UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Investigación del virus de importancia médica y veterinaria transmitidos por *Culicoides*

Juan Daniel Mosquera Bolaños

Sonia Zapata, Ph. D
Directora de Trabajo de Titulación

Trabajo de titulación de posgrado presentado como requisito
para la obtención del título de Magíster en Microbiología

Quito, 19 de diciembre de 2017

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

Investigación del virus de importancia médica y veterinaria transmitidos por *Culicoides*

Juan Daniel Mosquera Bolaños

Firmas

Sonia Zapata Ph. D.
Directora del Instituto de Microbiología,
Director del Trabajo de Titulación

Gabriel Trueba Ph. D
Director del Programa de Posgrados del
Instituto de Microbiología

Gustavo Spinelli Ph. D.
Comité de Tesis

Hugo Burgos, Ph.D
Decano del Colegio de Posgrados

Quito, 19 de diciembre de 2017

© Derechos de Autor

Por medio del presente documento certifico que he leído todas las Políticas y Manuales de la Universidad San Francisco de Quito USFQ, incluyendo la Política de Propiedad Intelectual USFQ, y estoy de acuerdo con su contenido, por lo que los derechos de propiedad intelectual del presente trabajo quedan sujetos a lo dispuesto en esas Políticas.

Asimismo, autorizo a la USFQ para que realice la digitalización y publicación de este trabajo en el repositorio virtual, de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior.

Firma del estudiante:	
Nombre:	Juan Daniel Mosquera Bolaños
Código de estudiante:	00133307
C. I.:	1720624251
Lugar, Fecha	Quito, 19 de diciembre de 2017

DEDICATORIA

Dedico este trabajo a todas las personas que me han apoyado durante la experiencia que ha sido la maestría. A mi familia, especialmente a mi madre, todo lo que haga siempre será por ti y sé que hubieras estado muy orgullosa. A Sonia, más que una jefa, un apoyo incondicional y un ejemplo a seguir. Y, finalmente, a mis amigos que son la familia que escogí.

AGRADECIMIENTOS

Agradezco a la Universidad San Francisco de Quito y a su Instituto de Microbiología por el financiamiento de este proyecto de investigación. A Gabriel y Antonio por responder todas mis dudas. A Sonia por su guía y apoyo. A Gustavo Spinelli por su paciencia y experiencia en el tema de los *Culicoides.* A Fernando Salas (Cotundo) por su apertura y cordialidad. A Darío, Estefanía, Camilo, Pedro y Luis; gracias a ustedes pude avanzar de la mejor manera con todo el trabajo.

Finalmente, a mi familia y resto de amigos por siempre estar conmigo, bajo cualquier circunstancia.

RESUMEN

El primer artículo comprende el estudio del virus de la lengua azul (BTV) y el virus de la enfermedad epizoótica hemorrágica (EHDV), dos orbivirus transmitidos por *Culicoides*, y agentes causales de enfermedades en rumiantes salvajes y domésticos. En Ecuador, se encontró evidencia serológica alarmante sobre la presencia de ambos virus en ganado vacuno, pero las especies vectores todavía no se conocen. En este estudio se tomó el primer paso para la implicación de *Culicoides insignis* como vector biológico de BTV en el Ecuador. Un total de 326 *C. inisgnis* hembras fueron colectadas en dos localidades donde se evaluaron previamente muestras serológicas positivas. Se encontró BTV en un espécimen de la localidad de Cotundo a través de RT-qPCR. Ninguno de los especímenes fue positivo para EHDV. Se necesita de estudios futuros para establecer de otras especies vectores candidatas para ambos virus.

El segundo artículo se enfoca en la fauna de *Culicoides* en el Ecuador. El género *Culicoides* abarca alrededor de 1400 especies mundialmente. Entre julio del 2010 y septiembre del 2012 se realizaron colecciones entomológicas en cuatro localidades en la región norte del Ecuador, incluyendo cuencas de la Amazonía, pies de montaña, y sierra de los Andes. Un total de 36 especies fueron identificadas, de las cuales 16 son récords nuevos para el Ecuador. Con estos hallazgos la fauna de *Culicoides* en el Ecuador suma 65 especies.

ABSTRACT

The first paper comprises the study of the Bluetongue virus (BTV) and Epizootic Hemorrhagic Virus (EHDV), two orbiviruses transmitted by *Culicoides* biting midges, and causative agents of disease in wild and domestic ruminants. In Ecuador, alarming serological evidence of the presence of both viruses in cattle has recently been found, but the vector species are still unknown. In this study we take the first step for the implication of *Culicoides insignis* as biological vector for BTV in Ecuador. A total of 326 female *C. insignis* were collected in two localities where serologically positive samples were previously assessed. We found BTV in one specimen from the locality of Cotundo through RT-qPCR. None of the specimens were positive for EHDV. Further studies are needed to stablish the presence of other candidate vector species for both viruses.

The second paper focus on the fauna of *Culicoides* in Ecuador. The *Culicoides* genus comprises about 1400 species of biting midges worldwide. Between July 2010 and September 2012, entomological collections were made at four locations in the north region of Ecuador including Amazon Basin, foothills and highlands of the Andes. A total of 36 species were identified, 16 of them are new records for Ecuador. With these findings, the fauna of *Culicoides* of Ecuador adds up to 65 species.

TABLA DE CONTENIDO

ÍNDICE DE TABLAS

Artículo 1

Artículo 2

ÍNDICE DE FIGURAS

Artículo 1

ARTÍCULO 1

C. insignis as possible vector of bluetongue virus in Ecuador

Juan D. Mosquera[a], Denis Augot[b], Sonia Zapata[a]

[a]Instituto de Microbiología, Universidad San Francisco de Quito. Diego de Robles y Vía Interoceánica, Quito, Ecuador

[b]USC ANSES « VECPAR » Faculté de Pharmacie Université de Reims Champagne-Ardenne Reims, France

INTRODUCTION

Culicoides biting midges belong to the Ceratopogonidae family and are among the smallest and most numerous hematophagous flies, with more than 1400 species described (Meiswinkel, Venter, Nevill, Coetzer, & Tustin, 2004). Some species of biting midges are biological vectors of arboviruses of great veterinary importance, like the bluetongue virus (BTV), and epizootic hemorrhagic disease virus (EHDV) (P. Mellor, Boorman, & Baylis, 2000). BTV and EHDV are closely related orbiviruses, with segmented double-stranded RNA (dsRNA) genomes, and causative agents of non-contagious infectious diseases in ruminants (Kienzle, Poulson, Ruder, & Stallknecht, 2017). Bluetongue disease (BTD) is considered as notifiable by the Office International des Epizooties (OIE) given its dramatic economic impact (Commission & Committee, 2008). Although there is not an exact number, losses are estimated to be around US$3 billion per year globally, due to cattle mortality, export restrictions of live animals, and derived products (Rushton & Lyons, 2015). On the other hand, there is limited information regarding the economic losses caused by epizootic hemorrhagic disease (EHD). In Israel, a pioneer study of the dairy cattle industry showed that EHD costs approximately US$ 2,491,000 in terms of mortality and milk loss (Kedmi, Van Straten, Ezra, Galon, & Klement, 2010).

