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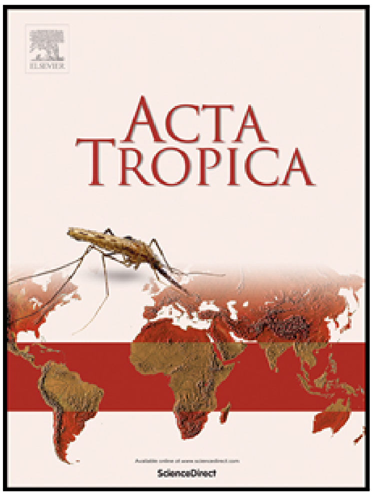

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Identification of mosquitoes (Diptera: Culicidae) from Mexico State, Mexico using morphology and *COI* DNA barcoding

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Abstract

Mosquitoes are commonly identified to species level using morphological traits, but complementary methods for identification are often necessary when specimens are collected as immature stages, stored inadequately or when delineation of species complexes is problematic. DNA-barcoding using the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene is one such tool used for the morphological identification of species. A comprehensive entomological survey of mosquito species in Mexico State identified by *COI* DNA barcoding and morphology is documented in this paper. Specimens were collected from all the physiographic provinces in Mexico State between 2017 and 2019. Overall, 2,218 specimens were collected from 157 localities representing both subfamilies Anophelinae and Culicinae. A species checklist that consists of 6 tribes, 10 genera, 20 subgenera, and 51 species, 35 of which are new records for Mexico State, is provided. Three hundred and forty-two *COI* sequences of 46 species were analysed. Mean intraspecific and interspecific distances ranged between 0% to 3.9% and from 1.2% to 25.3%, respectively. All species groups were supported by high bootstraps values in a Neighbour-Joining analysis and new *COI* sequences were generated for eight species: *Aedes chionotum* Zavortink, *Ae. vargasi* Schick, *Ae. gabriel* Schick, *Ae. guerrero* Berlin, *Ae. ramirezi* Vargas and Downs, *Haemagogus mesodentatus* Komp and Kumm, *Culex restrictor* Dyar and Knab, and *Uranotaenia geometrica* Theobald. This study provides a detailed inventory of the Culicidae from Mexico State and discusses the utility of DNA barcoding as a complementary tool for accurate mosquito species identification in Mexico.

Keywords: Mosquitoes, Culicidae, Species, DNA-Barcoding, Mexico State

1. Introduction

Mosquitoes are important vectors with enormous economic and public health importance. There are over 3,574 mosquitoes species worldwide (Harbach, 2020), and the hematophagous females of certain species are responsible for the transmission of numerous pathogens that cause millions of deaths annually (Burkett-Cadena, 2013; Harbach, 2020). Vector surveillance programs undertaken by health authorities at local or state levels are of paramount importance for the vector-borne disease controls administered by health authorities; hence, the success of these programs is dependent on the availability of rapid identification methods (Hernández-Triana et al. 2012; 2019). Unfortunately, the rapid and accurate identification of vector species is complicated because of the morphological homogeneity in certain life stages among species, the increasing lack of the necessary taxonomic expertise and the presence of species complexes (Cranston et al., 1987; Hernández-Triana et al., 2012, 2014; Murugan et al., 2016). In addition, the loss of diagnostic characters such as setae and scales during specimen collection, and poor storage condition can further make the morphological and DNA sequence identification complicated (Hebert et al., 2003).

As a result, complementary tools to species morphological identification such as enzyme electrophoresis (Chapman, 1982), cytotaxonomy (Subbarao et al., 2000), cross-breeding (Dix et al., 1992), and molecular techniques involving various gene markers such as *COI*, *COII*, *Cyt b*, *ITS1*, *ITS2* (Walton et al., 1999) have been postulated. Of these, the molecular method of the mitochondrial cytochrome *c*-oxidase subunit 1 (*COI*)-based DNA barcoding has been widely adopted to delineate many taxonomic groups across the animal kingdom (Hebert et al., 2003), including mosquitoes (for example, Cywinska et al., 2006; Kumar et al., 2007; Ashfaq et al., 2014; Chan et al., 2014; Beebe, 2018; Hernández-Triana et al., 2019; Hernández-Guevara et al., 2020). Mitochondrial markers are popular because of their abundance (1000's copies per cell), lack of introns, limited exposure to recombination and haploid mode of inheritance. *COI* in particular is popular because of the availability of the universal primers, enabling the recovery of the 5' end from most, if not all, animal phyla (Hebert et al., 2003). However, the COI-based barcoding approach has also been dismissed for its simplicity, the need for reference specimens to have been reliably identified by morphotaxonomists, the inability of *COI* to recognize hybrids due to its maternal inheritance, and the presence of introgression, heteroplasmy and pseudogenes. Despite these postulated limitations, the DNA barcoding has proven successful for species identification in many biodiversity-rich groups, even those in which the identification is obscured due to cryptic species, phenotypic plasticity, or unknown developmental life stages (see review of Hernández-Triana et al., 2012). In addition, several methods are now available that help in the establishment of species boundaries based on *COI* DNA barcode sequences such as the Automatic Barcode Gap Discovery and the Barcode Index Number (Chan-Chable et al., 2018b). In spite of the notable medical and veterinary importance, in combination with their utility as water quality indicators (e.g. Hernández-Triana et al., 2019), less than 40% of the known mosquitoes species have been barcoded worldwide (Chan-Chable et al., 2019).

