón and Ferreira 1991; Felippe-Bauer et al. 2003; Trindade and Gorayeb 2005, 2010)

Culicoides leopoldoi, the most abundant and widespread species, stands out due to its frequent occurrence in peridomiciliary areas (Bandeira et al. 2017; Silva et al. 2026). Although its vector competence remains unclear, its abundance, ecological plasticity and association with multiple vertebrate hosts suggest a potential epidemiological role that merits further investigation.

Despite the high abundance of specimens from the *Culicoides paraensis* species group, none of the 414 pools tested positive for Oropouche virus RNA. This finding suggests absent or low viral circulation during the sampling period, which may be influenced by seasonal variation, climatic factors or fluctuations in reservoir host populations (Travassos da Rosa et al. 2017; Sakkas et al. 2018).

The absence of detection does not exclude sporadic or focal transmission, as OROV circulation is often temporally and spatially heterogeneous. Previous studies have shown that OROV outbreaks can be intermittent and strongly dependent on local ecological conditions (Shelley and Coscarón 2001; Santarém and Felippe-Bauer 2024). Long-term surveillance and seasonal sampling are therefore essential to better understand viral dynamics in the region.

Blood meal analysis revealed a predominance of avian hosts, particularly *Gallus gallus*, reflecting host availability in peridomiciliary environments and the importance of birds in the feeding ecology of *Culicoides*. Similar patterns have been reported in Amazonian and other tropical settings, where domestic birds contribute to maintaining local vector populations (Carvalho and Silva 2014).

Since the study of blood meals is also an important component in understanding the pathogenic role of hematophagous insects, especially *Culicoides*, the present study indicates that the PCR direct sequencing system using universal primer sets for vertebrate *Cyt b* gene is a promising technique for blood meal identification

The detection of wild and domestic mammals, including *Homo sapiens* Linnaeus, demonstrates the feeding plasticity of species such as *C. leopoldoi* and *C. paraensis*. This opportunistic behavior may facilitate pathogen circulation between sylvatic and anthropogenic environments, increasing zoonotic risk (Lassen et al. 2012; Kasicová et al. 2021). Such feeding flexibility reinforces the epidemiological relevance of *Culicoides* species occurring in forest–human interfaces.

In conclusion, this study reveals high *Culicoides* diversity in Rondônia, including new state and national records, reinforcing western Amazonia as a key biodiversity hotspot for Ceratopogonidae. Forest fragments supported greater richness and diversity, whereas peridomiciliary environments concentrated fewer species but included taxa of recognized epidemiological importance.

Although Oropouche virus RNA was not detected, the widespread occurrence of confirmed and potential vector species across forest–human interfaces indicates sustained ecological conditions that may support arbovirus transmission. These results highlight that vector presence alone does not equate to active transmission, but underscores the need for continuous surveillance in environmentally dynamic regions.

From a One Health perspective, the findings emphasize that integrating taxonomic resolution, ecological data, and molecular surveillance is essential to anticipate arboviral emergence in the Amazon under ongoing land-use change.

Acknowledgements The authors thank the field and laboratory teams for technical assistance and the local communities for their collaboration during sample collection. This study was conducted as part of the Master's dissertation of Gabriel Rodrigues de Souza. Jansen F. Medeiros acknowledges Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the Productivity Fellowship. Emanuelle S. Farias acknowledges the postdoctoral fellowship granted by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Authors' contributions Gabriel Rodrigues de Souza (Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing – Original Draft), Emanuelle Sousa Farias (Investigation, Validation, Writing – Review & Editing), Isis Marinho Falcão Investigation, Methodology, Formal analysis, Data curation), Jordam William Pereira Silva (Formal Analysis, Validation), Victor Lourenço Silva

(Investigation, Methodology, Formal analysis, Data curation), Tivian Raphaella Melo Munis (Investigation, Methodology, Formal analysis, Data curation), Nadson Willian Felipe Gasparelo (Writing – Review & Editing, Methodology, Investigation, Data curation), Michelli Santos Silva (Investigation, Methodology, Formal analysis, Data curation), Jackson Alves da Silva Queiroz ((Investigation, Methodology, Formal analysis), Deusilene Souza Vieira Dall'Acqua (Writing – review & editing, Writing – original draft, Methodology, Formal analysis), Luis Paulo Carvalho (Investigation, Validation, Supervision, Writing – Review & Editing), Jansen Fernandes Medeiros (Conceptualization, Methodology, Supervision, Project Administration, Resources, Funding Acquisition, Writing – Review & Editing). All authors reviewed and approved the final manuscript.

Funding This study was supported by INCT-EpiAmO (National Institute of Epidemiology of the Western Amazon – CNPq grant: 465657/2014-1). Gabriel Rodrigues de Souza received a graduate fellowship from CNPq.

Conflicts of Interest The authors declare that they have no conflict of interest.

Declarations

Ethics Approval The study involving human samples was approved by the Research Ethics Committee of the Tropical Medicine Research Center (CEP/CEPEM-RO), under approval number 1,474,102.

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Table 1: Species of *Culicoides* recorded in the municipalities of Alta Floresta D'Oeste (AFL), Ariquemes (ARI), Cabixi (CBX), Cerejeiras (CER), Colorado do Oeste (CDO), Distrito do Abunã (DAB), Guajará-Mirim (GUM), Jaru (JAR), Ouro Preto do Oeste (OPO), and Vilhena (VHA), in the state of Rondônia.