BTD and EHDV are linked to the geographic distribution of their arboviruses, which in turn depend on climatic and environmental factors that affect the areas containing competent vector species, as well as susceptible hosts (Coetzee, Stokstad, Venter, Myrmel, & Van Vuuren, 2012; W. J. Tabachnick, 1996). BTV and EHDV have a virtually worldwide distribution in latitudes between 40°N and 35°S, and are usually detected circulating together (Dulac et al., 1989; Sailleau et al., 2012; Viarouge et al., 2014). However, BTD has managed to expand further away from its original range towards previously disease-free zones (P. S. Mellor, Carpenter, Harrup, Baylis, & Mertens, 2008; Saegerman et al., 2010). Certain changes in climate and weather conditions can contribute to the dispersal of vector-borne diseases by improving vector population sizes and their means of dispersal (Brand & Keeling, 2017; Purse et al., 2005). Accordingly, the expansion of BTD has been attributed principally to windborne spread of infected *Culicoides* females from enzootic areas, in addition to the geographical distribution of ruminants, and to changes in global climate factors (Purse et al., 2005).

Some species of *Culicoides* have been implicated in the transmission both BTV and EHD. Supported on epidemiological data, the main suspected vectors are relatively well defined by region, including: North America (*C. sonorensis* and *C. variipennis*), South America (*C. insignis*), Africa (*C. imicola*), Australia (*C. brevitarsis* and *C. wadai*), Asia and Indonesia (*C. fulvus*) and *Culicoides obsoletus* group in Northern Europe (Hoffmann et al., 2009; Ruder et al., 2012; W. Tabachnick, 2004; Wilson & Mellor, 2009). The importance of identifying the specific vectors throughout each region lays in the establishment of measures for the control and prevention of both diseases (Oem et al., 2013).

In Ecuador, the presence of *C. insignis*, the main vector of BTV in South America, was recorded in localities from the Amazon and Highland regions (Gualapuro & Rubén, 2013; Salazar Alekseyeva, 2014). Additionally, alarming serological evidence of the occurrence of BTV and

EHDV, was found in cattle and sheep from Ecuadorian slaughter houses and dairy farms (Verdezoto Velarde, 2016). In spite of this, there has not been a single report of BTD or EHD in Ecuador (Escandón Escandón, 2011). Therefore, the aim of this study is to detect and quantify the viral genomes of BTV and EHDV in *C. insignis* collected mainly from places where a previously high seroprevalence of both orbiviruses was found in cattle, as a first step in the implication of *C. insignis* as the biological vector of both viruses in Ecuador.

MATERIALS AND METHODS

Entomological collections

CDC-like light traps were placed at different points in localities from the provinces of Santo Domingo and Napo (Table 1). The traps were suspended about 5 to 6 feet above the ground at the end of secondary forests, near pastures, and avoiding areas close to other light sources, and sites exposed to strong winds. The traps were set in the afternoon at 6pm and were collected the next morning at 6am, to overlap with the times of mayor activity of midges. Collected samples were sent to the Laboratory of Parasitology and Vectors of the Institute of Microbiology at Universidad San Francisco de Quito, and conserved in 70% ethanol at -20ºC.

Morphological identification of Culicoides spp. *and C. insignis*

The specimens belonging to the *Culicoides* genus were first separated from other arthropods based on morphological keys previously described by various authors (Augot et al., 2010; Borkent & Spinelli, 2007; Wirth, 1985). For identification of species other than *C. insignis*, each specimen was placed on a slide. Then, the wings, alter, mesonotum, head, legs and the three last segments of the abdomen were separated from the body using sterile needles. The

head and the last 3 segments of the abdomen were cleared with Marc André solution (Chloral Hydrate 40% and acetic acid 30%). The remaining portions of the abdomen were stored at -20 °C in 1.5 ml Eppendorf tubes. A drop of chloral gum (Chloral Hydrate 30%, Arabic Gum 20%, Glycerin 13%) was then placed on a microscope slide, on which the wings, alter, mesonotum, head, legs and the three last segments of the abdomen of the female midge were settled. A cover glass slide was placed to protect the entomological parts.

C. insignis midges were identified based on the wing patterns described by Spinelli, *et al*. (1993) (Spinelli, Greiner, & Wirth, 1993). Engorged and unengorged midges (lacking abdominal pigments that develop after blood meals) were separated, and each specimen was placed in a 1.5 ml Eppendorf tube. The tubes were stored at -20 °C for the posterior molecular detection of BTV and EHDV.

Nucleic acids extraction

Total nucleic acids, including dsRNA, were extracted with a modified protocol based on metal chelating agent Chelex-100 resin (BioRad) (Casquet, Thebaud, & Gillespie, 2012). One hundred µl of Chelex 10% was added to each tube containing one specimen of a nulliparous *C. insignis*, and crushed with a sterile pestle. The tubes were centrifuged for 15 seconds at 2300 g. After that, 5 µl of proteinase K was added to the samples, and then incubated for 60 minutes at 56 °C. Later, the samples were vortexed, incubated for 30 min at 95 °C, and centrifuged for 15 seconds at 2300 g. Finally, the supernatant was transferred to another 1.5 ml Eppendorf tube and stored at -20ºC. β-actin gen amplification was used as quality control of DNA extraction and to confirm the absence of PCR inhibitors.

RT-qPCR for BTV and EHDV segment 9

BTV and EHDV were tested in separate reactions. The oligonucleotides were obtained from Macrogen (Seul, Korea). Tables 1-4 contain the primers and probes sequences, reactions composition, and RT-PCR conditions for segment 9 of all serotypes of BTV and EHDV (Maan et al., 2015; Viarouge, Breard, Zientara, Vitour, & Sailleau, 2015). The extracted viral RNA from sera of two different bovines, one

containing BTV and one EHDV, were quantified and diluted to serve as positive controls, and for the standard curves. The RT-qPCR reactions were prepared with one-step RT-PCR Kit (SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, Invitrogen), in a laminar flow hood to avoid contamination. Samples were first denatured by heating at 99°C for 5 minutes followed by cooling. The RT-qPCR was performed in a CFX96™Real Time System thermal cycler (Bio-Rad).