In Mexico, there are an estimated 234-250 mosquito species (Bond et al., 2014; Ortega-Morales et al., 2015; Rodríguez-Martínez et al., 2020), and mosquito-borne diseases such as malaria, dengue virus (DENV), zika virus (ZIKV), and chikungunya virus (CHIKV), which are being transmitted by species within the genera *Anopheles*, *Aedes* and *Culex*, are endemic (González-Ceron et al., 1999; Fernández-Salas et al., 2015; Elizondo-Quiroga et al., 2018; Danis-Lozano et al., 2019). Despite this knowledge, *COI* DNA barcoding has not been widely employed to characterize the biodiversity of mosquito species in Mexico. Studies have mostly been limited to the southeastern region of the country, where DNA Barcoding was used to confirm the presence of *Psorophora albipes* (Theobald) and *Anopheles veruslanei* Vargas in Quintana Roo State (Chan-chable et al., 2016, 2018a). These data support the presence of cryptic diversity in *Aedes taeniorhynchus* (Wiedemann) (Chan-Chable et al., 2018b, 2019), as well as supporting the morphological identification and the presence of cryptic diversity within seven species in Quintana Roo (*Aedes angustivittatus*. *Aedes serratus, Anopheles crucians* s.l., *Culex taeniopus, Haemagogus equinus, Culex erraticus*, *Psorophora ferox*, and *Anopheles apicimacula*) (Chan-Chable et al., 2018b, 2019).

Furthermore, most of the mosquito taxonomic studies in Mexico in the last 15 years that have analysed local fauna have frequently used morphological methods for data collection only (Muñoz-Cabrera et al., 2006; Ortega-Morales et al., 2010; Ordóñez-Sánchez et al., 2013; Ortega-Morales et al., 2013, 2015, 2018a; Dávalos-Becerril et al., 2019; Ortega-Morales et al., 2019a,b; Hernández-Amparan et al., 2020; Rodríguez-Martínez et al., 2020). However, reports of mosquito species from pristine habitats and high elevation forests in the State of Mexico have not been well represented (Ortega-Morales et al., 2018a). Previous to this study, only 16 mosquito species belonging to four genera have been recorded in Mexico State (Vargas and Martínez-Palacios, 1956; Díaz-Nájera and Vargas, 1973). Our study surveyed the mosquito fauna in Mexico State and used the DNA barcoding approach to support morphological identifications. In addition, the DNA barcode variability was assessed using genetic distance methods to detect cryptic diversity in some species. New distributional limits and their associated ecological and biological impacts are also discussed.

2. Materials and methods

2.1. Study area

Mexico State is located in the southern part of Mexico. It shares boundaries with seven other States: Queretaro and Hidalgo to the north; Mexico City, Morelos and Guerrero to the south; Michoacán to the west; Tlaxcala and Puebla to the east. It lies between latitude 19^o21'N and longitude 99^o38'W (Fig. 1) and has an estimated area of 22,500 Km^2. The state is largely mountainous with temprate forests with Nearctic affinities to the north and tropical and sub-tropical forests with Neotropical affinities to the south. It is divided into two physiographic provinces that include the Neo-Volcanic Axis and Sierra Madre Oriental. The description of the two physiographic provinces are provided in Table 1.

2.2. Sample collection and identification

Mosquito specimens were collected in the two physiographic provinces in Mexico State between September-October 2017, and February 2019, with similar sampling efforts in both regions. Specimen collection was conducted according to the protocols proposed by Berlin (1969). Briefly, immature stages were collected from any available water body and other potential habitats of mosquito larva like tree and rocks holes, flower vases in cemeteries, discarded tires, plastic containers, water storage in abandoned buildings, and open natural water bodies using the standard larval dipping procedure. Larvae and pupae were preserved alive in plastic cylindrical containers with the same water from larval habitats and transported to Parasitology Laboratory of the Universidad Autónoma Agraria Antonio Narro, Laguna unit, (UAAAN-UL) and reared to adult stages, storing the associated immature exuviae. Adult mosquitoes were collected using mouth and mechanical aspirators and killed in lethal chambers with triethylamine vapours. Larvae and exuviae were mounted on microscope slides using Euparal Mounting Medium (BioQuip® No. 6372). The male genitalia were dissected to assist with identification when required. All adult specimens were mounted on insect pins and stored in entomological boxes. Morphological identification was carried out using the taxonomic keys of Sirivanakarn (1982), Clark-Gil and Darsie (1983), Darsie and Ward (2005) and Burkett-Cadena (2013).The Arthropods and Mites of Medical Importance (CAIM) records, deposited at the Institute of Epidemiological Diagnosis and Reference (INDRE) in Mexico City were also reviewed for additional species records of Mexico State. The simplified aedine generic designationsdetailed in Wilkerson et al. (2015) was followed in this study.

