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Exploring the Key Actors in the Oropouche virus Transmission Cycle in the Ecuadorian Amazon: Expanding the Vector Range and Identifying New Bloodmeal Hosts

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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Exploring the Key Actors in the Oropouche virus Transmission Cycle in the Ecuadorian Amazon: Expanding the Vector Range and Identifying New Bloodmeal Hosts

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DEDICATORIA

A todos aquellos que creen en mí sin dudar, ustedes, que me levantan después de las caídas y me acompañan en cada aventura que decido emprender. Es para ustedes mi corazón y cada sueño que cumpla. Francisco, Janeth, Francisco Andrés, Sebastián, Blanchi y Sismo.

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RESUMEN

En 2024, más de 10,000 casos y las primeras dos muertes relacionadas con el virus Oropuche (OROV) fueron reportadas, evidenciando su impacto emergente. En este estudio, presentamos evidencia de la circulación de OROV en la Estación de biodiversidad Tiputini Orellana, Ecuador (Amazonía ecuatoriana) una región sin vigilancia epidemiológica en humanos y vectores. La extraordinaria biodiversidad del área favorece la aparición de nuevos vectores y reservorios. Mediante metagenómica de amplicones, identificamos material genético de OROV en pools de mosquitos Culicidae, con la presencia predominante del género *Culex* en todos los pools, destacando a *Culex pedroi* como un potencial vector emergente. Adicionalmente, el análisis de especímenes con sangre de *Culicoides* y mosquitos positivos para OROV sugirió a *Tapirus terrestris* y *Tamandua tetradactyla* como posibles reservorios clave en la ecología del virus. Aunque no se detectó OROV en *Culicoides paraensis*, principal vector conocido, se observó una notable diversidad de especies de *Culicoides*, respaldando la hipótesis de la participación de otros vectores en el ciclo de transmisión. Este trabajo representa un paso crucial hacia la comprensión del ciclo selvático del OROV en la Amazonía ecuatoriana y subraya la necesidad urgente de implementar vigilancia epidemiológica para prevenir futuros brotes en la región.

Key words: Virus de Oropuche, Culicoides, Culicidae, Amazonia Ecuatoriana, reservorio

ABSTRACT

In 2024, more than 10,000 cases and the first two deaths related to Oropouche virus (OROV) were reported, highlighting its emerging impact. This study presents evidence of OROV circulation in the Tiputini Biodiversity Station in Orellana, Ecuador (Ecuadorian Amazon), a region lacking epidemiological surveillance in humans and vectors. The extraordinary biodiversity of this area favors the emergence of new vectors and reservoirs. Through amplicon-based metagenomics, we identified OROV genetic material in pools of Culicidae mosquitoes, with the predominant presence of the *Culex* genus in all pools, highlighting *Culex pedroi* as a potential emerging vector. Additionally, the analysis of blood-fed *Culicoides* and mosquitoes positive for OROV suggested *Tapirus terrestris* and *Tamandua tetradactyla* as potential key reservoirs in the virus's ecology. Although OROV was not detected in *Culicoides paraensis,* the primary known vector, a remarkable diversity of *Culicoides* species was observed, supporting the hypothesis that other vectors may be involved in the transmission cycle. This work represents a critical step toward understanding the sylvatic cycle of OROV in the Ecuadorian Amazon and highlights the urgent need to implement epidemiological surveillance to prevent future outbreaks in the region.

Key words: Oropouche virus, Culicoides, Culicidae, Ecuadorian amazon, host

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ARTICULO CIENTIFICO

HIDDEN PLAYERS IN OROPOUCHE VIRUS TRANSMISSION: NEW INSIGHTS INTO RESERVOIR HOSTS AND CULEX SP. AS THE POTENTIAL VECTOR IN THE ECUADORIAN AMAZON

INTRODUCTION

Oropouche virus (OROV), fist identified in Trinidad in 1955, belongs to the Peribunyaviridae family, Orthobunyavirus genus within the Simbu serogroup (Wesselmann et al., 2024).It causes Oropouche fever, an emerging underreported illness similar to other vector-borne diseases like DENV, ZIK and CHIKV, with an estimated 140 000 cases annually across Latin America (World Health Organization, 2024). This region, known for its rich biodiversity, faces increasing human-vector contact and expanding reservoir host ranges due to socioeconomic factors, population growth, urbanization, and agricultural development (Pereira-Silva et al., 2021) .In the last years, OROV has caused over 30 febrile outbreaks across Brazil, Ecuador, Peru, Guyana, Colombia and Venezuela (Walsh et al., 2021) (Wesselmann et al., 2024) (Sakkas et al., 2018) (Manock et al., 2009)(Wise et al., 2018). Although previously considered self-limiting, in 2024, two deaths attributed to OROV were reported, along with four cases of microcephaly in Brazil (Riccò et al., 2024)(World Health Organization, 2024)

Both Culicoides biting midges and Culicidae mosquitoes are involved in the OROV transmission cycle, which comprises sylvatic and urban components. The urban component occurs in areas where humans, acting as the unique vertebrate hosts, are in close proximity. Conversely, the sylvatic cycle remains less understood, involving various reservoirs such as sloths (*Bradypus tridactylus)*, wild primates (*Callithrix spp.*, *Alouatta spp.*, *Sapajus spp.*,

Cebus spp.), domestic birds (*Fringillidae spp.*, *Columbina spp.*), and rodents (*Proechimys spp.)*, although the full range of reservoirs has yet to be determined (Pinheiro et al., 1981)(Pinheiro et al., 1976) (Pinheiro FP, 1961)(Batista et al., 2013) (Roberto Teixeira Nunes et al., 2005) (Travassos Da Rosa et al., 2017) (Wesselmann et al., 2024).