Fig 1. Study area, sampling sites, and collection method of *Culicoides* (Diptera: Ceratopogonidae) in the state of Rondônia, northern Brazil. Black dots indicate the sampled municipalities: Alta Floresta d'Oeste, Ariquemes, Cabixi, Colorado do Oeste, Cerejeira, Guajará-Mirim, Jaru, Ouro Preto do Oeste, Vilhena, and the Abunã district (Porto Velho municipality). The modified HP light trap used for specimen collection is shown, in which the original collection bag was replaced by a 50mL Falcon tube containing 80% ethanol, connected by polyamide-elastane cone allowing direct preservation of specimens without compromising airflow

Fig 2. Wing patterns of *Culicoides* species (Diptera: Ceratopogonidae) from Rondônia, Brazil, arranged by subgenus. *Anilomyia* (1): 1, *C. efferus*. *Avaritia* (2): 2*, C. pusillus*. *Haematomyidium* (3–12): 3*, C. austroparaensis*; 4*, C. crucifer*; 5, *C. darlingtonae*; 6, *C. debilipalpis*; 7, *C. ginesi*; 8, *C. paraensis*; 9, *C. peruvianus*; 10, *C. quasiparaensis*; 11, *C. glabrior*; 12, *C. spurius*. *Hoffmania* (13-28): 13, *C. batesi*; 14, *C. brownei*; 15, *C. coutinhoi*; 16, *C. diabolicus*; 17, *C. ferreyrai*; 18, *C. foxi*; 19, *C. franklini*; 20, *C. guttatus*; 21, *C. ignacioi*; 22, *C. insignis*; 23, *C. lutzi*; 24, *C. ocumarensis.*

Fig 3. Wing patterns of *Culicoides* species (Diptera: Ceratopogonidae) from Rondônia, Brazil, arranged by subgenus and informal species groups. *Hoffmania* (25–28): 25, *C. paraignacioi*; 26, *C. parauapebensis*; 27, *C. plaumanni*; 28, *C. pseudodiabolicus*. *Mataemyia* (29): 29, *C. aldomari*. *carpenteri* group (30): 30, *C. carpenteri*. *dasyophrus* group (31–32): 31, *C. dasyophrus*; 32, *C. guerrai*. *fluvialis* group (33–34): 33, *C. leopoldoi*; 34, *C. tetrathyris*. l*eoni* group (35–39): 35, *C. benarrochi*; 36, *C. fieldi*; 37, *C. gabaldoni*; 38, *C. glabellus*; 39, *C. trifidus*. l*imai* group (40–44): 40, *C. galindoi*; 41, *C. limai*; 42, *C. santanderi*; 43, *C. vernoni*; 44, *C. willinki*. *reticulatus* group (45–46): 45, *C. forattinii*; 46, *C. rhombus*.

Fig 4. Individual-based rarefaction curves showing *Culicoides* species richness by municipality in Rondônia, Brazil, based on the combined samples from forest fragments and peridomiciliary environments.

Fig 5. Rényi diversity profiles comparing *Culicoides* assemblages between forest fragment and peridomiciliary environments in Rondônia, Brazil. Profiles were generated using pooled data from all municipalities.

Fig 6. Individual-based rarefaction curves showing the cumulative species richness of *Culicoides* in peridomiciliary and forest fragment environments in the state of Rondônia, Brazil, based on pooled municipal data.

Fig 7. Rényi diversity profiles of *Culicoides* assemblages sampled in ten municipalities of the state of Rondônia, Brazil. Alpha (α) = 0 corresponds to log-transformed species richness, $\alpha = 2$ corresponds to the Simpson diversity index, and $\alpha \rightarrow \infty$ corresponds to the Berger–Parker dominance index.

Fig 8. RT-qPCR detection plot showing the absence of Oropouche virus (OROV) RNA in *Culicoides* specimens collected by protected human attraction in municipalities of the state of Rondônia, Brazil.

Fig 9. Agarose gel electrophoresis (2.0%) stained with GelRed showing PCR-cytb amplification products from engorged female *Culicoides* specimens collected in municipalities of the state of Rondônia, Brazil, used for vertebrate blood meal identification.