2.3. DNA extraction, PCR amplification, and sequencing

Only adult specimens were used for DNA extraction. Immature stages were not for molecular analysis as their exuviae were mounted in Euparal for morphological identification. Two legs were taken from individual specimens of each mosquito species collected for DNA extraction, with intentional selection to include all localities that were sampled, and the remaining part of the specimen kept as a voucher. DNA was extracted using a slightly modified hotshot method of DNA extraction (Truett et al., 2000). The *COI* barcoding region was amplified using the primers LCOI490 and HCO2198 (Folmer et al., 1994). PCR reactions were performed in a total volume of 50 μL using 2 μL of genomic DNA, 1 X NH_4

buffer, 2 pmol/µL dNTPs, 1.5 mM $MgCl_2$, 10 pmol/µL of each primer, 0.6U *Taq* DNA polymerase (Bioline) and 20 mg/mL bovine serum albumin. The thermal profile consisted of 1-min initial cycle at 94°C followed by a pre-amplification 5 cycles of 94°C for 1 min, 45°C for 1.5 min, 72°C for 1.5 min, and an amplification step of 35 cycles of 94°C for 1 min, 57°C for 1.5min, 72°C for 1.5 min with a final extension of 72°C for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gel and samples showing the expected band size were purified using the QIAquick PCR purification kit and sequenced in both directions using the ABI PRISM® BigDye® Terminator sequencing kit (Applied Biosystems) following the manufacturer´s instructions.

2.4. Sequence analysis

DNA sequences generated in both directions were edited manually using the BioEdit sequence alignment Editor version 7.0.5.3 (Hall, 1999) and a consensus sequence was generated using ClustalW (Larkin et al., 2007). The full data set was analyzed in MEGA v.7 (Kumar et al. 2016), and genetic relationships between species was analyzed using three methods: Neighbor Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP). The NJ tree analysis was carried out using the K2P distance metric to represent their clustering pattern sites (Saitou and Nei, 1987); bootstrap values were calculated to test the robustness of the tree and were obtained by conducting 1000 pseudoreplicates; only groups with more than 80% bootstrap support are shown on the NJ tree (Hernández-Triana et al., 2012; 2014). Maximum likelihood analysis was performed on the RAxML web server version (https://raxml-ng.vital-it.ch/#/) (Kozlov et al., 2019). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm, the initial trees were obtained by the random addition of sequences (10 replicates). For the NJ, tML and MP phylogenetic analyses, a *COI* DNA barcode sequence of a black fly, *Simulium weji* Takaoka (Accession no. KF289451) was used as an outgroup.

Detailed specimen field records and sequences (including trace files) were uploaded to the BOLD database (http://www.boldsystems.org) within the Working Group 1.4 Initiative on Human Pathogens and Zoonoses, and the project titled "Mosquitoes of Mexico State "MOSMEX18". The Digital Object Identifier (DOI) for the publicly available project in BOLD is dx.doi.org/10.5883/10.5883/DS-MOSMEX18. All sequences have been submitted to GenBank (accession numbers: MT108552 - MT108679). Reference sequences were downloaded from the BOLD database to represent species collected in the state from which we were unable to obtain sequences (Table 2). All sequences analyzed in this study are archived in MEGA and they are available upon request from the corresponding author.

3. Results

3.1. Faunistic survey

Overall, 2,218 specimens including 789 adult mosquitoes (510 females and 279 males), and 1,429 immature stages (1,304 larvae and 125 pupae) were retrieved from 157 field collections. The identified specimens belong to the two subfamilies of Culicidae, Anophelinae and Culicinae, present in Mexico. In this study a total of six tribes, 10 genera, 20 subgenera, and 51 species are now reported in Mexico State. As a whole, 40 species were collected during our field trips, including seven new genera for Mexico State (*Haemagogus, Limatus, Lutzia, Psorophora, Wyeomyia, Toxorhynchites* and *Uranotaenia*), 12 new sub-genera (*Aztecaedes, Howardina, Protomacleaya, Haemagogus, Janthinosoma, Anoedioporpa, Microculex, Phenacomyia, Lutzia, Wyeomyia, Lynchiella* and *Uranotaenia*) and 35 new records were found (Table 2). Eleven species that are also reported here were only obtained from previous records: seven from CAIM collection records: *Ae. scapularis* (Rondani), *Ae.*

zoosophus Dyar and Knab, *Cx. bidens* Dyar and Knab, *Cx. lactator* Dyar and Knab, *Limatus durhamii* Theobald, *Uranotaenia lowii* Theobald, and *Ur. sapphirina* (Osten Sacken), and four species from the literature: *Anopheles aztecus* Hoffman, *Ae. muelleri* Dyar, *Cx. erythrothorax* Dyar, and *Cx. apicalis* (Adams) (see Table 2).