RESULTS

Culicoides and C. insignis collection

A total of 3684 specimens of female *Culicoides* were collected, from which 326 (8.85%) were *C. insignis*. A higher percentage of *C. insignis* females was found in the locality of Cotundo (26.50%), in contrast with Paraiso Escondido (0.57%) (Table 5).

Detection and quantification of BTV and EHDV via RT-qPCR

BTV was detected in one specimen from Cotundo. None of the specimens were positive for EHDV. The quantification assay found a viral load of 10^5 copies of the virus per 4 µl of RNA, meaning that the sample of 100 µl contained a total of 10^6 copies of the virus. (Figure 2). The

presence of BTV was confirmed by sequencing (Functional Biosciences, Inc). Sequence identity was confirmed with BLAST (NCBI), showing correspondence with segment 9.

DISCUSSION

BTV and EHDV can infect ruminant hosts by means of certain species of *Culicoides* hematophagous biting midges. *C. insignis* is one of the competent vectors of BTV and has been deemed as a possible candidate for the transmission of EHDV (Smith et al., 1996; Tanya, Greiner, & Gibbs, 1992). In Ecuador, the species of midges involved in the transmission of BTV and EHDV are still unknown. *C. insignis* has been found in Ecuadorian localities of the Amazon and Highland regions. Also, the circulation of BTV and EHDV in domestic ruminants has been reported by serological tests, and the etiological agents have been isolated from blood samples in cattle (Verdezoto et al., 2017; Verdezoto Velarde, 2016). In this study, *C. insignis* females were identified based on morphological characteristics and according to the patterns of their wings. The midges were collected inhabiting the same places where high seroprevalence of BTV and EHDV was found in cattle, and they were tested for both viruses by RT-qPCR.

Different criteria are used to consider an arthropod as a biological vector of arboviruses. The presence of the virus and the abundance of the suspected vector are two of them (DeFoliart, Grimstad, & Watts, 1987). In Cotundo, where the midge carrying BTV was found, *C. insignis* accounted for more than one fourth of the collected *Culicoides*. The occurrence of both host and midge in the same area strengthens the evidence of *C. insignis* as a vector of BTV in Ecuador. However, BTV remains to be isolated from *C. insignis*.

BTV have also been isolated from other species of *Culicoides* in South America, such as *C. filarifer* and *C. pusillus* (Lager, 2004). The presence of *C. filarifer* in Ecuador has been reported in a study conducted by Gualapuro (2013) (Gualapuro & Rubén, 2013). These species should also be considered as possible vectors in further studies in localities, like Paraiso Escondido, where *C. insignis* is not present in considerable numbers, and in the case of EHDV, since none of the specimens tested were positive for this virus.

The quantification of the viral copies serves as an indicator of the level of infection in adult *Culicoides.* Titers greater than 10^3 have been associated with a disseminated infection in individual midges. *Culicoides* can bear concentrations up to 10^6 of BTV for their entire lifetime and high viral loads could be related to transmissibility (P. Mellor, 1990; Veronesi et al., 2008).

In conclusion, the present study shows preliminary evidence to incriminate *C. insignis* as a possible vector for BTV in Ecuador. EHDV's vector remains to be unknown, so further studies should be conducted to determinate the *Culicoides* species involved in its transmission. The detection of the virus in field specimens is also crucial for the understanding of the epidemiology of the transmitted diseases, information that can contribute to the implementation of control and prevention programs, especially in countries like Ecuador where BTV and EHDV have only been recently reported.

ACKNOWLEDGMENTS

This study was supported by a Collaboration Grant from the San Francisco de Quito University. We would also like to thank Gustavo Spinelli for the information provided for the identification of *Culicoides* spp.

REFERENCES

Meiswinkel, R., et al., *Vectors: Culicoides spp.* Infectious diseases of livestock, 2004. **1**: p. 93-136.

Mellor, P., J. Boorman, and M. Baylis, *Culicoides biting midges: their role as arbovirus vectors.* Annual review of entomology, 2000. **45**(1): p. 307-340.

Kienzle, C., et al., *Virus Isolation and Molecular Detection of Bluetongue and Epizootic Hemorrhagic Disease Viruses from Naturally Infected White-Tailed Deer (Odocoileus virginianus).* Journal of Wildlife Diseases, 2017.

Commission, I.O.o.E.B.S. and I.O.o.E.I. Committee, *Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees*. Vol. 2. 2008: Office international des épizooties.

Rushton, J. and N. Lyons, *a review of the effects on production.* Veterinaria italiana, 2015. **51**(4): p. 401-406.

Kedmi, M., et al., *Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds.* Journal of dairy science, 2010. **93**(6): p. 2486-2495.

Tabachnick, W.J., *Culicoides variipennis and bluetongue-virus epidemiology in the United States.* Annual review of entomology, 1996. **41**(1): p. 23-43.

Coetzee, P., et al., *Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa.* Virology journal, 2012. **9**(1): p. 198.

Dulac, G., et al., *British Columbia. Incursion of bluetongue virus type 11 and epizootic hemorrhagic disease of deer type 2 for two consecutive years in the Okanagan Valley.* The Canadian Veterinary Journal, 1989. **30**(4): p. 351.

Sailleau, C., et al., *Co-circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in Reunion Island.* Veterinary microbiology, 2012. **155**(2): p. 191-197.

Viarouge, C., et al., *Identification of bluetongue virus and epizootic hemorrhagic disease virus serotypes in French Guiana in 2011 and 2012.* Veterinary microbiology, 2014. **174**(1): p. 78-85.

Saegerman, C., et al., *The most likely time and place of introduction of BTV8 into Belgian ruminants.* PloS one, 2010. **5**(2): p. e9405.

Mellor, P.S., et al., *Bluetongue in Europe and the Mediterranean Basin: history of occurrence prior to 2006.* Preventive veterinary medicine, 2008. **87**(1): p. 4-20.

Purse, B.V., et al., *Climate change and the recent emergence of bluetongue in Europe.* Nature Reviews Microbiology, 2005. **3**(2): p. 171-181.

Brand, S.P. and M.J. Keeling, *The impact of temperature changes on vector-borne disease transmission: Culicoides midges and bluetongue virus.* Journal of The Royal Society Interface, 2017. **14**(128): p. 20160481.

Ruder, M.G., et al., *Vector competence of Culicoides sonorensis (Diptera: Ceratopogonidae) to epizootic hemorrhagic disease virus serotype 7.* Parasites & vectors, 2012. **5**(1): p. 236.

Wilson, A.J. and P.S. Mellor, *Bluetongue in Europe: past, present and future.* Philosophical Transactions of the Royal Society of London B: Biological Sciences, 2009. **364**(1530): p. 2669-2681.

Tabachnick, W., *Culicoides and the global epidemiology of bluetongue virus infection.* Vet Ital, 2004. **40**(3): p. 144-150.