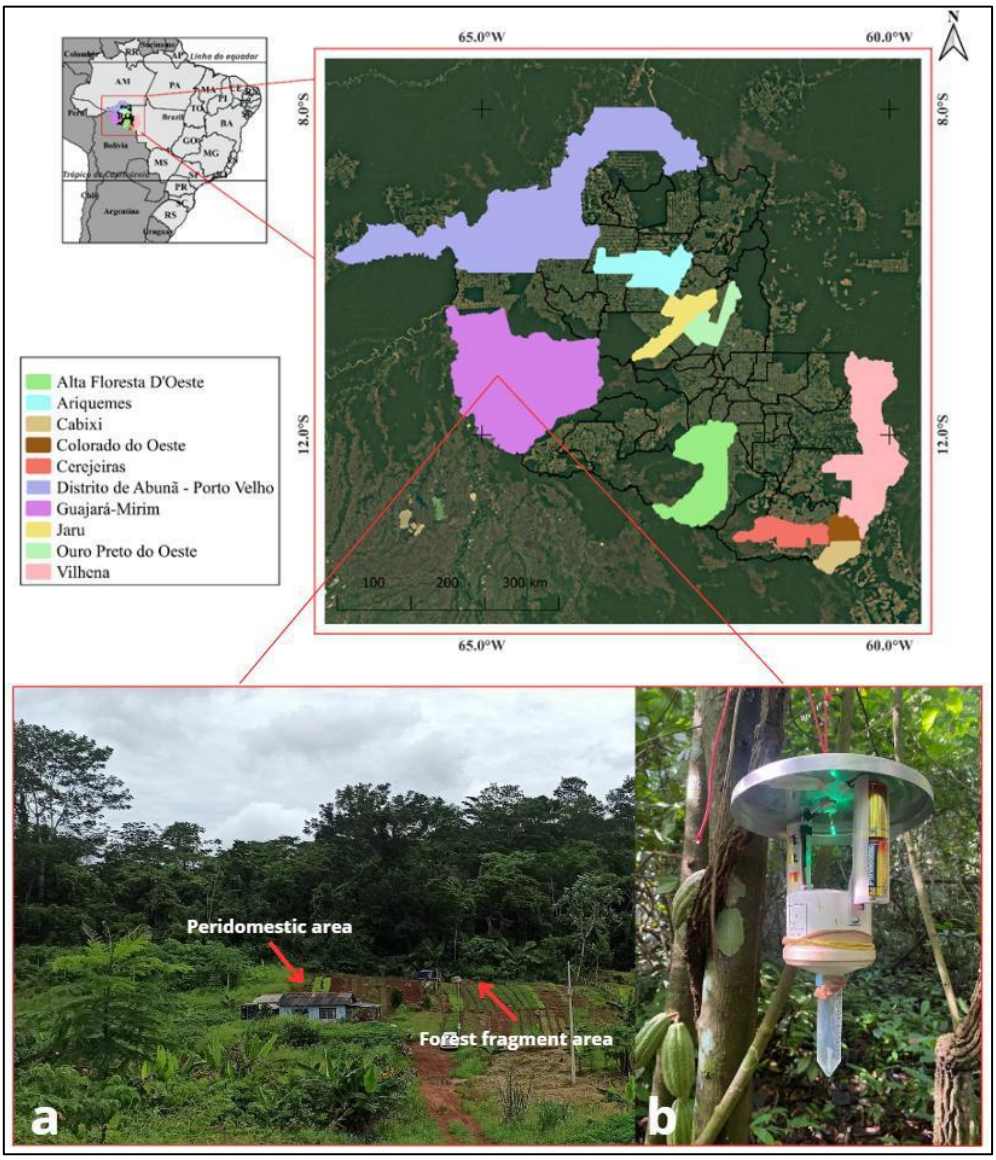


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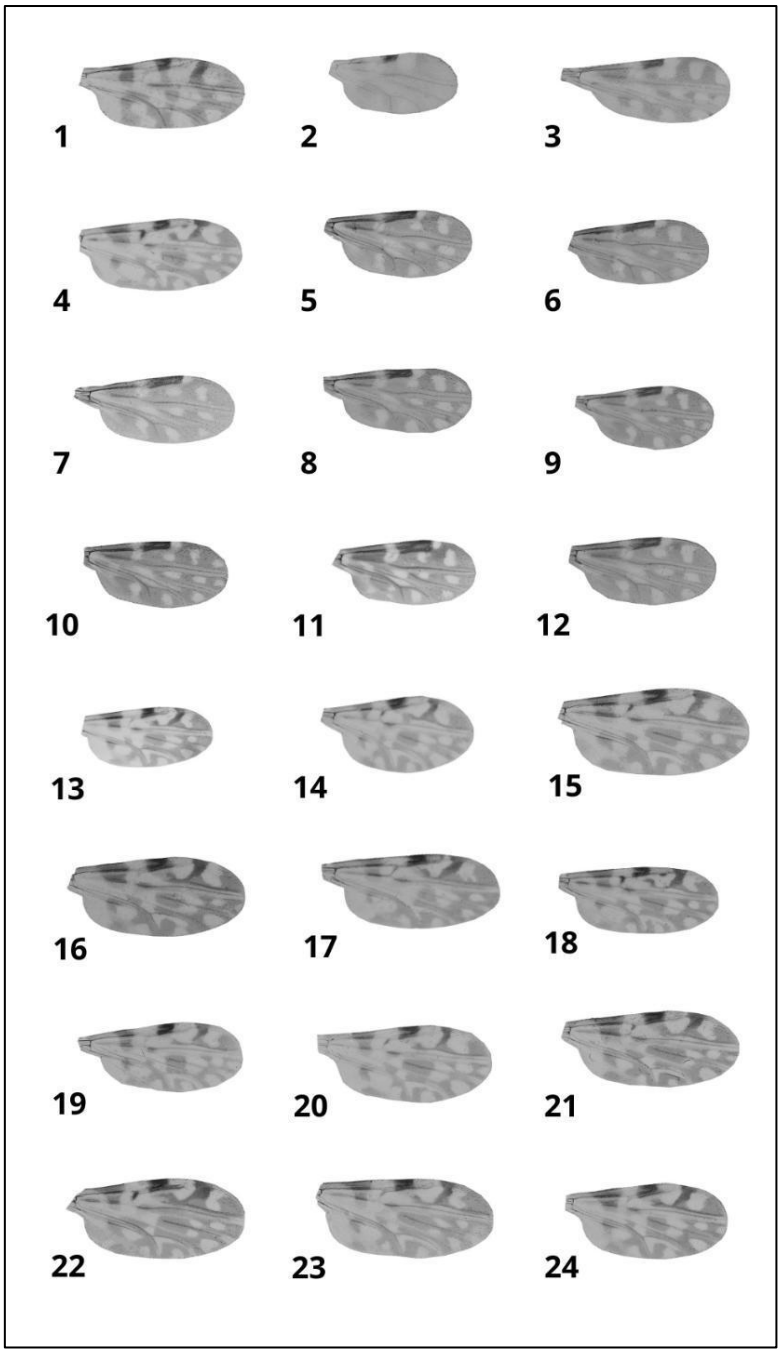


***Fig.* 2** Wing patterns of Culicoides species (Diptera: Ceratopogonidae) from Rondônia, Brazil, arranged by subgenus. *Anilomyia (1): 1, C. efferus. Avaritia (2): 2, C. pusillus. Haematomyidium (3–12): 3, C. austroparaensis; 4, C. crucifer; 5, C. darlingtonae; 6, C. debilipalpis; 7, C. ginesi; 8, C. paraensis; 9, C. peruvianus; 10, C. quasiparaensis; 11, C. glabrior; 12, C. spurius. Hoffmania (13-28): 13, C. batesi; 14, C. brownei; 15, C. coutinhoi; 16, C. diabolicus; 17, C. ferreyrai; 18, C. foxi; 19, C. franklini; 20, C. guttatus; 21, C. ignacioi; 22, C. insignis; 23, C. lutzi; 24, C. ocumarensis.*