3.1.1. Biological notes. New State records were mostly found associated with other species in several of the habitats sampled as shown in Table 3, and new distributional limits are provided for these species.

3.1.1.1. Anopheles (Anopheles) franciscanus McCracken

This species occurs in USA and Mexico, and it is considered a potential vector of *Plasmodium vivax* Grassi and Feletti, causing malaria (Gaffigan et al., 2020). The larvae are usually found in shallow pools of receding streams that have green algae. In Mexico State, this species was collected in only one site, a swamp in Zumpahuacán in association with *An. pseudopunctipennis* Theobald and *Cx. pseudostigmatosoma* Strickman.

3.1.1.2. Aedes (Aztecaedes) ramirezi

The subgenus *Aztecaedes* and its monobasic species *Ae. ramirezi* is endemic to Mexico. The species has only been previously reported in Jalisco, Morelos, Sinaloa, Sonora, and Veracruz (Villegas-Trejo et al., 2010). Immature stages of *Ae. ramirezi* were collected from three volcanic rock holes at ground level with clean water and few leaves at the base. It was found in the same location from one flower vase in a cemetery associated with *Ae. epactius* Dyar and Knab.

3.1.1.3. The subgenus Howardina of Aedes

The Neotropical subgenus *Howardina* of *Aedes* is found and reported for first time in Mexico State. This subgenus includes six species in Mexico, but in Mexico State we reported four species: *Ae. allotecnon* Kumm, Komp, and Ruiz, *Ae. guerrero* Berlin, *Ae. lorraineae* Berlin, and *Ae. quadrivittatus* (Coquillett). Most species in this subgenus develop their immature stages in bromeliad axils, which are filled with rainwater. *Aedes allotecnon* has been previously reported from Costa Rica, El Salvador, Guatemala, Nicaragua, Panama, and Mexico (Gaffigan et al., 2020). In Mexico State, immature stages of this species were found in axils of bromeliads two meters above the ground level. *Aedes guerrrero* has been reported in Mexico and Guatemala; a single female of this species was collected in Mexico State approaching humans with biting intention during the day in a sub-tropical forest. This evidence suggests that this is one of the most uncommon species within the *Howardina* subgenus in Mexico.

Another poorly known mosquito species endemic in Mexico is *Ae. lorraineae*. This species has been collected in the states of Guerrero, Oaxaca and Chiapas (Berlin, 1969; Díaz-Nájera and Vargas, 1973; Heinemann and Belkin, 1977). Immature stages of this species were collected from an unused water tank with clean water and some leaves at the base of the tank, while adult females were also collected in a diurnal landing on humans in an oak forest. This is the northernmost distributional rank of *Ae. lorraineae*. *Aedes quadrivittatus* that occurs in Colombia, Costa Rica, Guatemala, Honduras, Mexico, and Panama (Gaffigan et al., 2020); and is the most common species within the subgenus *Howardina* in Mexico. Immature stages of *Ae. quadrivittatus* were collected from axils of bromeliads 1.5 to four meters above the ground level, and adult females were collected biting humans at day in an oak forest.

3.1.1.4. The subgenus Ochlerotatus of Aedes

Although the subgenus *Ochlerotatus* is the most common subgenus of *Aedes* in Mexico, only three species have been found in Mexico State, and two are new records for the State. *Aedes scapularis* (Rondani) was obtained from CAIM records as this species was not collected during any collection trips. Immature stages of this species were collected in 1995 from a large pond in Tejupilco and Tatlaya municipalities (DG-5576-95, DG7921-95). *Aedes shannoni* Vargas and Downs is another endemic species of Mexico, which has been recorded in the states of Michoacán, Morelos, and Querétaro (Díaz-Nájera and Vargas, 1973). Adult female of *Ae. shannoni* were collected biting humans during the day in a sub-tropical forest.