Culicoides paraensis, are considered the primary vectors, and *Culex quinquefasciatus, considered* probable vector, in both urban and sylvatic environments due to its adaptability and preference for human blood (Pereira-Silva et al., 2021). In the sylvatic component, other suspected vectors exist such as several mosquitoes from the *Culicidae* family, such as *Culex sp.*, *Coquillettidia venezuelensis*, and *Aedes serratus (Walsh et al., 2021)* (Sakkas et al., 2018). The females of these species are hematophagous, facilitating virus maintenance among human and non-human vertebrate hosts. However, their host-feeding preferences, particularly in sylvatic environments, remain poorly understood (Purse et al., 2015)

In Ecuador, OROV has been detected in human sera from both the Coastal and Amazon regions (Wise et al., 2018) (Manock et al., 2009). However, there is not a real number of cases due to symptom overlap with other arboviruses, limited epidemiological surveillance and poor diagnostic tools (Riccò et al., 2024). It is possible that many cases testing negative for DENV, ZIKV and CHIKV may actually be OROV or OROV-like (Pereira-Silva et al., 2021).

Despite the recognized importance of entomological surveillance and host-vector dynamics in managing arbovirus outbreaks, to date, no studies have analyzed OROV presence in vectors while carrying out blood meal analyses in *Culicoides* bitting midges and *Culicidae* mosquitoes within Ecuador amazon forest. This study aims to detect viral genome of OROV in probable vectors: *Culicoides paraensis* and mosquitoes belong to *Culicidae* family, as well

as to identify the blood host preferences of engorged females collected in the Tiputini National Reserve in Orellana, Ecuador.

METHODS

Entomological Sampling and study sites

Specimens were collected at the Tiputini Biodiversity Station (TBS) in Orellana, Ecuador, over two distinct approaches. The first involved monthly sampling from November 2022 to October 2023, with traps deployed at three different heights on a 20-meter tower. In the second, collections were conducted during four separate expeditions in July 2022, January 2023, March 2023, and November 2023. During the four collections, traps were placed across 57 different sites within TBS, with 40 traps positioned near salt licks and remote paths with minimal human activity and 17 traps placed closer to human structures and paths frequently accessed by researchers and tourists.

For two approaches, we used standard CDC miniature light traps set to operate from approximately one hour before sunset until one hour after sunrise. In the tower the lights traps were positioned at 1.5 meter, 10 m and 20 m from the ground. In the individual collections were positioned at a height of 1.5 meters from the ground. Specimens were captured in entomological nets attached to each trap and subsequently transferred to sterile containers

filled with 70% ethanol. Samples were then stored at 4°C until transport to the Parasitology and Vectors Laboratory at Universidad San Francisco de Quito.

The traps were placed with the permission of Tiputini Biodiversity Station, San Francisco University and the Ministry of Environment, Water and Ecological Transition of Ecuador (MAATE-ARSFC-2022-2357)

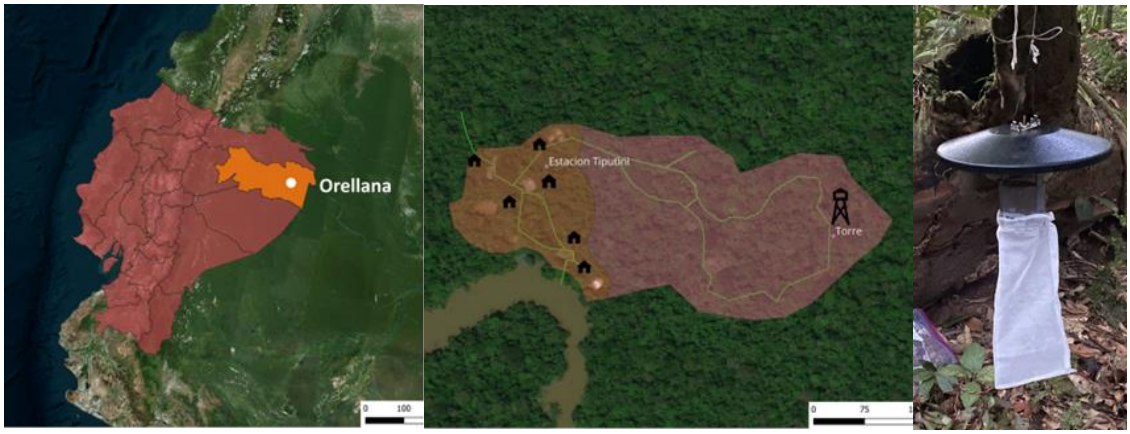

Figure 1. A) Map of Ecuador highlighting the province of Orellana, where the sampling took place. B) Detailed view of the study area, showing the location of the traps: yellow area near human settlements and pink area remote areas, away from human activity. C) CDC light traps used in this study

Morphological identification of Culicoides and Culicidae

All *Culicoides* specimens and *Culicidae* family mosquitoes were separated from each trap content using a stereomicroscope (Leica e24). Specimens were categorized by sex and feeding status: males, engorged females, and nulliparous females.