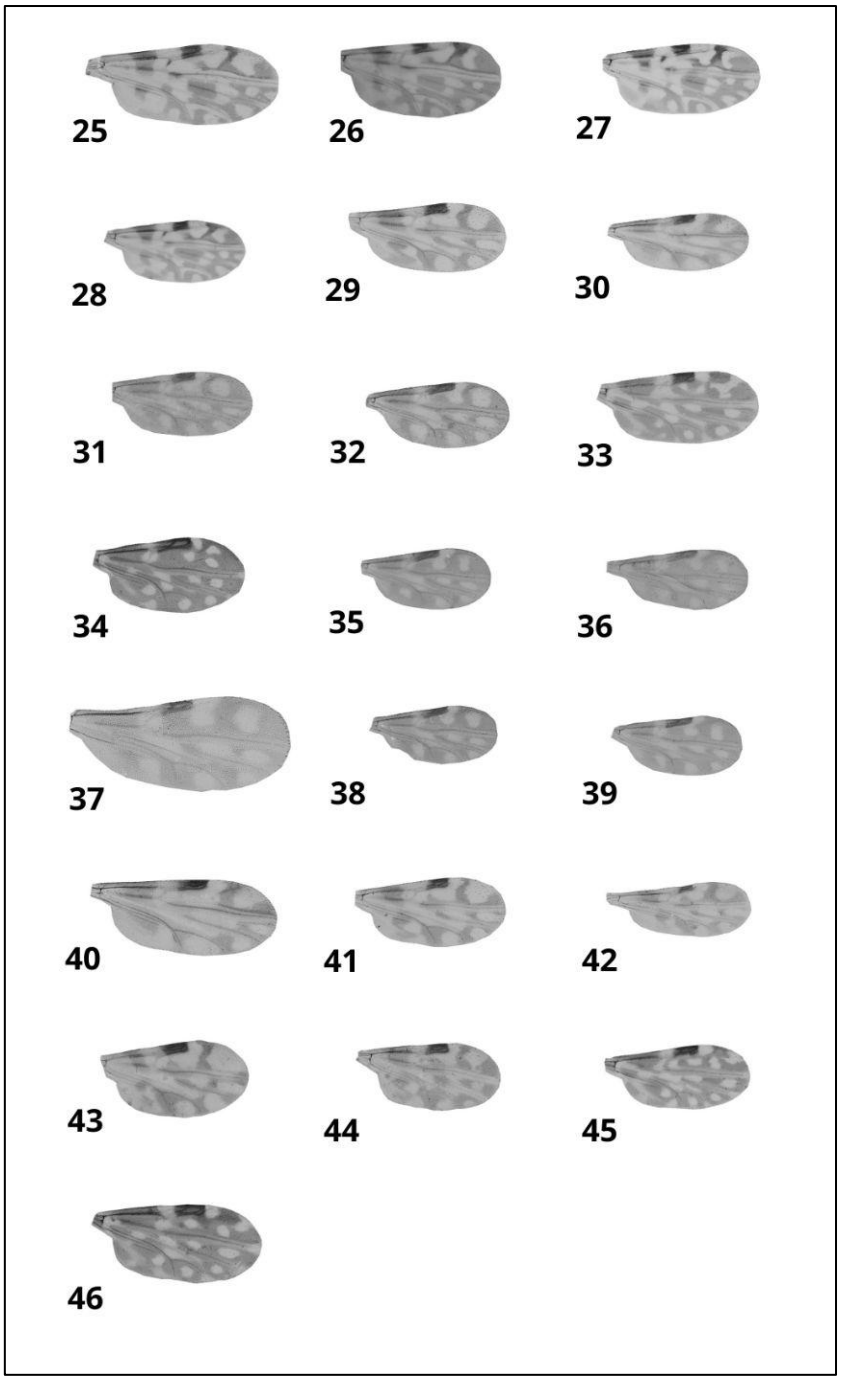


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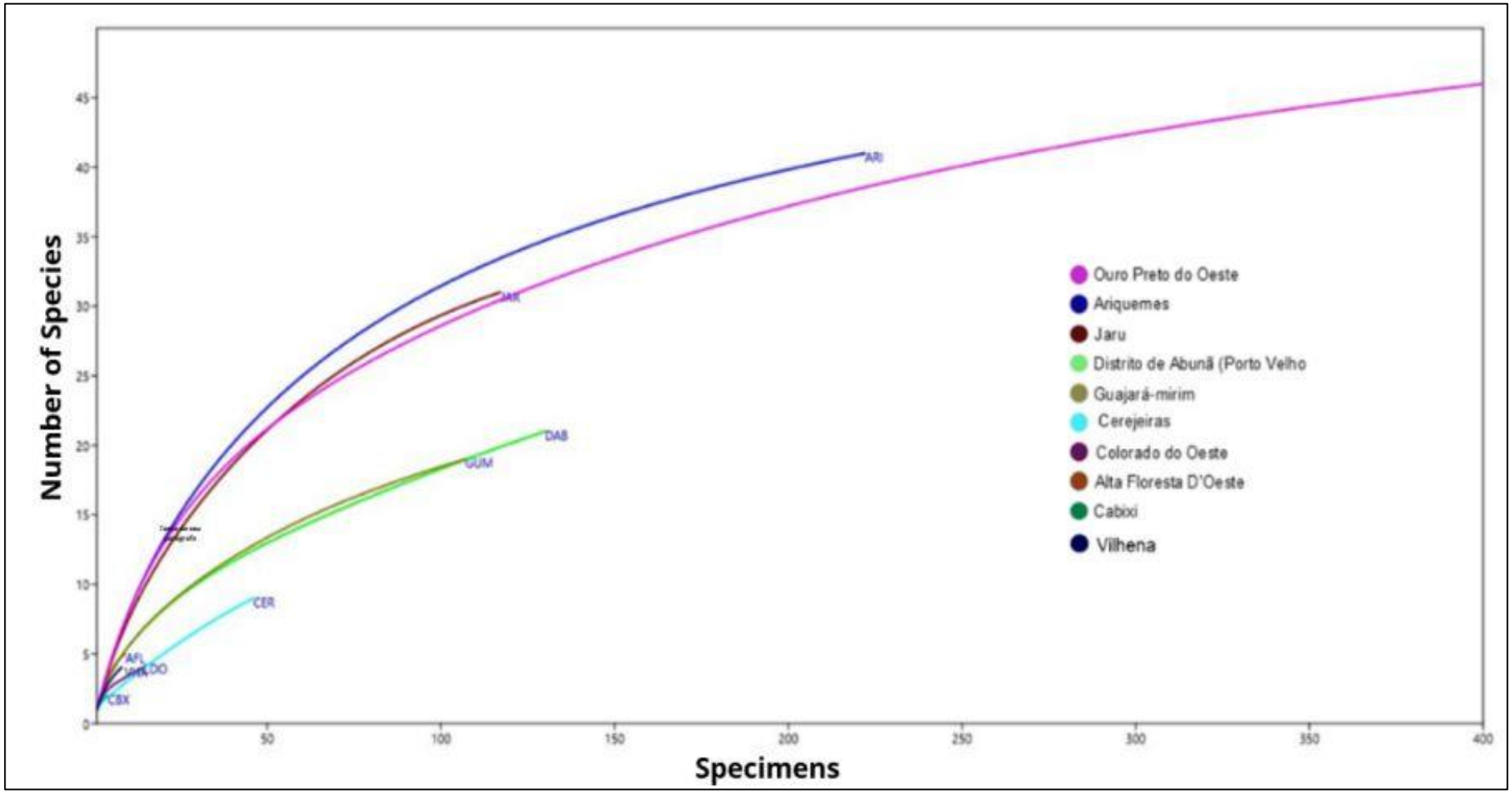


Fig. 4 Individual-based rarefaction curves showing Culicoides species richness by municipality in Rondônia, Brazil, based on the combined samples from forest fragments and peridomiciliary environments.