3.1.1.5. The subgenus Protomacleaya of Aedes

The subgenus *Protomacleaya* of *Aedes* is the most representative in Mexico State. It includes six species all of which are new records for Mexico State: *Ae. chionotum* Zavortink, *Ae. gabriel* Schick, *Ae. idanus* Schick, *Ae. kompi* Vargas and Downs, *Ae. vargasi* Schick, and *Ae. zoosophus* Dyar and Knab. In Mexico, the subgenus *Protomacleaya* is sub-divided into four groups, three of which are present in Mexico State: the Kompi group including *Ae. chionotum* and *Ae. kompi*; the Terrens group which includes *Ae. gabriel*, *Ae. idanus*, and *Ae. vargasi*; and the Triseriatus group which includes *Ae. zoosophus*. *Aedes chionotum* is an endemic species of Mexico, from where it has been collected in Morelos and Oaxaca states (Díaz-Nájera and Vargas, 1973; Zavortink, 1972), this collection is the northernmost distributional rank for this species. Immature stages of *Ae. chionotum* were collected from tree-holes with clear and colored water at 1-2 m above the ground level, while adult females were collected approaching humans diurnally in three separate locations of oak-pine forests.

Aedes gabriel is another endemic species of Mexico. It has been reported in the states of Morelos, Jalisco, Zacatecas, and Hidalgo (Schick, 1970; Heinemann and Belkin, 1977; Ortega-Morales et al., 2019c). Immature stages of *Ae. gabriel* were collected from tree-holes at ground level, and 1 m above ground level in sub-tropical forests. In addition, it was also found in discarded tires with colored water, and from an artificial container with colored water at ground level. Adult females were collected biting humans at day and at dusk in four separate locations of sub-tropical forests. *Aedes idanus* is one of the rarest endemic Mexican mosquito species. It has been reported in the states of Guerrero, Morelos, Nayarit, and Jalisco (Schick, 1970; Heinemann and Belkin, 1977). Immature stages of *Ae. idanus* were collected from a tree hole in a single location in a sub-tropical forest with colored water at 1.5 meters above the ground level. This is the most uncommon species belonging to the *Protomacleaya* subgenus in Mexico State.

Aedes kompi Vargas & Downs is another endemic species of Mexico. This species has been previously reported only in Morelos State (Díaz-Nájera and Vargas, 1973; Heinemann and Belkin, 1977), and this is the northern most distributional limit of the species. Immature stages of *Ae. kompi* were collected from a tree hole with colored water at 1 meter above the ground level in a single location in an oak-forest. *Aedes vargasi* is another endemic species of Mexico. This species has been reported in the states of San Luis Potosí and Veracruz (Schick, 1970; Heinemann and Belkin, 1977). This is the southernmost distributional rank of *Ae. vargasi*. *Aedes gabriel* and *Ae. vargasi* are the most common species in the subgenus *Protomacleaya* in Mexico State. Immature stages of *Ae. vargasi* were collected from three tree-holes with clean water at 0.5-1 m above the ground level; from discarded tires with clean water in a sub-tropical forest; and from an artificial container with colored water, all within the sub-tropical forest region of the state. *Aedes zoosophus* is the only member of the Triseriatus group of *Protomalceya* found in Mexico State. This species record was obtained from CAIM. Immature stages of this species were collected from discarded tires in Tatlaya municipality on 2nd.August .1995 (DG-5268-95).

3.1.1.6. Aedes (Stegomyia) albopictus Skuse

This invasive species continues its global expansion into new locations. In Mexico, *Ae. albopictus* has now been reported in a total of fifteen states (out of 32 states) in addition to this report. Originally found in Tamaulipas (Francy et al., 1990), subsequent reports were from Coahuila (Rodríguez and Ortega, 1994), Nuevo León (Orta-Pesina et al., 2005), Veracruz (Flisser et al., 2002), Chiapas (Casas-Martínez and Torres-Estrada, 2003), Morelos (Villegas-Trejo et al., 2010), Quintana Roo (Salomón-Grajales et al., 2012), Sinaloa (Torres-Avendaño et al., 2015), San Luis Potosí (Ortega-Morales and Rodríguez, 2016), Hidalgo (Ortega-Morales et al., 2016), Tabasco and Yucatán (Ortega-Morales et al., 2018b), Mexico City (Dávalos-Becerril et al., 2019), and Guerrero (González-Acosta et al., 2019). Immature stages of *Ae. albopictus* were collected from a discarded tire and an artificial container at ground level with clean water, while adult females were collected biting diurnally, in all locations within a sub-tropical forest.

3.1.1.7. The genus Haemagogus

In Mexico, there are four species belonging to *Haemagogus* genus, two are found in Mexico State: *Hg. equinus* Theobald and *Hg. mesodentatus* Komp and Kumm. Although the distribution patterns of the species of *Haemagogus* are unknown in Mexico, *Hg. equinus* is possibly the most common species within the genus in Mexico. Immature stages of *Hg. equinus* were collected from ovitraps placed in sub-urban areas of Tlatlaya municipality (Vicente Sánchez-Reyes, pers. comm.), while adult females were collected biting during the day in one location of sub-tropical forest region. Adult females of *Hg. mesodentatus* were collected biting at dusk in a sub-tropical forest of Sultepec municipality.