For species-level identification within the *Culicoides* genus, we mounted the head, wings, and spermatheca on slides and used morphological keys to distinguish species

(Mosquera et al., 2022), (Felippe-Bauer et al., 2003) (Felippe-Bauer et al., 2008) (Felippe-Bauer et al., 2013) (Spinelli et al., 1973) (Felippe-Bauer & Wirth, 1988) (Wirth, 1989) (Wirth & Blanton, 1973).

Due to ethanol preservation at the time of collection, morphological identification of Culicidae mosquitos was not feasible, as essential identification criteria were compromised. Thus, Culicidae species were identified exclusively using molecular techniques.

RNA extraction

The heads and thoraces of nulliparous *Culicoides paraensis* and Culicidae mosquitoes were pooled in sterile tubes with 10-15 specimens per tube. Just Culicidae mosquitoes collected during the last three collections were processed. In total we have eight Culicoides paraensis pools and 40 Culicidae mosquitoes' pools.

Pooled specimens were homogenized using sterile pestles and then incubated for 2 hours at 37 °C with proteinase K. RNA extraction was performed using the Quick-RNA Viral Kit (Zymo) according to the manufacturer's instructions. RNA was eluted in a final volume of 50 µL. The same protocol was used for individual specimens.

DNA extraction

Engorged females from *Culicoides* biting midges and Culicidae mosquitoes were individually ground using sterile pestles and then incubated for 3 hours at 56 °C with proteinase K. DNA extractions were performed using the Genomic DNA from Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. DNA was eluted in the final volume of 80 µL.

Molecular detection of OROV

All females identify as *Culicoides paraensis,* and mosquitoes belong to Culicidae family (both engorged and nulliparous) were screened for OROV. For the detection of OROV virus a qRT-PCR was performed using the Superscript III Platinum One-Step Quantitative RT-PCR Kit on an ABI 7500 Fast PCR system. Amplification of the OROV segment S fragment was conducted following the protocol by (Wise et al., 2020) with primers OROV F, OROV Ec2 R, and the OROV P probe. The final reaction volume was 20 µL, containing 3 µL of RNA sample. A synthetic positive control and a negative (no-template) control were included with each test pool. Samples with a Ct < 42 were considered positive and were sequenced bidirectionally by Macrogen, Korea, using the same primers.

Molecular identification of blood meal sources

Bloodmeal analysis was conducted using two markers: vertebrate *cytochrome b* (*CytB*) and *prepronociceptin* (*PNOC*) genes. Amplifications of *PNOC* and *CytB* were performed following the protocol described by (Hadj-Henni et al., 2015). Distilled water served as the negative control, while mammalian DNA was used as the positive control. Amplicons of 333 bp for *PNOC* and 360 bp for *CytB* were visualized on a 1.5% agarose gel.

Molecular identification of Culicidae species

To identify engorged Culicidae mosquitoes species a conventional PCR was used using *COI* marker with LepR and Lep F. The PCR conditions were based in (Augot et al., 2017). Distilled water was used as negative control and *Culicoides sp*. DNA was as a positive control. Amplicons of 650pb were examined in a 1.5% agarose gel.

To explore the Culicidae species present in potentially OROV-positive pools, an amplicon-based metagenomic analysis was performed on mosquitoes collected during the 2nd, 3rd, and 4th collection. Due to preservation issues, mosquitoes from the 1st collection

were excluded. Culicidae collected from the tower were excluded from this analysis because of financial constraints.

Sequencing and Molecular analysis

The positive pools for OROV, COI, as well as the PNOC and CytB amplicons, were bidirectionally sequenced using Sanger technology with the same primers described above. The sequencing was performed by Macrogen (Korea). All sequences were visualized and edited using the Staden package version 2.0. The resulting consensus sequences were analyzed using the Nucleotide BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Homology for vertebrate host sequences ranged from 98% to 100% with entries in GenBank. The identified species were compared with documented fauna of the Amazon region.

Genomic DNA from pools of Culicidae was sent to Macrogen, Korea, where 300 bp paired-end Illumina sequencing targeting the 18S V4 region was conducted. After removing primers, the resulting sequences were collapsed into amplicon sequence variants (ASVs) using DADA2 version 1.8.1. Taxonomic assignment of each ASV was performed using the Naïve Bayes k-mer method, which was implemented using the MOTHUR package and the Silva 138 database. All samples were rarefied to 60,000 reads per sample before analysis. AVS classified as Diptera were clustered with the centroid method with 95% identity threshold in the UCLUST tool (version 11.0.667). DADA2 and ggplot2 packages were used in RStudio with the R version 4.1.

RESULTS

Entomological collections and Molecular detection of OROV

A total of 3,983 female *Culicoides spp.* was collected using two methods: 1,650 specimens were obtained from the tower collections, while 2,333 were gathered through

individual entomological collections. Out of the total, 121 specimens (3.03%) were identified as *Culicoides paraensis*. Of these, 96 were nulliparous individuals, and 6 were engorged females. Detailed information on the two collection approaches is presented in Table 1. None of the pools, including both nulliparous and engorged *C. paraensis* specimens, tested positive for OROV.

Table 1. Number of specimens Culicoides paraensis and Culicidae mosquitoes collected in each approach separated by sex and feeding status.