Hoffmann, B., et al., *Monitoring of putative vectors of bluetongue virus serotype 8, Germany.* Emerging infectious diseases, 2009. **15**(9): p. 1481.
Oem, J.-K., et al., *Abundance of biting midge species (Diptera: Ceratopogonidae, Culicoides spp.) on cattle farms in Korea.* Journal of veterinary science, 2013. **14**(1): p. 91-94.
Gualapuro, G. and M. Rubén, *Contribución al estudio de la fauna de Culicoides (Díptera: Ceratopogonidae) en la zona norte del Ecuador*. 2013, Quito: USFQ, 2013.
Salazar Alekseyeva, K.J., *Diversidad genética de Culicoides insignis (Diptera: Ceratopogonidae) en cuatro provincias de la zona norte y centro del Ecuador*. 2014, Quito: USFQ, 2014.
Verdezoto Velarde, J.E., *Investigación de Orbivirus en ganado vacuno y sus posibles vectores*. 2016, Quito: USFQ, 2016.
Escandón Escandón, A.M., *Lengua azúl en bovinos*. 2011.
Augot, D., et al., *Discrimination of Culicoides obsoletus and Culicoides scoticus, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis.* Infection, genetics and evolution, 2010. **10**(5): p. 629-637.
Borkent, A. and G.R. Spinelli, *Neotropical Ceratopogonidae (Diptera, Insecta): Ceratopogonidae Neotropicales (Diptera, Insecta)*. Vol. 4. 2007: Pensoft Publishers.
Wirth, W.W., *An atlas of wing photographs, with a summary of the numerical characters of Nearctic species of Culicoides (Diptera: Ceratopogonidae).* Contrib. Am. Entomol. Inst., 1985. **22**: p. 1-46.
Spinelli, G.R., E. Greiner, and W. Wirth, *The Neotropical bloodsucking midges of the Culicoides guttatus group of the subgenus Hoffmania (Diptera: Ceratopogonidae).* Contributions of the American Entomological Institute (USA), 1993.
Casquet, J., C. Thebaud, and R.G. Gillespie, *Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders.* Molecular Ecology Resources, 2012. **12**(1): p. 136-141.
Maan, N.S., et al., *A quantitative real-time reverse transcription PCR (qRT-PCR) assay to detect genome segment 9 of all 26 bluetongue virus serotypes.* Journal of virological methods, 2015. **213**: p. 118-126.
Viarouge, C., et al., *Duplex real-time RT-PCR assays for the detection and typing of epizootic haemorrhagic disease virus.* PloS one, 2015. **10**(7): p. e0132540.
Smith, K.E., et al., *Monitoring of Culicoides spp. at a site enzootic for hemorrhagic disease in white-tailed deer in Georgia, USA.* Journal of wildlife diseases, 1996. **32**(4): p. 627-642.
Tanya, V., E. Greiner, and E. Gibbs, *Evaluation of Culicoides insignis (Diptera: Ceratopogonidae) as a vector of bluetongue virus.* Veterinary microbiology, 1992. **32**(1): p. 1-14.
Verdezoto, J., et al., *Novel serotype of bluetongue virus in South America and first report of epizootic haemorrhagic disease virus in Ecuador.* Transboundary and Emerging Diseases, 2017.
DeFoliart, G., P. Grimstad, and D. Watts, *Advances in mosquito-borne arbovirus/vector research.* Annual review of entomology, 1987. **32**(1): p. 479-505.
Lager, I., *Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features.* Vet. ital, 2004. **40**(3): p. 89-93.
Veronesi, E., et al., *Quantifying bluetongue virus in adult Culicoides biting midges (Diptera: Ceratopogonidae).* Journal of medical entomology, 2008. **45**(1): p. 129-132.
Mellor, P., *The replication of bluetongue virus in Culicoides vectors*, in *Bluetongue viruses*. 1990, Springer. p. 143-161.

Perruolo, G.J., *Clave de las especies de Culicoides (Diptera: Ceratopogonidae) asociadas con la ganadería en la región Neotropical.* Revista Científica, 2009. **19**(002).

Wittmann, E., P. Mellor, and M. Baylis, *Effect of temperature on the transmission of orbiviruses by the biting midge, Culicoides sonorensis.* Medical and veterinary entomology, 2002. **16**(2): p. 147-156.

Blanton, F.S. and W.W. Wirth, *Arthropods of Florida and neighbouring land areas. Volume 10. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae).* Arthropods of Florida and neighbouring land areas. Volume 10. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae). 1979.

Ducheyne, E., et al., *Quantifying the wind dispersal of Culicoides species in Greece and Bulgaria.* Geospatial Health, 2007. **1**(2): p. 177-189.

Beckenbach, A.T. and A. Borkent, *Molecular analysis of the biting midges (Diptera: Ceratopogonidae), based on mitochondrial cytochrome oxidase subunit 2.* Molecular phylogenetics and evolution, 2003. **27**(1): p. 21-35.

Mihn Hoan, T., *Contribution a l'étude des Ceratopogonidae de nouveau monde: le genre Culicoides Latreille, 1809 (Diptera: Ceratopogonidae) morphologie, importance médicale et vétérinaire, description des espèces de la Guyane Française.* Universite Paris: Paris, 2010.

Santamaría, E., et al., *Preliminary evaluation of the Culicoides biting nuisance (Diptera: Ceratopogonidae) in the province of Boyacá, Colombia.* Biomedica, 2008. **28**(4): p. 497-509.

Roberts, D.R., et al., *Oropouche virus.* The American journal of tropical medicine and hygiene, 1981. **30**(1): p. 165-171.

Bass, M.S., et al., *Global conservation significance of Ecuador's Yasuní National Park.* PloS one, 2010. **5**(1): p. e8767.

Zook, D., *Tropical rainforests as dynamic symbiospheres of life.* Symbiosis, 2010. **51**(1): p. 27-36.

da Rosa, J.F.T., et al., *Oropouche Virus: Clinical, Epidemiological, and Molecular Aspects of a Neglected Orthobunyavirus.* The American journal of tropical medicine and hygiene, 2017. **96**(5): p. 1019-1030.

Manock, S.R., et al., *Etiology of acute undifferentiated febrile illness in the Amazon basin of Ecuador.* The American journal of tropical medicine and hygiene, 2009. **81**(1): p. 146-151.

Diaz, F., M.M. Ronderos, and G.R. Spinelli, *The immatures of the Neotropical species Culicoides venezuelensis Ortiz and Mirsa (Diptera: Ceratopogonidae).* Transactions of the American Entomological Society, 2005: p. 375-385.

Aybar, C.A.V., et al., *Spatial and temporal distribution of Culicoides insignis and Culicoides paraensis in the subtropical mountain forest of Tucumán, northwestern Argentina.* Florida entomologist, 2011. **94**(4): p. 1018-1025.