3.1.1.8. Psorophora (Janthinosoma) ferox (von Humboldt)

Seventeen species of the genus *Psorophora* occur in México. This is the first record of this genus and the subgenus *Janthinosoma* in Mexico State. Adult females of *Ps. ferox* were collected biting at day in a single location in a sub-urban area of Malinalco municipality. Although only this species of *Psorophora* was found in Mexico State, surely there are more species of this genus including subgenus *Grabhamia* and *Psorophora* inhabiting Mexico State, which are also common in surrounding states.

3.1.1.9. Culex (Anoedioporpa) restrictor

There are two species of the subgenus *Anoedioporpa* of *Culex* in Mexico: *Cx. conservator* Dyar and Knab and *Cx. restrictor*, which is the most common species within the subgenus *Anoedioporpa* in Mexico. Immature stages of *Cx. restrictor* were collected from tree-holes with turbid water from ground level to 2 m above in eight locations; and four discarded tires with water, all located in sub-tropical, oak forests, and sub-urban areas; while adult females and males were collected resting into a tree-hole in one oak forest.

3.1.1.10. The subgenus Culex of Culex

Six species of the subgenus *Culex* have been previously reported in Mexico State. In this study, five additional new records within this subgenus are reported: *Cx. bidens* Dyar and Knab, *Cx. coronator* Dyar and Knab, *Cx. nigripalpus* Theobald, *Cx. pseudostigmatosoma* Strickamn, and *Cx. salinarius* Coquillett. The species-record of *Cx. bidens* was obtained from CAIM. Immature stages of this species were collected in 1997 from clean and colored water in a discarded tire in Tlatlaya municipality, and from a pond and unused water tank in 2017, both sites are located Teoloyucan municipality. *Culex coronator* is a common species in Mexico, immature stages of this species were collected from three discarded tires with turbid water in a sub-tropical forest, and sub-urban areas; from two artificial containers at ground level with clean water in a sub-tropical forest; from flower vases with colored water in a cemetery in Sultepec municipality. *Culex nigripalpus* could be the most uncommon species within the subgenus *Culex* in Mexico State. Although this species is very common in tropical regions of southeastern Mexico, the collection of this species in our survey was represented by a single record. These were two larvae collected from a pond with brackish water and abundant aquatic and emerging vegetation and green algae in Jilotepec municipality. Since *Cx. pseudostigmatosoma* was discovered and described by Strickman (1989), very few occurrence records of this species have been published in Mexico, although it has been found in tropical states of Mexico such as Chiapas and Veracruz (Strickman, 1989). Apparently, *Cx. pseudostigmatosoma* is mostly common in the plains of the Mexico Valley. Immature stages of *Cx. pseudostigmatosoma* were collected from a swamp with clear water and abundant emerging vegetation and brown algae in a valley of Zumpahuacán municipality; and from a pond with clear water and abundant emerging vegetation in a valley of Temazcaltepec municipality. *Culex salinarius* is another uncommon species in Mexico State, immature stages of this species were collected from a single site in a discarded water tank with clear water in Lerma municipality.

3.1.1.11. Culex (Microculex) rejector

In Mexico, two species within the subgenus *Microculex* that have been reported are *Cx. daumastocampa* Dyar & Knab and *Cx. rejector*, the latter the most common and the one found in Mexico State. Immature stages of *Cx. rejector* were collected from bromeliad axils with colored water at 1.5-four meters above the ground level in five locations of sub-tropical and oak forests; adult males were collected resting in bromeliads bracts at 1.5 meters above the ground level in a sub-tropical forest.

3.1.1.12. The subgenus Phenacomyia of Culex

Culex corniger Theobald and *Cx. lactator* Dyar and Knab are the two species known within the subgenus *Phenacomyia*, both occurring in Mexico. A female adult of *Cx. corniger* Theobald was collected approaching humans at night in a sub-urban area of Malinalco municipality. No immature stages of this species were collected. The species record of *Cx. lactator* Dyar and Knab was obtained from CAIM records. Immature stages of this species were collected in 1997 from unused water tank in Tejupilco municipality.

3.1.1.13. Lutzia (Lutzia) bigoti (Bellardi)

In Mexico, the genus *Lutzia* is represented by two species, the one found in Mexico State is *Lt. bigoti*, which is the most common species within the genus in Mexico. Immature stages of this species were collected from two artificial containers with colored water at ground level in sub-tropical region; and from discarded tires with clear and colored water in sub-tropical regions, oak forests, urban and sub-urban areas. Fourth instar larvae of *Lt. bigoti* were collected in association with *Ae. epactius*, *Ae. gabriel*, *Ae. vargasi*, *Cx. restrictor*, *Cx. coronator*, *Cx. quinquefasciatus* Say, *Cx. thriambus*, *Cx. arizonensis*, *Cs. particeps* (Adams), and *Tx. moctezuma* (Dyar and Knab); and with the exception of the later three species, all species were predated by *Lt. bigoti*.