	TOWER			COLLECTIONS			TOTAL
	Males	Females	Engorged	Males	Females	Engorged	
Culicoides paraensis	4	42	3	15	54	3	121
***Culicidae* mosquitos**	3	95	7	23	698	8	808

In other hand, a total of 808 female Culicidae mosquitoes were collected during both sampling phases, including 15 engorged specimens. Nulliparous Culicidae mosquitoes from the last three individual collections (n=449) were analyzed. Seven pools tested positive for OROV. These sequences exhibited >98% similarity to the Oropouche virus strain OROV/EC/Esmeraldas segment S sequence, as recorded in GenBank (Accession number: MK506820.1)

All engorged *Culicoides* specimens were tested for OROV to identify potential reservoir hosts of the virus. Among these, 10 individuals tested positive, comprising *Culicoides guttatus* (n=6), *Culicoides fusipalpis* (n=3), and an unidentified *Culicoides* species (n=1). Analysis of OROV-positive samples revealed that 90% (n=9/10) of the engorged

females had fed on *Tapirus indicus*, while the remaining individual had fed on Tamandua tetradactyla. Figure 2.

Among the engorged mosquitoes analyzed, 20% (n=3) were confirmed positive for OROV through molecular analysis. These specimens were taxonomically identified as *Ochlerotatus serratus*, *Culex vaxus*, and *Culex eastor* based on *COI* gene sequencing (Figure 4). Among the OROV-positive mosquitoes, 33.33% (n=1/3) were determined to have fed on *Bos taurus*. The remaining two samples failed to yield successful PCR amplification, preventing the identification of their vertebrate hosts.

Molecular identification of blood meal sources

The six engorged *C. paraensis* specimens were analyzed, revealing four vertebrate host species: *Bos taurus* (n=2), *Alouatta sara* (n=1), and *Lagothrix cana* (n=1) (Figure 3).

Among the total collection, 72 engorged non-*paraensis Culicoides* were identified, distributed across six subgroups, with *Hoffmania fox* being the most frequent (65.27%, n=47). These subgroups included 18 species, with *Culicoides guttatus* being the most prevalent.

Eight specimens could not be conclusively identified to either subgroup or species level based on wing morphology, highlighting the need for further molecular analysis (Figure 2).