Ander, M., K. Troell, and J. Chirico, *Barcoding of biting midges in the genus Culicoides: a tool for species determination.* Medical and veterinary entomology, 2013. **27**(3): p. 323-331.

TABLES

Table 1: Collection points of *Culicoides*

Province	Locality
Napo	Cotundo
Santo Domingo	Paraiso escondido

Table 2: List of oligonucleotides for BTV and EHDV

Name	Sequence (5'-3')
BTV-S9(1000-1023) P	**FAM**-CACCTCTAAAGGGTCCAGGGTACC-**BHQ-1**
BTV-S9(956-978) FP1	GYRCGGGNGGDGAYRYDAARAYG
BTV-S9(956-977) FP2	GTACAGGCGGAGATGTGAAAACG
BTV-S9(1048-1026) RP1	RWRBRAAATCGCMCTACGTCAAG
BTV-S9(1048-1026) RP2	GTGTAAAACCGCTATATGCCGTG
EHDV-S9(29-44) P	**FAM**- TTTGCTCGCACCCGGT -**BHQ-1**
EHDV-S9(7-25) FP	AATTGCGCATGTCAGCTGC
EHDV-S9(76-55) RP	TTTAATTCCTCGGTCGAACGTT

Table 3: RT-PCR conditions for BTV and EHDV

Number of cycles	Temperature (°C)	Time (minutes)
1	95	5.0
1	55	30.0
1	95	10.0
40	95	0.5
40*	60*	1.0*
* Annealing/elongation step in which fluorescence was measured		

Table 4: RT-PCR reaction composition for the BTV and EHDV

BTV			EHDV		
Reagent	**Volume (µl)**	**Final concentration**	**Reagent**	**Volume (µl)**	**Final concentration**
Probe	0.5	0.1 µM	Probe	1.0	0.1 µM
Forward primer 1	1.0	0.8 µM	Forward primer	0.5	0.8 µM
Forward primer 2	1.0	0.8 µM	Reverse primer	0.5	0.8 µM
Reverse primer 1	1.0	0.8 µM	ROX	0.5	1.0 nM
Reverse primer 2	1.0	0.8 µM	$MgSO_4$*	1.0	
ROX	0.5	1.0 nM	2x reaction mix*	12.5	
$MgSO_4$*	1.0		Superscript III RT/platinum Taq mix*	0.5	
2x reaction mix*	12.5		DEPC treated water	4.5	
Superscript III RT/platinum Taq mix*	0.5		dsRNA	4.0	
DEPC treated water	2.0				
dsRNA	4.0				

* SuperScript III/Platinum Taq One-Step qRT-PCR Kit (Invitrogen)

Table 5: Number of *Culicoides* specimens collected by locality

Locality	***Culicoides spp.* females**	***C. insignis* females**
Cotundo	860	310 (26.50%)
Paraíso Escondido	2824	16 (0.57%)
Total	**3684**	**326 (8.85%)**

FIGURES

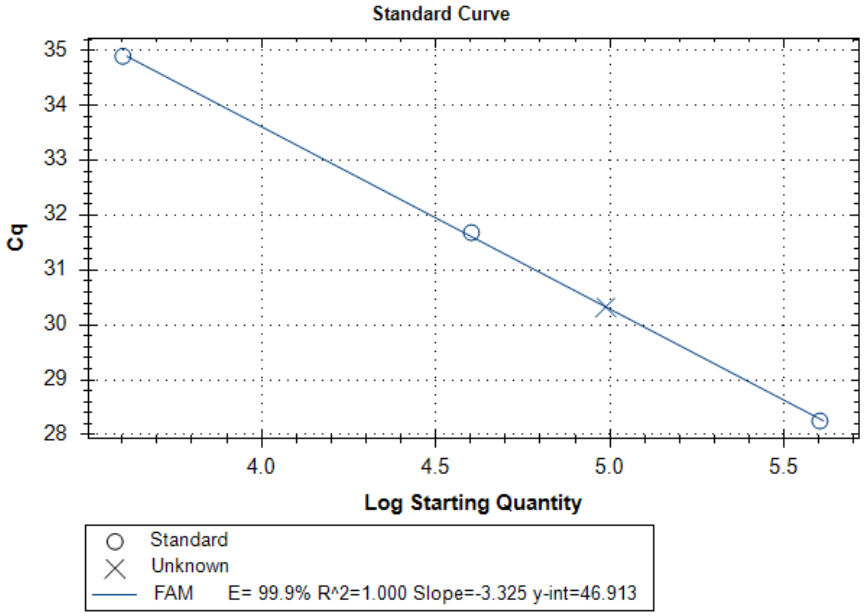

Figure 1: Quantification curve of the positive sample for BTV from Cotundo

ARTÍCULO 2

New records of *Culicoides* (Diptera: Ceratopogonidae) from Ecuador

Juan D. Mosquera[a]. Sonia Zapata[a], Moises Gualapuro[a], Renato León[a] and Denis Augot[b]

[a]Instituto de Microbiología, Universidad San Francisco de Quito. Diego de Robles y Vía Interoceánica, Quito, Ecuador

[b]USC ANSES « VECPAR » Faculté de Pharmacie Université de Reims Champagne-Ardenne Reims, France

INTRODUCTION

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are the smallest (1-3 mm) haematophagous flies, broadly distributed throughout the world (Borkent & Spinelli, 2007). The identification of *Culicoides* is based on morphological and morphometrical parameters, mainly based on their wing patterns (Borkent & Spinelli, 2007; Perruolo, 2009; Wirth, 1985). Some species of *Culicoides* are involved in the transmission of viruses, protozoans and filarial worms to a variety of hosts. Some of the transmitted diseases, such as the Oropouche fever (OF) and the Bluetongue disease (BTD), are of great medical, veterinary, and economic importance (Wittmann, Mellor, & Baylis, 2002).

The ability of *Culicoides* to serve as biological vectors is linked to their considerable abundance and population sizes. Only females feed on blood. Although there are species that feed during the day, most of them have crepuscular habits (P. Mellor et al., 2000). The life cycle of *Culicoides* is comprised by the stages of egg, larva (4 stages), pupa, and imago. Biting midges

breed near water sources and rotten vegetation, since moisture is needed for the development of their immatures (Blanton & Wirth, 1979). Their flying capacity is limited, so they can only reach a couple hundred meters away from their breeding sites. On the other hand, wind currents can drastically improve their range of dispersal (Ducheyne et al., 2007).