3.1.1.14. Culiseta (Culiseta) incidens (Thomson)

Four species within the genus *Culiseta* are known in Mexico, three occurring in Mexico State but with only *Cs. incidens* as a new record for the state. Immature stages of this species were collected from a swamp with clean water and abundant aquatic emerging and floating vegetation and brown algae, and from one water channel in the same location in a sub-urban area of San Mateo Texcalyac municipality

3.1.1.15. Limatus durhamii Theobald

Two species belonging to the Sabethini tribe were collected in Mexico State, the first which is *Li. durhamii* was obtained from CAIM records. Immature stages of *Li. durhami*i were collected in 1995 from an unused water tank in Tejupilco municipality.

3.1.1.16. Wyeomyia (Wyeomyia) mitchellii (Theobald)

This is the second species belonging of the Tribe Sabethini collected in Mexico State. Adult females of *Wy. mitchellii* were collected in a diurnal biting at a single site in a sub-urban area of Malinalco municipality. No immature stages of this species were collected.

3.1.1.17. Toxorhynchites (Lynchiella) Moctezuma (Dyar and Knab)

The genus *Toxorhynchites* is recorded for first time in Mexico State, and is represented by the species *Tx. moctezuma*, which is the most common species within the genus in Mexico. Immature stages of *Tx. moctezuma* were collected from three discarded tires with turbid water in a sub-urban and sub-tropical forest area; from a tree-hole with clean water at 1 meter above the ground level in a sub-tropical forest; and from a flower vase with clean water at ground level in a sub-urban area. Larvae of *Tx. moctezuma* were found in association with *Ae. gabriel*, *Ae.*

vargasi, *Ae. aegypti aegypti* (Linnaeus), *Cx. restrictor*, and *Lt. bigoti*, and with the exception of later species, all species were predated by *Tx. moctezuma*.

3.1.1.18. The genus Uranotaenia

In Mexico, ten species of the genus *Uranotaenia* have been reported and this genus is reported for first time in Mexico State. It is represented by three species: *Ur. geometrica*, *Ur. lowii* Theobald, and *Ur. sapphirina* (Osten Sacken). *Uranotaenia geometrica* rarely bites warm-blooded animals, including humans. Immature stages of this species were collected from a plastic water tank with clean water containing few leaves at the base in a single site of sub-urban area of Temzcaltepec municipality. The collection records of *Ur. lowii* Theobald and *Ur. sapphirina* (Osten Sacken) were obtained from CAIM. Immature stages of *Ur. lowii* were collected in 1997 from one discarded tire with clean water in Otzoloapan municipality, while immature stages of *Ur. sapphirina* were collected in 1994 from unused water tank in Tejupilco municipality.

3.2. COI DNA barcoding

A dataset with 342 *COI* DNA barcode sequences representing 46 morpho-species of mosquitoes found in the State of Mexico was analysed. Of these, 139 *COI* sequences from 31 species were obtained from samples collected in this study. Genetic diversity was analysed using the NJ, ML and MP methods. The phylogenetic trees obtained showed similar topology and support values, thus, only the NJ tree (Fig. 4) is shown (see Additional Files Figs S1, S2 for ML and MP trees). Overall, more than three DNA barcode sequences were obtained for 36 morpho-species. It was not possible to obtain *COI* barcode sequences for *Aedes alloctecnon*, *Ae. idanus*, *Ae. lorraineae* Berlin, *Ae. shannoni*, and *An. aztecus* as they were either only identified by morphology using few immature specimens, PCR amplification failures or specimen records were retrieved from CAIM had no available sequences in any public database (Table 2).

Intraspecific distance ranged between 0% to 3.9% with the highest divergence found in *An. pseudopunctipennis*, while the interspecific distance ranged between 1.2% to 25.3% with the lowest divergence found between *Cx. pseudostigmatosoma* and *Cx. nigripalpus*. The pairwise genetic distance ranged from 0% to 27.7% with taxa belonging to different genera or sub-genera showing higher values of genetic distance with the most divergent pair being *An. pseudopunctipennis/Tx. moctezuma* (25.3%) and *Cx. erythrothorax/Ae. vargasi* (24.9%). Conversely, low genetic distance was observed among species of the same genus or sub-genus as seen in *Cx. pseudostigmatosoma/Cx. nigripalpus* (1.2%) and *Cx. nigripalpus/Cx. lactator* Dyar and Knab (1.4%) (Additional File TableS1).

Individuals belonging to the same species clustered together in the NJ analysis, including specimens collected at different locations within the state as well as sequences from other countries. However, a deep division was observed within *An. pseudopunctipennis* (average 3.95%, maximum of 13.8% between 23 specimens) and *Cs. particeps* (average 2.51%, maximum of 4.76% between four specimens) in the NJ tree (Fig 5). Thirty-four BINs were assigned to the barcode sequences generated in this study (Table 2). Each taxonomic name was consistent with a single BIN except *Ae. trivittatus* (Coquillett) (BOLD:AAC9486 and BOLD:ADP6375) and *Cs. particeps* (BOLD:ADM1783 and BOLD:ADF3447), which were divided into two BINs each.