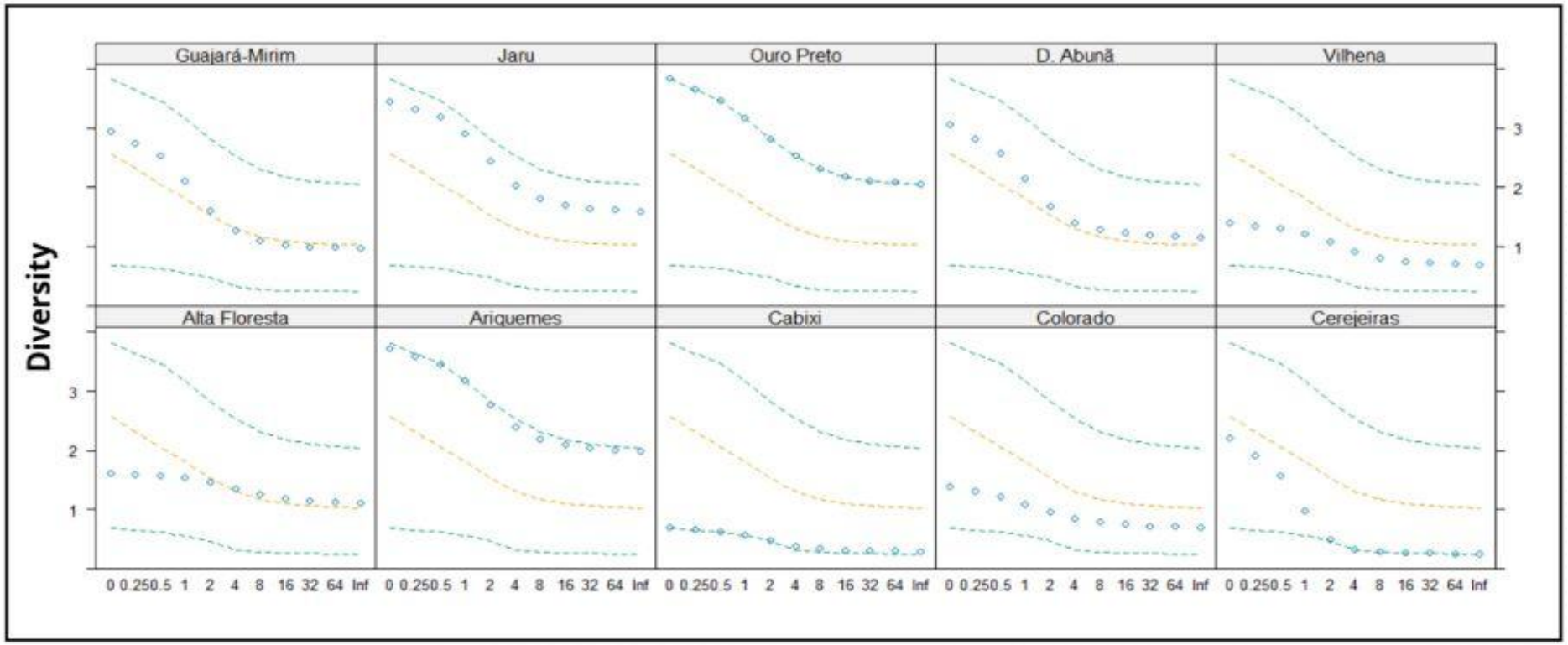


Fig. 5 Rényi diversity profiles comparing *Culicoides* assemblages between forest fragment and peridomiciliary environments in Rondônia, Brazil. Profiles were generated using pooled data from all municipalities.