In the Neotropical region, a total of 285 species have been identified. from which 49 can be found in Ecuador, 114 in Colombia, 20 in Perú, 111 in Brazil, and 52 in Venezuela (Beckenbach & Borkent, 2003; Borkent & Spinelli, 2007; Mihn Hoan, 2010). Factors such as recent changes in global climate have been held responsible for variations in the distribution of *Culicoides,* and therefore, the distribution of the diseases they carry (Purse et al., 2005). In Ecuador, evidence of the advent of BTD and OF was recently found, but, information concerning the distribution of *Culicoides*, including candidate vector species, is very limited.

Therefore, the purpose of this study is to determine the new distribution of species of the genus *Culicoides* in Northern Provinces of Ecuador, information that can contribute to the prevention of transmitted diseases and the control of possible vectors.

MATERIALS AND METHODS

Entomological collections

Biting midges were captured using CDC-like light traps, between July 2010 and September 2012, in the localities of Tiputini and Cofán Dureno (Amazon Basin); Pacto (Foothills); Urkusiki and Tambillo (Highlands of the Andes). The traps where set around 6pm and collected at 8am, since the peaks of activity of most *Culicoides* are higher at sunset and sunrise. Specimens from the genus *Culicoides* were separated from other arthropods based on

morphological keys, using a stereo microscope, and stored in 1.5 ml eppendorf tubes with 70% ethanol at -20°C.

Species identification

Females' wings, head, alter, mesonotum, legs and the last three segments of the abdomen were mounted on slides in chloral gum media and were examined with a stereo microscope. Morphological and morphometrical patterns described by various authors were used to identify the biting midges at species level. Samples were sent to Dr. Gustavo Spinelli (Argentina) for confirmation of the identified species. All specimens were deposited in the collections of the San Francisco de Quito University.

RESULTS

Entomological collections

A total of 4,453 specimens of the genus *Culicoides* were collected, from which 3838 were captured in Yasuní-Tiputini, 73 in Cofán-Dureno, 323 in Pacto, and 219 in Urkusiki. Females accounted for 77.9% of the specimens.

Species identification

Thirty-six species were identified in this study. They belonged to 5 different subgenres: *Anilomyia, Haematomyidium, Hoffmania, Mataemyia, Psychophaena*; and 9 species group: *acotylus, carpenteri, eublepharus, fluvialis, limai, pachymerus, reticulatus, stigmalis* and *leoni* (Table 1). Sixteen species were found to be new records for Ecuador, two of them being *C. insignis*, a known vector of BTD, and *C. pachymerus*, causative agent of dermatozoonosis

(Santamaría et al., 2008; Tanya et al., 1992). The presence of *C. paraensis*, vector of OF, was also confirmed (Roberts, Hoch, Dixon, & Llewellyn, 1981). *C. venezuelensis* was found for the first time in the Amazon (Sucumbios) and Highlands (Pichincha and Imbabura). From the new records, only *C. insignis* and *C. pseudoheliconiae* were found in both regions.

DISCUSSION

The new records presented in this study add up to a total of 65 species of *Culicodes* in Ecuador. Some species are also found in the neighboring countries of Colombia, Peru and in the of Brazilian Amazon. The most number of species was found in the Amazon region, in the locality of Tiputini. This supports other studies, in which Tiputini was attributed the most diversity in terms of insects in the world (100,000 species/Ha) (Bass et al., 2010; Zook, 2010).

Among the records, there are some species of medical and veterinary importance, such as *C. paraensis, C. insignis. C. paraensis* is one of the known vectors of the Oropouche virus (OROV). It is estimated that more than half million people have been affected by the OROV since the it was first isolated in Brazil (da Rosa et al., 2017). In Ecuador, a study conducted in patients from the province of Pastaza (Amazon) found serological evidence of the OROV (Amazon region) (Manock et al., 2009). In this study, *C. paraensis* was found in Orellana, a province that is located north of Pastaza, and therefore could also be present in Pastaza.

C. insignis is the main vector of BTD in South America, a disease of cattle that causes loses of around USS$3 billion per year worldwide (Rushton & Lyons, 2015). The presence of the Bluetongue virus (BTV) has previously been reported in blood samples from Ecuadorian cattle (Verdezoto et al., 2017). The vector of BTD for the country is not yet known, but *C. insignis* has become the most probable candidate.

Some species like *C. insignis* were found in both Amazon and Highland regions. It is possible that they have a greater adaptation capacity since their broad distribution throughout South America (Aybar, Juri, De Grosso, & Spinelli, 2011; Diaz, Ronderos, & Spinelli, 2005). On the other hand, the seemingly same midges present in both sides of the Andes mountain range could be cryptic species, product of geographic isolation. The main tool for *Culicoides* species identification are morphological keys, but they are unable to distinguishing between cryptic species, together with being difficult and time-consuming. With the advent of molecular entomology, techniques such as barcoding could be used to characterize sister species and shed light to their taxonomic relationships (Ander, Troell, & Chirico, 2013).

Sixteen new records are reported for Ecuador in this study. With the finding of *C. insignis* and the confirmation of C. *paraensis*, further studies involving the isolation of BTV and OROV are needed to stablish their role as vectors in the country. Also, expanding the information about the distribution of *Culicoides* and vector candidate species could prove useful in terms implementing control and prevention measures for diseases such as BTD and OF.

ACKNOLEDGMENTS

We would like to thank Dr. Gustavo Spinelli for helping with the identification confirmation of the species found in this study.

REFERENCES

Ander, M., Troell, K., & Chirico, J. (2013). Barcoding of biting midges in the genus Culicoides: a tool for species determination. *Medical and veterinary entomology, 27*(3), 323-331.

Augot, D., Sauvage, F., Jouet, D., Simphal, E., Veuille, M., Couloux, A., . . . Depaquit, J. (2010). Discrimination of Culicoides obsoletus and Culicoides scoticus, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. *Infection, genetics and evolution, 10*(5), 629-637.

Aybar, C. A. V., Juri, M. J. D., De Grosso, M. S. L., & Spinelli, G. R. (2011). Spatial and temporal distribution of Culicoides insignis and Culicoides paraensis in the subtropical mountain forest of Tucumán, northwestern Argentina. *Florida entomologist, 94*(4), 1018-1025.

Bass, M. S., Finer, M., Jenkins, C. N., Kreft, H., Cisneros-Heredia, D. F., McCracken, S. F., . . . Villa, G. (2010). Global conservation significance of Ecuador's Yasuní National Park. *PloS one, 5*(1), e8767.

Beckenbach, A. T., & Borkent, A. (2003). Molecular analysis of the biting midges (Diptera: Ceratopogonidae), based on mitochondrial cytochrome oxidase subunit 2. *Molecular phylogenetics and evolution, 27*(1), 21-35.