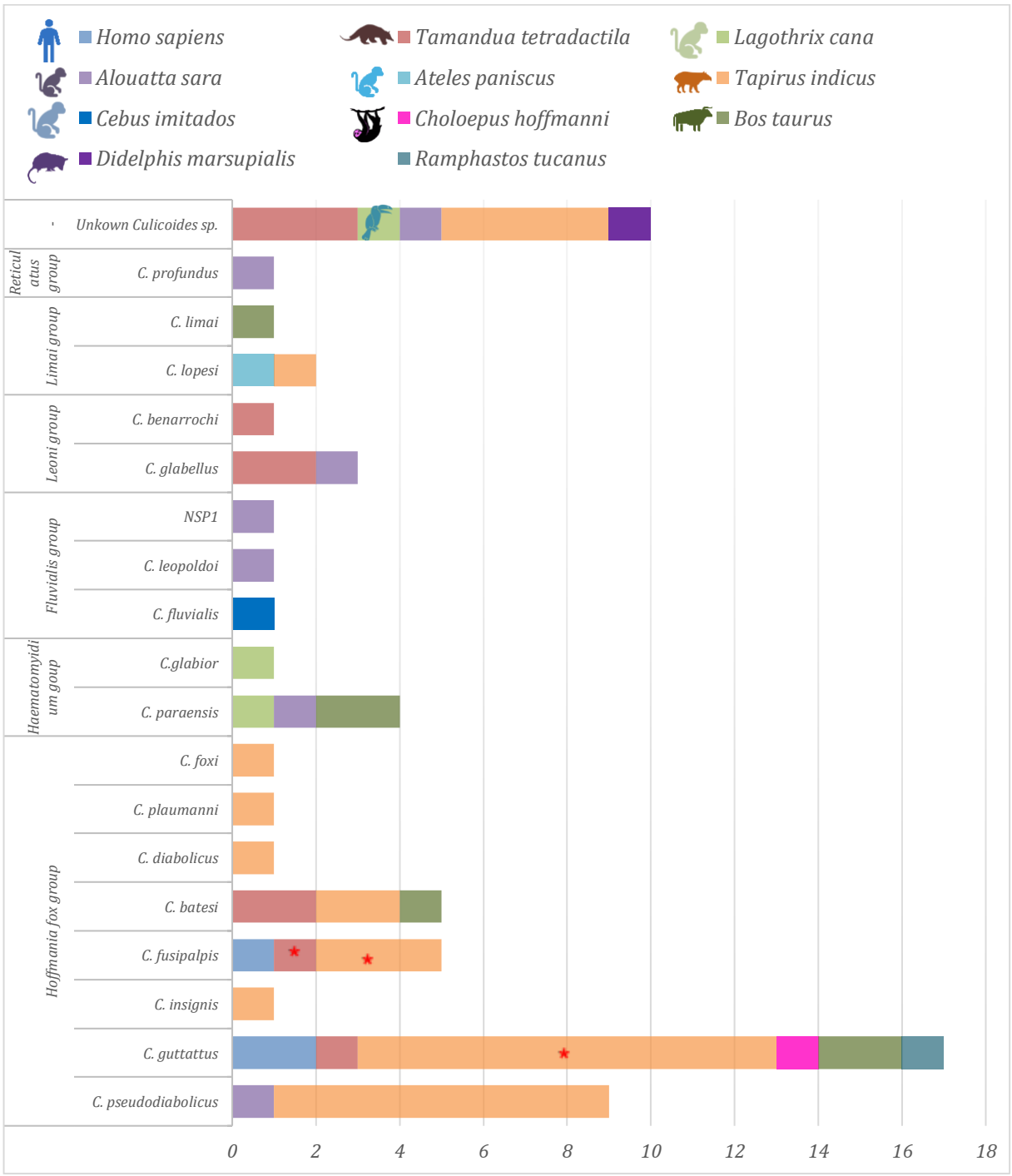

Figure 2 Distribution of Culicoides spp. and Their Bloodmeal Sources The chart illustrates the proportion of vertebrate hosts identified for various Culicoides species, grouped into six subgroups. An asterisk (*) indicates that at least one specimen within the group tested positive for OROV.

Host identification was successfully performed for 66 blood-fed *Culicoides* specimens using PNOC or CytB markers, while PCR amplification was unsuccessful for the remaining 12 samples. Analysis of the PNOC and CytB markers revealed that the blood meals were derived from nine vertebrate species, including eight mammals and one bird species. Notably, 48,48% (n=32) of the *Culicoides* specimens fed on *Tapirus terrestris*, followed by *Tamandua tetradactyla* (n=11, 16,66%). Avian blood was detected in only one specimen, identified as *Ramphastos tucanus* (n=1, 1.54%). Further details are presented in Figure 2.

A total of 15 engorged mosquitoes from the family Culicidae were collected (Table 1), representing four genera: *Culex* (n=6), *Aedes* (n=1), *Phosphora* (n=1), and *Ochlerotatus* (n=1). Additionally, several specimens (n=6) could not be identified due to poor sequencing quality. Figure 3.

Molecular analysis using PCR with the PNOC marker was successful for 7 of the engorged specimens, while amplification failed for the remaining 8 samples. Among the successfully identified mosquitoes, all detected vertebrate hosts were mammals. The

identified hosts included *Homo sapiens* (n=1), *Bos taurus* (n=3), *Tapirus indicus* (n=1), and *Sus scrofa* (n=2). Figure 3.

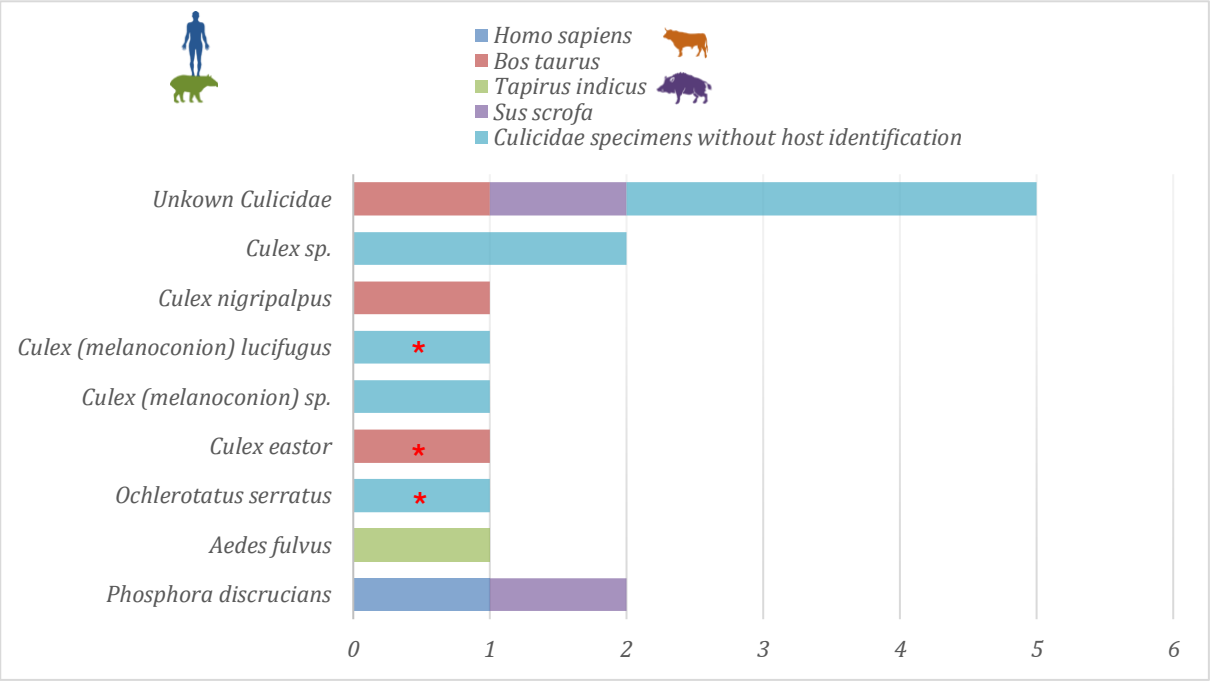

Figure 3 Distribution of Culicidae species and Their Bloodmeal Sources. The chart illustrates the proportion of vertebrate hosts identified for various Culicidae species. An (*) indicates that at least one specimen within the group tested positive for OROV

Bioinformatic analysis

The metagenomic analysis revealed that six of the seven pools consisted entirely of (100%) of taxa from the phylum Arthropoda, while one pool included 1% representation of other taxa, including Nematoda and Ascomycota (see Supplementary Material 1). The analysis of amplicon sequence variants (ASVs) revealed significant patterns in the composition of Culicidae species within the seven pools. By clustering sequences at a 95% similarity threshold, four primary clusters were identified, accounting for 99% of the total Diptera abundance across all pools. Clusters 1 and 2 dominated in most pools, highlighting their prevalence across the sampled mosquito populations. However, Pool 3 displayed a

markedly distinct composition compared to the other pools, with a high relative abundance of Cluster 3. Fig 4.