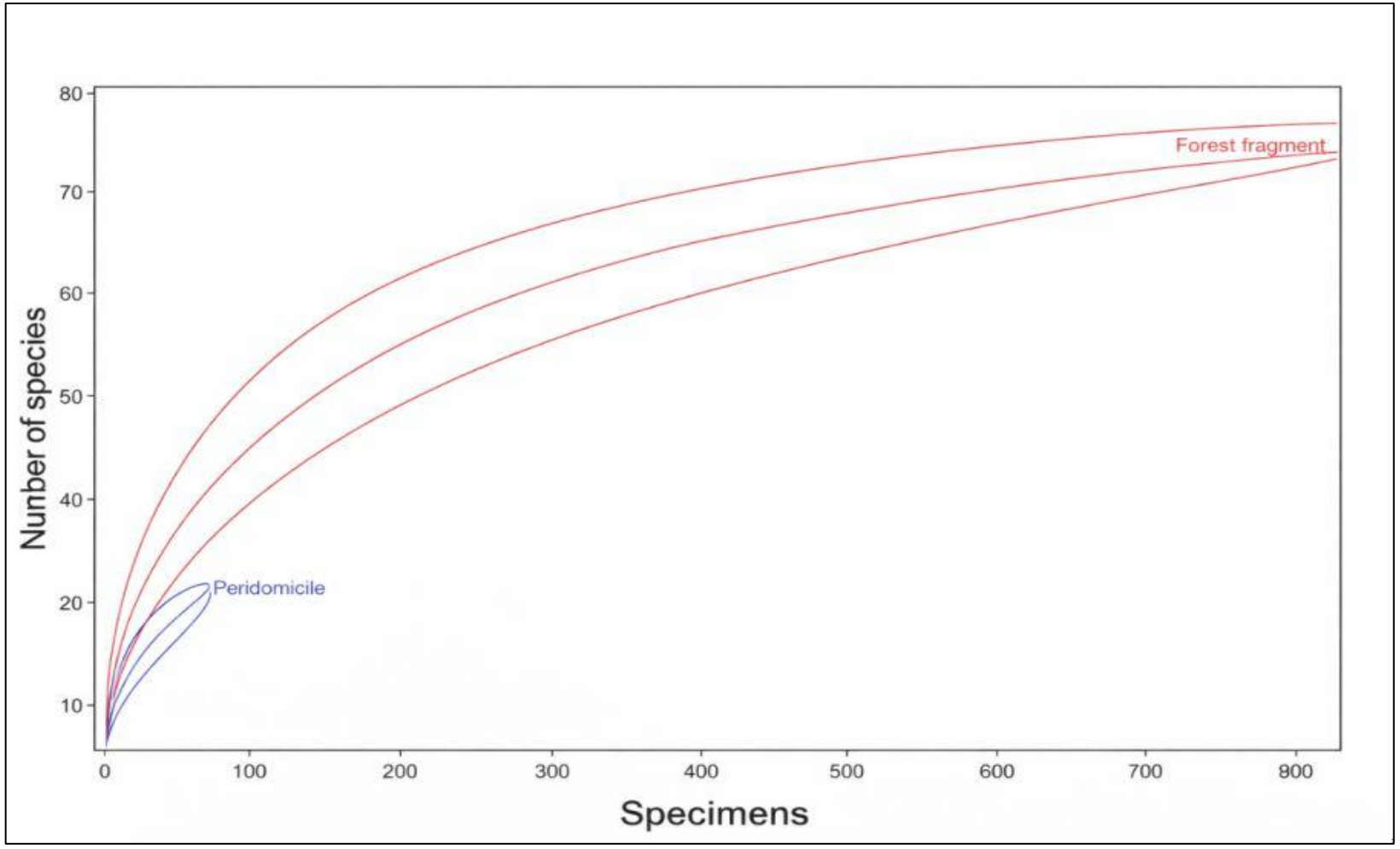


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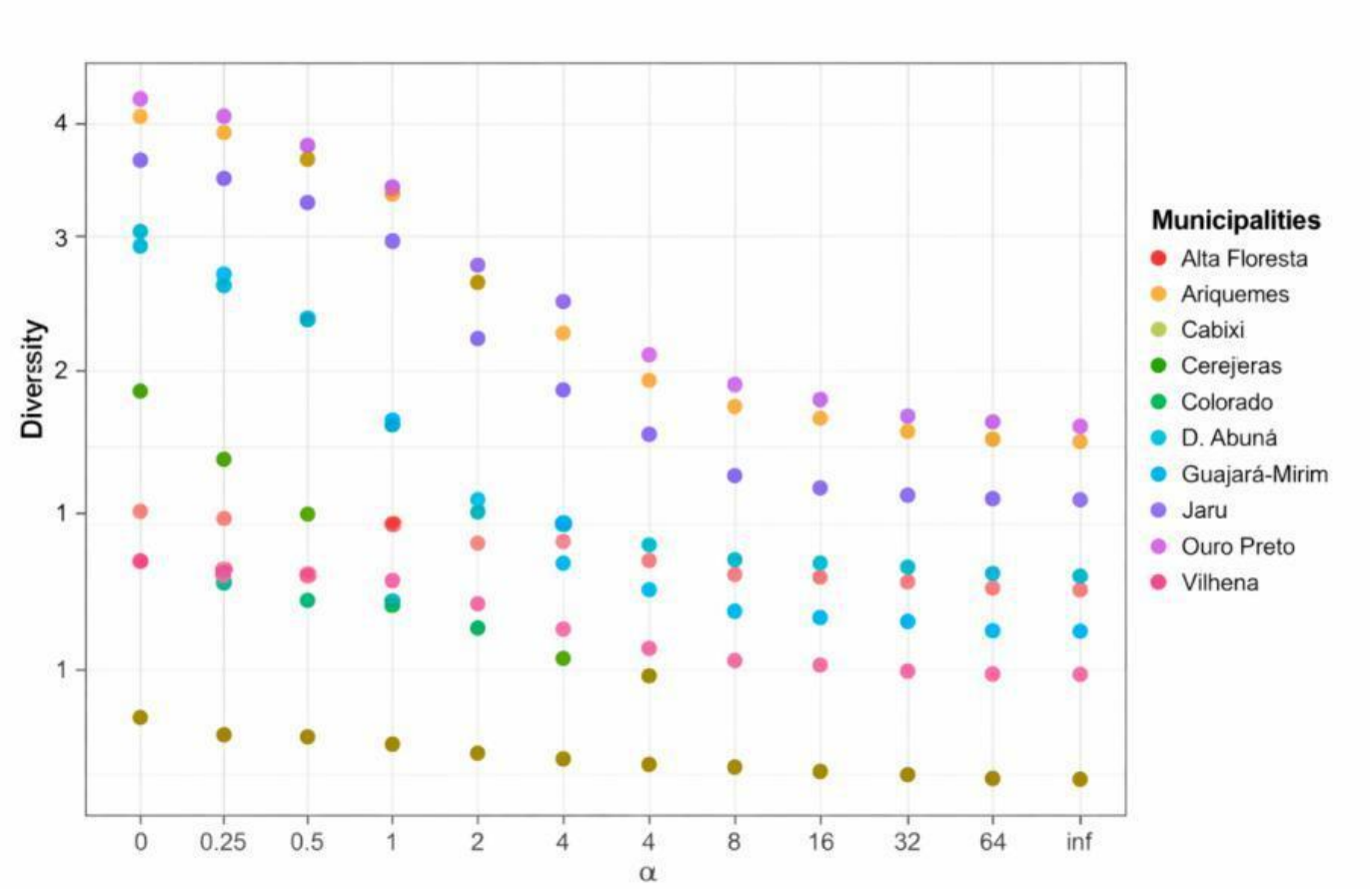


Fig. 7 Rényi diversity profiles of *Culicoides* assemblages sampled in ten municipalities of the state of Rondônia, Brazil. Alpha (α) = 0 corresponds to log-transformed species richness, α = 2 corresponds to the Simpson diversity index, and α → ∞ corresponds to the Berger–Parker dominance index.