Blanton, F. S., & Wirth, W. W. (1979). Arthropods of Florida and neighbouring land areas. Volume 10. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae). *Arthropods of Florida and neighbouring land areas. Volume 10. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae).*

Borkent, A., & Spinelli, G. R. (2007). *Neotropical Ceratopogonidae (Diptera, Insecta): Ceratopogonidae Neotropicales (Diptera, Insecta)* (Vol. 4): Pensoft Publishers.

Brand, S. P., & Keeling, M. J. (2017). The impact of temperature changes on vector-borne disease transmission: Culicoides midges and bluetongue virus. *Journal of The Royal Society Interface, 14*(128), 20160481.

Casquet, J., Thebaud, C., & Gillespie, R. G. (2012). Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Molecular Ecology Resources, 12*(1), 136-141.

Coetzee, P., Stokstad, M., Venter, E. H., Myrmel, M., & Van Vuuren, M. (2012). Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa. *Virology journal, 9*(1), 198.

Commission, I. O. o. E. B. S., & Committee, I. O. o. E. I. (2008). *Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees* (Vol. 2): Office international des épizooties.

da Rosa, J. F. T., de Souza, W. M., de Paula Pinheiro, F., Figueiredo, M. L., Cardoso, J. F., Acrani, G. O., & Nunes, M. R. T. (2017). Oropouche Virus: Clinical, Epidemiological, and Molecular Aspects of a Neglected Orthobunyavirus. *The American journal of tropical medicine and hygiene, 96*(5), 1019-1030.

DeFoliart, G., Grimstad, P., & Watts, D. (1987). Advances in mosquito-borne arbovirus/vector research. *Annual review of entomology, 32*(1), 479-505.

Diaz, F., Ronderos, M. M., & Spinelli, G. R. (2005). The immatures of the Neotropical species Culicoides venezuelensis Ortiz and Mirsa (Diptera: Ceratopogonidae). *Transactions of the American Entomological Society*, 375-385.

Ducheyne, E., De Deken, R., Bécu, S., Codina, B., Nomikou, K., Mangana-Vougiaki, O., . . . Hendrickx, G. (2007). Quantifying the wind dispersal of Culicoides species in Greece and Bulgaria. *Geospatial Health, 1*(2), 177-189.

Dulac, G., Dubuc, C., Myers, D., Afshar, A., Taylor, E., Ward, D., & Sterritt, W. (1989). British Columbia. Incursion of bluetongue virus type 11 and epizootic hemorrhagic disease of deer type 2 for two consecutive years in the Okanagan Valley. *The Canadian Veterinary Journal, 30*(4), 351.

Escandón Escandón, A. M. (2011). *Lengua azúl en bovinos.*

Gualapuro, G., & Rubén, M. (2013). *Contribución al estudio de la fauna de Culicoides (Díptera: Ceratopogonidae) en la zona norte del Ecuador.* Quito: USFQ, 2013.

Hoffmann, B., Bauer, B., Bauer, C., Bätza, H.-J., Beer, M., Clausen, P.-H., . . . Liebisch, G. (2009). Monitoring of putative vectors of bluetongue virus serotype 8, Germany. *Emerging infectious diseases, 15*(9), 1481.

Kedmi, M., Van Straten, M., Ezra, E., Galon, N., & Klement, E. (2010). Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. *Journal of dairy science, 93*(6), 2486-2495.

Kienzle, C., Poulson, R. L., Ruder, M. G., & Stallknecht, D. E. (2017). Virus Isolation and Molecular Detection of Bluetongue and Epizootic Hemorrhagic Disease Viruses from Naturally Infected White-Tailed Deer (Odocoileus virginianus). *Journal of Wildlife Diseases*.

Lager, I. (2004). Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features. *Vet. ital, 40*(3), 89-93.

Maan, N. S., Maan, S., Belaganahalli, M., Pullinger, G., Montes, A. J. A., Gasparini, M. R., . . . Mertens, P. P. (2015). A quantitative real-time reverse transcription PCR (qRT-PCR) assay to detect genome segment 9 of all 26 bluetongue virus serotypes. *Journal of virological methods, 213*, 118-126.

Manock, S. R., Jacobsen, K. H., de Bravo, N. B., Russell, K. L., Negrete, M., Olson, J. G., . . . Quist, B. K. (2009). Etiology of acute undifferentiated febrile illness in the Amazon basin of Ecuador. *The American journal of tropical medicine and hygiene, 81*(1), 146-151.

Meiswinkel, R., Venter, G., Nevill, E., Coetzer, J., & Tustin, R. (2004). Vectors: Culicoides spp. *Infectious diseases of livestock, 1*, 93-136.

Mellor, P. (1990). The replication of bluetongue virus in Culicoides vectors *Bluetongue viruses* (pp. 143-161): Springer.

Mellor, P., Boorman, J., & Baylis, M. (2000). Culicoides biting midges: their role as arbovirus vectors. *Annual review of entomology, 45*(1), 307-340.

Mellor, P. S., Carpenter, S., Harrup, L., Baylis, M., & Mertens, P. P. (2008). Bluetongue in Europe and the Mediterranean Basin: history of occurrence prior to 2006. *Preventive veterinary medicine, 87*(1), 4-20.

Mihn Hoan, T. (2010). Contribution a l'étude des Ceratopogonidae de nouveau monde: le genre Culicoides Latreille, 1809 (Diptera: Ceratopogonidae) morphologie, importance médicale et vétérinaire, description des espèces de la Guyane Française. *Universite Paris: Paris*.

Oem, J.-K., Chung, J.-Y., Kwon, M.-S., Kim, T.-K., Lee, T.-U., & Bae, Y.-C. (2013). Abundance of biting midge species (Diptera: Ceratopogonidae, Culicoides spp.) on cattle farms in Korea. *Journal of veterinary science, 14*(1), 91-94.

Perruolo, G. J. (2009). Clave de las especies de Culicoides (Diptera: Ceratopogonidae) asociadas con la ganadería en la región Neotropical. *Revista Científica, 19*(002).

Purse, B. V., Mellor, P. S., Rogers, D. J., Samuel, A. R., Mertens, P. P., & Baylis, M. (2005). Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology, 3*(2), 171-181.

Roberts, D. R., Hoch, A. L., Dixon, K. E., & Llewellyn, C. H. (1981). Oropouche virus. *The American journal of tropical medicine and hygiene, 30*(1), 165-171.

Ruder, M. G., Howerth, E. W., Stallknecht, D. E., Allison, A. B., Carter, D. L., Drolet, B. S., . . . Mead, D. G. (2012). Vector competence of Culicoides sonorensis (Diptera: Ceratopogonidae) to epizootic hemorrhagic disease virus serotype 7. *Parasites & vectors, 5*(1), 236.

Rushton, J., & Lyons, N. (2015). a review of the effects on production. *Veterinaria italiana, 51*(4), 401-406.