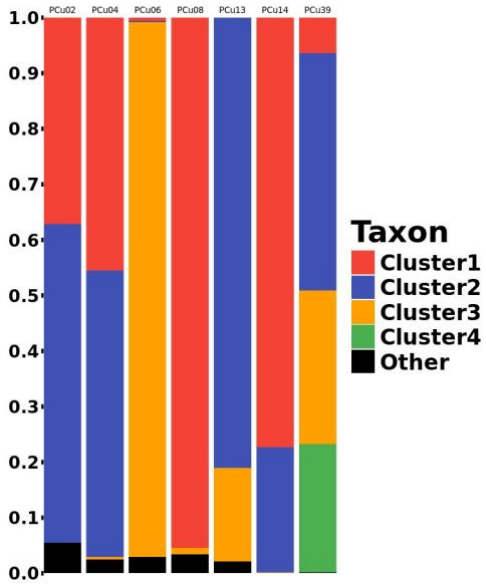

Figure 4. Relative abundance of clusters in Oropouche virus (OROV) positive mosquito pools. The bar chart illustrates the composition of four principal clusters identified through metagenomic analysis of the seven OROV-positive mosquito pools.

The phylogenetic relationships inferred using *V4 18S* rRNA sequences revealed variable resolution for species differentiation within the Culicidae family. While this marker demonstrated limitations in resolving some species-level distinctions, the four clusters were placed in well-supported genus clades (Figure 3). Cluster 2 displayed a high similarity (99%) to *Culex pedroi* (subgenus *Melanoconion*), by comparison with GenBank references, supporting this clade. Cluster 1 were grouped within a broader clade associated with *Culex* spp.; however, the identification of this cluster at the species level was not possible due to the lack of finer taxonomic resolution, suggesting the need for complementary molecular markers. Figure 5.

Among the identified genera, Culex was the most predominant, appearing consistently across all seven OROV-positive pools and representing the dominant taxon in terms of relative abundance. The genus *Mansonia* was also detected but exhibited a more restricted

distribution, being identified in pools 2, 3, 4, 5, and 7. In contrast, *Sabethes* was found exclusively in pool 7. Figure 5.