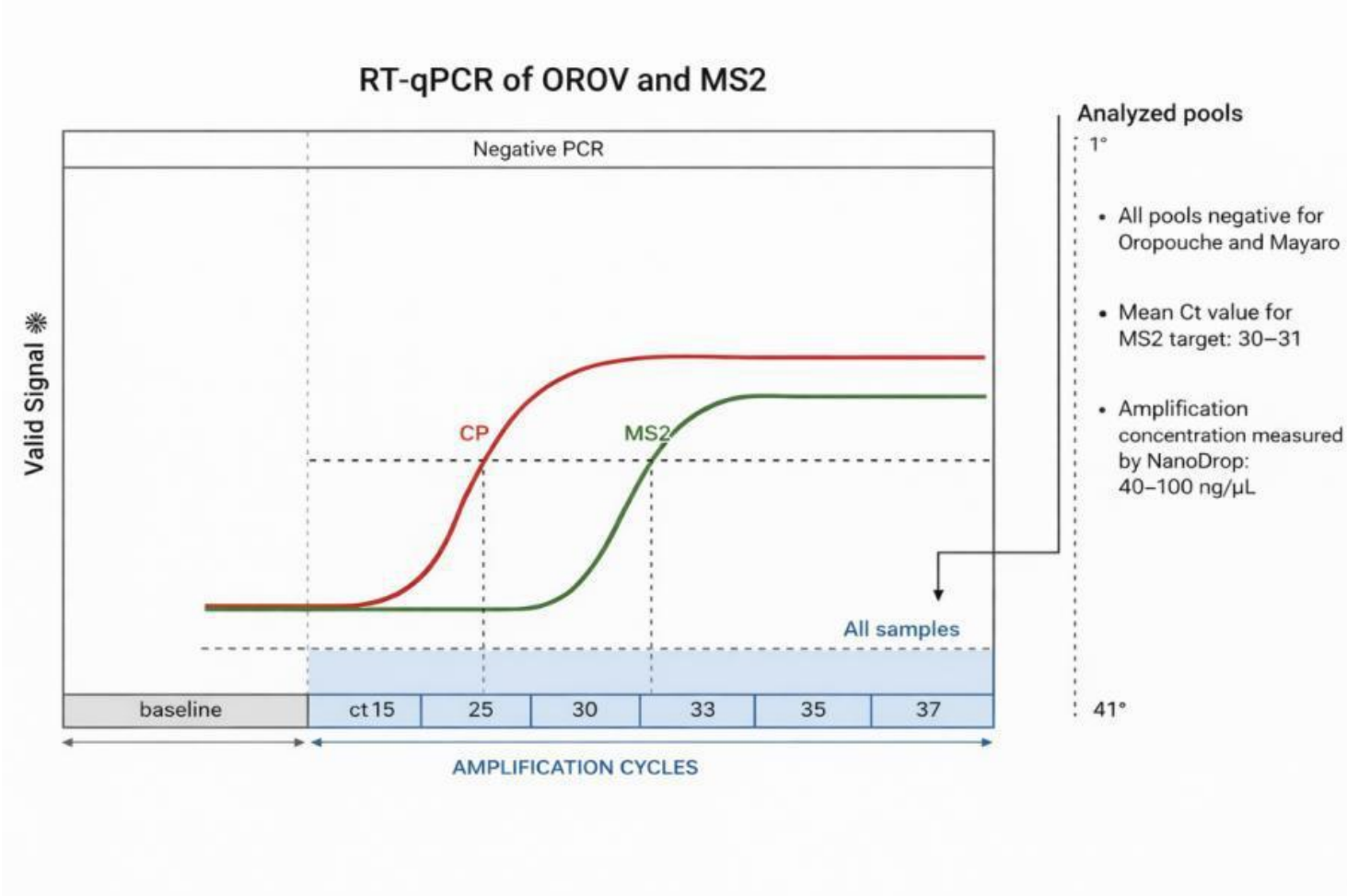


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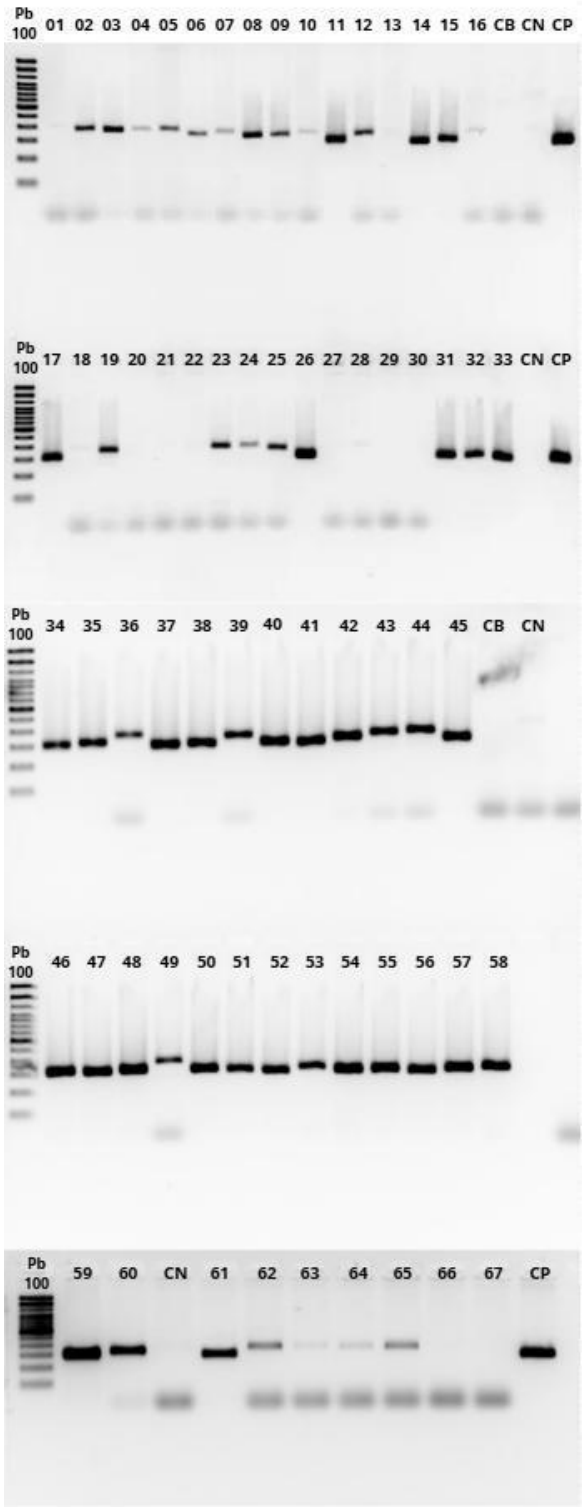


Fig. 9 Agarose gel electrophoresis (2.0%) stained with GelRed showing PCR-cytb amplification products from engorged female *Culicoides* specimens collected in municipalities of the state of Rondônia, Brazil, used for vertebrate blood meal identification.