Saegerman, C., Mellor, P., Uyttenhoef, A., Hanon, J.-B., Kirschvink, N., Haubruge, E., . . . Vandenbussche, F. (2010). The most likely time and place of introduction of BTV8 into Belgian ruminants. *PloS one, 5*(2), e9405.

Sailleau, C., Zanella, G., Breard, E., Viarouge, C., Desprat, A., Vitour, D., . . . Bakkali-Kassimi, L. (2012). Co-circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in Reunion Island. *Veterinary microbiology, 155*(2), 191-197.

Salazar Alekseyeva, K. J. (2014). *Diversidad genética de Culicoides insignis (Diptera: Ceratopogonidae) en cuatro provincias de la zona norte y centro del Ecuador.* Quito: USFQ, 2014.

Santamaría, E., Cabrera, O. L., Zipa, Y., Ferro, C., Ahumada, M. L., & Pardo, R. H. (2008). Preliminary evaluation of the Culicoides biting nuisance (Diptera: Ceratopogonidae) in the province of Boyacá, Colombia. *Biomedica, 28*(4), 497-509.

Smith, K. E., Stallknecht, D. E., Sewell, C. T., Rollor, E. A., Mullen, G. R., & Anderson, R. R. (1996). Monitoring of Culicoides spp. at a site enzootic for hemorrhagic disease in white-tailed deer in Georgia, USA. *Journal of Wildlife Diseases, 32*(4), 627-642.

Spinelli, G. R., Greiner, E., & Wirth, W. (1993). The Neotropical bloodsucking midges of the Culicoides guttatus group of the subgenus Hoffmania (Diptera: Ceratopogonidae). *Contributions of the American Entomological Institute (USA)*.

Tabachnick, W. (2004). Culicoides and the global epidemiology of bluetongue virus infection. *Vet Ital, 40*(3), 144-150.

Tabachnick, W. J. (1996). Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annual review of entomology, 41*(1), 23-43.

Tanya, V., Greiner, E., & Gibbs, E. (1992). Evaluation of Culicoides insignis (Diptera: Ceratopogonidae) as a vector of bluetongue virus. *Veterinary microbiology, 32*(1), 1-14.

Verdezoto, J., Breard, E., Viarouge, C., Quenault, H., Lucas, P., Sailleau, C., . . . Zapata, S. (2017). Novel serotype of bluetongue virus in South America and first report of epizootic haemorrhagic disease virus in Ecuador. *Transboundary and Emerging Diseases*.

Verdezoto Velarde, J. E. (2016). *Investigación de Orbivirus en ganado vacuno y sus posibles vectores.* Quito: USFQ, 2016.

Veronesi, E., Mertens, P. P., Shaw, A. E., Brownlie, J., Mellor, P. S., & Carpenter, S. T. (2008). Quantifying bluetongue virus in adult Culicoides biting midges (Diptera: Ceratopogonidae). *Journal of medical entomology, 45*(1), 129-132.

Viarouge, C., Breard, E., Zientara, S., Vitour, D., & Sailleau, C. (2015). Duplex real-time RT-PCR assays for the detection and typing of epizootic haemorrhagic disease virus. *PloS one, 10*(7), e0132540.

Viarouge, C., Lancelot, R., Rives, G., Bréard, E., Miller, M., Baudrimont, X., . . . Sailleau, C. (2014). Identification of bluetongue virus and epizootic hemorrhagic disease virus serotypes in French Guiana in 2011 and 2012. *Veterinary microbiology, 174*(1), 78-85.

Wilson, A. J., & Mellor, P. S. (2009). Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 364*(1530), 2669-2681.

Wirth, W. W. (1985). An atlas of wing photographs, with a summary of the numerical characters of Nearctic species of Culicoides (Diptera: Ceratopogonidae). *Contrib. Am. Entomol. Inst., 22*, 1-46.

Wittmann, E., Mellor, P., & Baylis, M. (2002). Effect of temperature on the transmission of orbiviruses by the biting midge, Culicoides sonorensis. *Medical and veterinary entomology, 16*(2), 147-156.

Zook, D. (2010). Tropical rainforests as dynamic symbiospheres of life. *Symbiosis, 51*(1), 27-36.

TABLES

Table 1: Distribution of species identified in this study

Subgenres	Species	Sites of collection	Provinces
Anilomyia	*C. metagonatus*	Pa, Ti	Pichincha, Orellana
Haematomyidium	*C. ginesi*	Ti, CD	Orellana, Sucumbíos
	C. glabrior	Ti, CD	Orellana, Sucumbíos
	C. neoparaensis	Ti	Orellana
	C. paraensis	Ti, CD	Orellana, Sucumbíos
	*C. youngi**	Pa	Pichincha
Hoffmania	*C. diabolicus*	Pa, Ti	Pichincha, Orellana
	*C. foxi**	Ti	Orellana
	C. heliconiae	Pa, Ti	Pichincha, Orellana
	*C. insignis**	Pa, CD	Pichincha, Sucumbíos
	C. hylas	Pa, Ti, CD	Pichincha, Orellana, Sucumbíos
	C. guttatus	Ti	Orellana
	*C. pseudoheliconiae**	Pa, Ti	Pichincha, Orellana
	C. verecundus	Pa	Pichincha
Mataemyia	*C. bricenoi*	Ti	Orellana
Psychophaena	*C. venezuelensis*	Ur, Uy, CD	Imbabura, Pichincha, Sucumbíos
Species Group			
Group *acotylus*	*C. acotylus**	Ti	Orellana
Group *carpenteri*	*C. camposi*	Ti	Orellana
	*C. belemensis**	Ti	Orellana
Group *eublepharus*	*C. eublepahrus*	Ti	Orellana
Group *fluvialis*	*C. castillae*	Ti	Orellana
	*C. fluvialis**	Ti	Orellana
	C. leopoldoi	Ti	Orellana
	C. tetrathyris	Ti	Orellana
Group *leoni*	*C. leoni*	Ti	Orellana
Group *limai*	*C. carvalhoi**	Ti	Orellana
	C. limai	Ti	Orellana
	*C. lopesi**	Ti	Orellana
	*C. vernoni**	Pa	Pichincha
Group *pachymerus*	*C. pachymerus**	Ti	Orellana
Group *reticulatus*	*C. lyrinotatus**	Ti	Orellana
	*C. pauscienfuscatus**	Ti	Orellana
	*C. pifanoi**	Ti	Orellana
	*C. reticulatus**	Ti	Orellana
Group *stigmalis*	*C. deanei**	Ti	Orellana

*New records of species in Ecuador; Pa: Pa, Cofán-Dureno: CD, Ti: Ti, Urkisiki:Ur, Uyumbicho: Uy