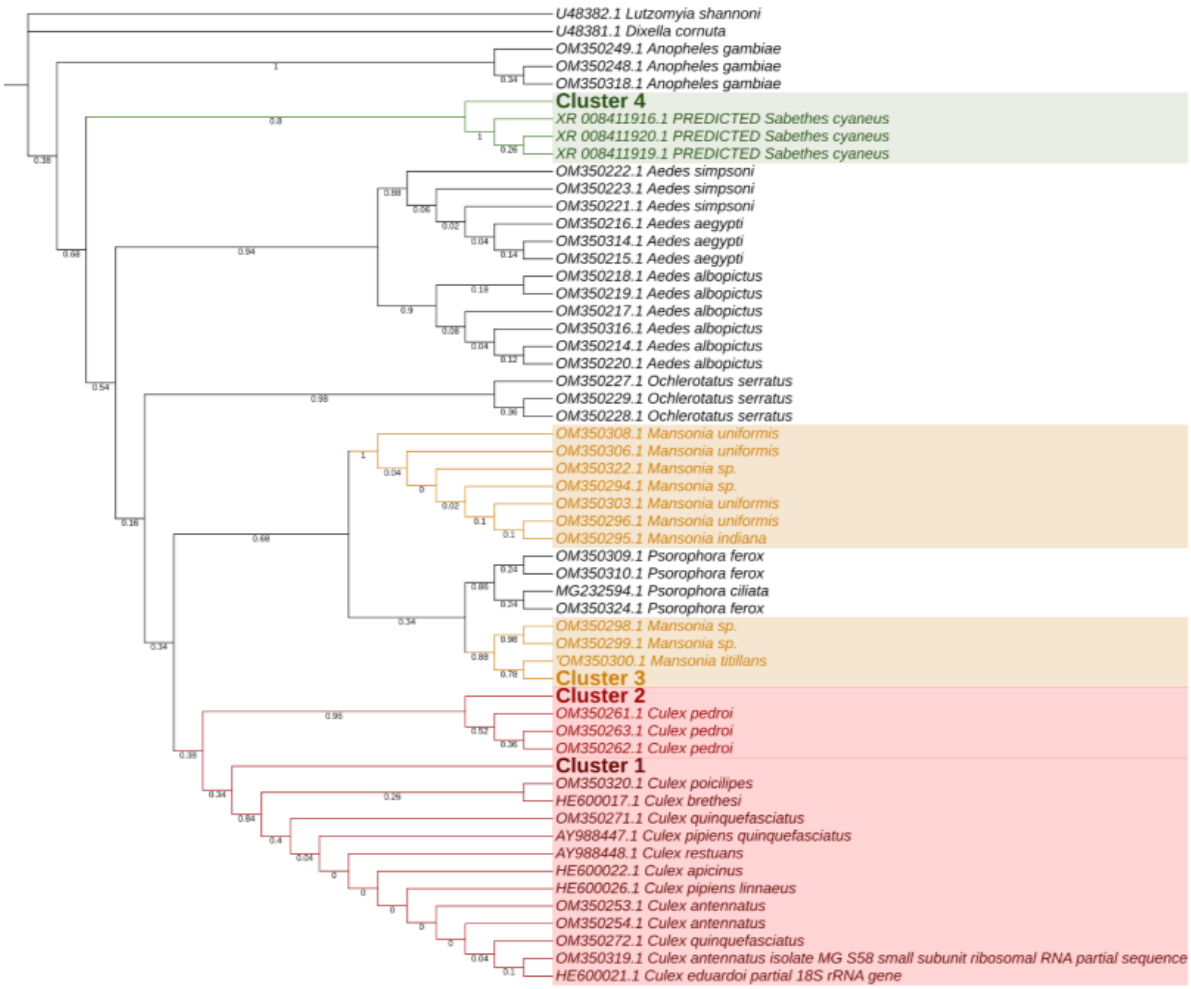

Figure 5. Phylogenetic tree based on V4 18S rRNA gene sequences of Culicidae mosquito species present in OROV positive pools. The tree shows the phylogenetic relationships among the four clusters, focusing on Culex (red), Sabethes (green), Mansonia (Yellow)

DISCUSSION

The absence of OROV detection in *Culicoides paraensis* in our study raises important questions, particularly since this species is widely regarded as the primary vector of OROV

(Sakkas et al., 2018). OROV is known to be circulating in multiple Latin American countries, many of which have begun monitoring this neglected disease, particularly in humans, following the 2024 outbreak that resulted in two fatalities (WHO, 2024). However, in Ecuador, studies on OROV are scarce, with limited research on human cases and no previous studies investigating its vectors. This is surprising given Ecuador's status as a megadiverse country, which could facilitate a broader range of potential vectors and vertebrate hosts (Peña-García et al., 2017a)

In this study, we provide the first evidence of OROV circulation in the Ecuadorian Amazon. Remarkably, our findings suggest that *Culicoides paraensis* might not be the primary vector in this sylvatic area, in contrast to its established role in other regions. The overall abundance of C. *paraensis* in our sampling was low (3.03%), especially when compared to its dominance during outbreaks in other regions. For instance, during an OROV outbreak in Pará, Brazil, *C. paraensis* represented over 95% of the collected midges (Pinheiro et al., 1976).This disparity in abundance may be attributed to ecological preferences, as *C. paraensis* is more commonly associated with peri-urban and urban environments, where its preference for human blood is notable ((Hoch A., 1990); (Carpenter et al., 2013)Studies have speculated that additional species might contribute to the sylvatic cycle of the virus such as *Culicoides sonorensis that* has demonstrated the ability to become infected, disseminate the virus, and moderately transmit it in in vivo analyses (Pinheiro et al., 1981)(McGregor et al., 2021). Thus, the role of other *Culicoides* species in OROV transmission remains poorly understood.

In this study, we found that all seven OROV-positive pools contained mosquitoes of the *Culex* genus. This recurrent presence highlights the potential role of Culex mosquitoes as key vectors in the transmission of OROV within the studied ecosystem. However, due to the presence of multiple species in each pool, it was not possible to confirm the exact species

infected with OROV and further analysis is imperative. Despite this limitation, our findings provide valuable insights into the genera and species potentially involved in the virus transmission sylvatic cycle. In sylvatic environments, several mosquito species within the Culicidae family, including *Coquillettidia venezuelensis, Aedes serratus, Culex quinquefasciatus*, and other *Culex* spp., are considered plausible vectors (ANDERSON et al., 1961) . Notably, *Culex* was the most abundant genus, and there is evidence supporting the role of certain *Culex* species as potential OROV vectors. For instance, *Culex quinquefasciatus* has been demonstrated to have the capacity to become infected, disseminate, and transmit OROV (Pereira-Silva et al., 2021) (McGregor, 2021). This species was also isolated from both patients and vectors during previous outbreaks, reinforcing its vectorial significance (Cardoso et al., 2015)

Our phylogenetic analysis confirms the presence of *Culex pedroi*, a species previously reported in the Ecuadorian Amazon and a member of the medically important Melanoconion subgenus (Ponce et al., 2021).This group is significant as several species within it are established or potential vectors for arboviruses such as the Eastern equine encephalitis virus (EEEV) and the Venezuelan equine encephalitis virus (VEEV) (Navarro & Weaver, 2004). The detection of *Culex pedroi* aligns with its known distribution in tropical ecosystems and its ecological role in arbovirus transmission (Ponce et al., 2021). These findings underscore the potential involvement of *Culex pedroi* in the transmission cycle of the Oropouche virus (OROV), emphasizing the need for further research into its vector competence. The second most abundant genus in our study was *Mansonia*, another species of significant public health importance. This mosquito genus has been implicated in the transmission of Venezuelan equine encephalitis (VEEV), St. Louis encephalitis virus and western equine encephalitis and is present in Ecuador territory (Beranek et al., 2018) (Ponce et al., 2021) While *Culex* and