Table 1 Species of *Culicoides* recorded in the municipalities of Alta Floresta D'Oeste (AFL), Ariquemes (ARI), Cabixi (CBX), Cerejeiras (CER), Colorado do Oeste (CDO), Distrito do Abunã (DAB), Guajará-Mirim (GUM), Jaru (JAR), Ouro Preto do Oeste (OPO), and Vilhena (VHA), in the state of Rondônia.

Species	AFL		ARI		CBX		CER		OPO		CDO		DBA		GUM		JAR		VHA		Total
	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	FF	PD	
Culicoides aldomari Felippe-Bauer & Trindade	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
C. austroparaensis Spinelli	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
C. batesi Wirth e Blanton	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	1	5
C. benarrochi Ortíz & Mirsa	0	0	8	1	0	0	0	0	25	0	1	0	8	0	0	0	2	0	0	0	45
C. brownei Spinelli	0	0	1	0	0	0	0	0	5	0	0	0	0	0	0	0	1	0	0	0	7
C. carpenteri Wirth & Blanton	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. coutinhoi Barretto	0	0	3	0	0	0	0	0	18	0	0	0	1	0	3	0	9	0	0	0	34
C. crucifer Clastrier	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. darlingtonae Wirth & Blanton	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
C. dasyophrus Macfie	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
C. debilipalpis Lutz	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	17
C. diabolicus Hoffman	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
C. efferus Fox	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
C. ferreyrai Ronderos e Spinelli	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	4	5
C. fieldi Wirth & Blanton	0	0	21	0	0	0	0	0	3	0	0	0	8	0	0	0	0	0	0	0	32
C. forattinii Ortíz	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	2	0	0	0	5
C. foxi Ortíz	0	0	6	0	0	0	0	0	10	2	0	0	7	0	1	0	2	0	0	0	28
C. franklini Spinelli, Greiner & Wirth	0	0	4	0	0	0	0	0	2	0	0	0	1	0	1	0	0	0	0	0	8
C. gabaldoni Ortíz	0	0	7	0	0	0	1	0	2	1	0	0	35	0	0	4	1	0	0	0	51
C. galindoi Wirth & Blanton	0	0	5	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0	0	0	11
C. ginesi Ortíz	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
C. glabellus Wirth e Blanton	0	0	30	1	0	0	0	0	5	0	0	0	41	0	3	4	7	0	0	1	92

C. glabrior Macfie	0	0	29	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	30
C. guerrai Wirth & Blanton	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
C. guttatus Coquillett	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
C. ignacioi Forattini	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3
C. insignis Lutz	0	0	1	0	0	0	1	1	0	0	0	0	2	5	1	2	2	0	0	0	15
C. leopoldoi Ortíz	3	0	2	0	0	0	2	34	31	0	7	0	7	0	41	0	24	0	0	2	153
C. limai Barretto	1	0	0	0	0	0	0	0	3	0	1	0	1	0	15	0	4	0	0	0	25
C. lutzi Costa Lima	2	0	2	1	0	0	2	0	0	0	0	0	0	1	0	0	16	0	0	0	24
C. ocumarensis Ortíz	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	6
C. paraensis Goeldi	0	0	1	2	0	0	0	1	0	0	0	0	0	0	0	2	2	0	0	0	8
C. paraignacioi Spinelli	0	0	0	0	0	0	0	0	34	0	0	0	0	0	0	0	0	0	0	0	34
C. parauapebensis Trindade & Felippe-Bauer	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3
C. peruvianus Felippe-Bauer	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	2	0	0	0	5
C. plaumanni Spinelli	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	9
C. pseudodiabolicus Fox	0	0	5	0	1	0	0	0	38	0	0	0	0	1	17	0	11	0	0	0	73
C. pusillus Lutz	0	0	5	1	0	3	0	0	10	5	5	0	0	0	0	0	2	0	0	0	31
C. quasiparaensis Clastrier	0	0	1	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	4
C. rhombus Santarém, Felippe-Bauer & Castellón	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	2
C. santanderi Browne	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
C. spurius Wirth & Blanton	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	5
C. tetrathyris Wirth & Blanton	0	0	15	0	0	0	0	0	4	0	0	0	0	0	0	0	1	0	0	0	20
C. trifidus Spinelli e Borkent	0	0	4	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	6
C. vernoni Wirth & Blanton	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
C. willinki Spinelli e Veggiani	1	0	5	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	8
Morphotype 1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	4
Morphotype 2	0	0	5	0	0	0	0	0	2	0	0	0	2	0	0	0	0	0	0	0	9
Morphotype 3	0	0	1	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	4
Morphotype 4	0	0	1	1	0	0	1	0	4	1	0	0	0	0	0	2	0	0	0	0	10

Morphotype 5	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	2	0	0	4		
Morphotype 6	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	6		
Morphotype 7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1		
Morphotype 8	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2		
Morphotype 9	0	0	0	0	0	0	0	0	24	0	0	0	1	0	0	0	0	0	0	0	25		
Morphotype 10	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1		
Morphotype 11	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0	0	0	0	0	0	22		
Morphotype 12	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1		
Morphotype 13	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1		
Morphotype 14	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2		
Morphotype 15	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3		
Morphotype 16	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	14		
Morphotype 17	0	0	0	0	0	0	0	0	52	0	0	0	0	0	0	0	0	0	0	0	52		
Morphotype 18	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	10		
Morphotype 19	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2		
Morphotype 20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Morphotype 21	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	5		
Morphotype 22	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3		
Morphotype 23	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	15		
Morphotype 24	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	10		
Morphotype 25	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1		
Morphotype 26	0	0	8	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	11		
Morphotype 27	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1		
Morphotype 28	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3		
Morphotype 29	0	0	3	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	5		
Morphotype 30	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	5		
Total	9	0	216	7	1	3	8	38	390	10	14	0	123	7	87	20	115	2	0	8	1058		
Abundance	9		223		4		46		400		14		130		107		117		8		1058	963	95

Riqueza	5	41	2	9	46	4	21	19	31	4	76	76	21
H’	1,52	3,16	0,56	0,96	3,17	1,09	2,13	2,11	2,89	1,21		3,53	2,3
J’	0,94	0,85	0,81	0,43	0,82	0,78	0,7	0,71	0,84	0,87		0,81	0,8

Abundance, richness, diversity (H'), and evenness (J') indices calculated for *Culicoides* species collected in the municipalities.

FF – Forest fragment. PD - Peridomicilie