Mansonia emerged as the most abundant genera, the involvement of other species cannot be ruled out, particularly given the biodiversity of the Amazon basin and the adaptability of arboviruses to multiple vectors and reservoirs (Peña-García et al., 2017b)

Clusters 1 and 5 were successfully classified within the Culex genus, demonstrating a clear affinity with this group. However, it was not possible to identify these sequences at the subgenus or species level due to the low resolution of the molecular marker employed. This highlights a technical limitation that needs to be addressed in future studies incorporating additional markers such as the ribosomal 28S gene or the mitochondrial COI gene (Koh et al., 2023) (Shepard et al., 2006)

We tested OROV in engorged *Culicoides* biting midges and Culicidae mosquitoes to identify potential reservoirs present in the area. It is important to note that detecting the virus in engorged vectors does not confirm their role as competent vectors, as the virus may simply be in transit through infected blood from the host (Franz et al., 2015).Our results highlight the diverse range of vertebrate blood-feeding sources utilized by both *Culicoides* and *Culicidae* mosquitoes, reflecting their broad feeding behavior (Tomazatos et al., 2020) (dos Santos Silva et al., 2012) (Mucci et al., 2015) (Ninio et al., 2011). Notably, blood meal sources for Culicoides species included a sloth (*Choloepus hoffmanni)*, a marsupial (*Didelphis marsupialis*), and several non-human primates (*Alouatta sara, Lagothrix cana, Ateles paniscus*, and *Cebus imitator*. Many of these vertebrates, particularly species within this genus, have been previously recognized as potential reservoirs for OROV and another arbovirus (Wesselmann et al., 2024)(de Thoisy et al., 2004)These findings highlight the need for further studies with larger sample sizes to better understand the potential roles of these vertebrates in the OROV transmission cycle .

In the case of *Culicoides spp.*, the predominant blood source identified was *Tapirus terrestris*. Most OROV-positive engorged *Culicoides* were found to have fed on this host.

While tapirs are not currently recognized as reservoirs of OROV, their potential role in the virus's transmission cycle warrants further investigation. Tapirs are large, herbivorous mammals that inhabit sylvatic environments. Experimental and serological studies of T. *terrestris* are necessary to determine whether they can support OROV replication and infect vectors and act as incidental hosts or serve as amplification hosts. Additionally, one *Culicoides fusipalpis* specimen was positive for OROV and had fed on *Tamandua* t*etradactyla,* a mammal from the order Xenarthra (sloths). Interestingly, other arboviruses, such as MAYV, have been isolated from mammals within this order (Muñoz & Navarro, 2012), raising questions about their role in other arbovirus transmission.

In the Culicidae family, three engorged specimens tested positive for OROV: *Ochlerotatus serratus* and two *Cule*x spp. Notably, *Oc. serratus* has been reported as a natural host for OROV, highlighting its role in the virus's sylvatic cycle (Pereira-Silva et al., 2021). The significance of the *Culex* genus in OROV transmission, previously discussed, is further highlighted by their consistent presence in OROV-positive pools. Of the three positive specimens, only one blood source was successfully identified, corresponding to *Bos taurus.* However, *Bos taurus* is not naturally present within the TBS or its surroundings, raising questions about the accuracy of the blood source identification.

A limitation of this study is the lack of reference sequences for Amazonian species when using PNOC gene as a marker, which may avoid precise identification. Given that *Bos taurus* and *Sus scrofa* belongs to the order Artiodactyla, the most plausible local vertebrates in this order are *Pecari tajacu, Tayassu Pecari, Mazama nemorivaga or Mazama americana* which are present within the study area (D. , B. J. Mosquera, 2017)This suggests that the blood source might have been misidentified due to limited genetic reference databases and poor molecular marker resolution (Hadj-Henni et al., 2015). Additional studies incorporating alternative molecular markers are needed. Finally, the methodology used in this study, based

on PCR targeting the S region of OROV, has a key limitation: it does not differentiate between OROV and its reassortants. These variants may vary in their vectors, reservoirs, and virulence, highlighting the need for more specific molecular tools to achieve precise identification and a better understanding of viral ecology (Naveca et al., 2024)(Navarro & Weaver, 2004)

In our knowledge for the first time in the Ecuadorian Amazon, we provide evidence of OROV genetic material in both nulliparous *Culicidae* mosquitoes and engorged *Culicoides* spp. and *Culicidae* mosquitoes. Among the Culicidae positive pools, Culex was identified as the predominant genus. *Culex (melanoconion) pedroi* and *Mansonia sp.* may play a potential role as vectors in the sylvatic cycle of OROV, warranting further investigation. A wide range of vertebrate blood sources were identified in both *Culicoides* and *Culicidae* mosquitoes, highlighting the role of *Tapirus terrestris* and Tamandua *tetradactyla*, which remain key questions for future studies. Understanding these interactions between vectors and hosts is crucial for clarifying the dynamics of the sylvatic and urban cycles of OROV, offering valuable insights into the virus's ecology and its potential for causing future outbreaks in the country.

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ÍNDICE DE ANEXOS

ANEXO A. …… 39

Anexo A: OROV positive pools order relative abundance