4. Discussion

This study assessed the use of an integrated approach based on morphology and *COI* DNA barcoding to delineate the mosquito fauna from Mexico State. It represents a comprehensive mosquito survey of the state and expands biological notes documented for the mosquito fauna in Mexico. These data enhance the existing collation of *COI* sequences for the further development of a reference library of mosquitoes in Mexico.

The 51 mosquito species reported in this study represents about 21% of the total mosquito fauna in Mexico and detailed an extensive species diversity coverage in the state. Although a similar number of species (n=46 spp.) was recorded in the State of Morelos (Villegas-Trejo et al., 2010), higher species diversity has been recorded in Hidalgo (n=57 spp.) (Ortega-Morales et al., 2018a), Tamaulipas (n=82 spp.) (Ortega-Morales et al., 2015), Quintana Roo (n=82 spp.) (Ortega-Morales et al., 2010; Salomón-Grajales et al., 2012; Ordóñez-Sánchez et al., 2012; Chan-Chablé et al., 2016), Veracruz (n=140 spp.) (Ibañez-Bernal et al., 2011), Tabasco (n=104 spp.) (Ortega-Morales et al., 2019c); and Nuevo León (n=65 spp.) (Ortega-Morales et al., 2019b). Mosquito diversity tends to be higher in tropical habitats (Harbach, 2007), and unlike the southern tropical and lesser altitude states with higher species diversity, most regions of Mexico State are composed of a high plateau where mosquito diversity seems to be low such as Tlaxcala State (n=26 spp.) (Muñoz-Cabrera et al., 2006).

Thirty-five species recorded in Mexico State are new state records, including species of relevant medical importance such as *Ae. albopictus*, with this being the fifteenth state in Mexico from which the species is being recorded. Immature stages were collected in Malinalco municipality which is less than 3km from Morelos State, where it has been previously reported (Villegas-Trejo et al., 2010). The presence of *Ae. albopictus, Ae. scapularis, Hg. equinus, Hg. mesodentatus, Cx. nigripalpus*, and *Ps. ferox* as new vectors in Mexico State increases the risk of pathogen transmission. These species are known vectors of numerous arboviruses such as WNV, CHKV and DENV, which have a recurrent transmission pattern with annual cases across Mexico and the Neotropical Region (Dirección General de Epidemiología, 2019). This underpins the importance of ongoing vector surveillance programs by state health authorities in the Mexico.

Regardless of some of the arguments against the use of *COI* barcoding gene in phylogenetic studies, the *COI* gene carries a phylogenetic signal (Hebert et al., 2003; Hernández-Triana et al., 2012; Beebe, 2018). In this study, NJ profile as well as the ML and MP analyses (Additional Files Figs S1, S2) exhibited some degree of concordance with the phylogenetic concepts proposed by de Oliveira Aragão *et al.* (2018) and Harbach (2020) for certain clades within Anophelinae, Aedini, Culicini and Sabethini. However, a combination of other mitochondrial markers, such as ND4, *COII* with nuclear region markers (e.g. ITS2), might enable the reconstruction of the deeper phylogenetic relationships in future studies.

The limitations of the use of *COI* DNA barcode sequences with regards to species identification has been well documented. Hernández-Triana et al. (2012) provided a review on the subject highlighting the controversies surrounding the approach. More recently, Beebe (2018) provided an appraisal of the literature and documented the advantages and disadvantages on the utility of this approach. In spite of the controversies, the application of *COI* DNA barcoding as a molecular tool to complement morphological identification of mosquitoes in Mexico State provided an identification congruence that is comparable to other studies (e.g. Chan et al., 2014; Cywinska et al., 2006; Hernández-Triana et al., 2020). With the exception of *Cx. pseudostigmatosoma* and *Cx. nigripalpus*, all morphologically identified species were separated by their *COI* DNA barcodes. Intraspecific genetic divergence of most species was within the ≤2% proposed limit for insects (Hebert et al., 2003), except *An. pseudopunctipennis* which separated in two groups with a divergence of 3.95% and with high bootstrap support values. *Anopheles pseudopunctipennis* is a malaria vector that can survive and transmit *Plasmodium vivax* at altitudes higher than other malaria vectors (*ca*. 3,000 mals) (Malaria Atlas Project, 2019). The observed genetic divergence is suggestive of the presence of a cryptic diversity and this is consistent with earlier reports that this species is a species complex (Coetzee et al., 1999; Estrada-Franco et al., 1993). However, there is low genetic variation within the population collected within Mexico State (Additional material TableS1) suggesting a unique gene flow, similar to what was reported in an Argentine population (Dantur Juri et al., 2014). A slightly higher intraspecific divergence was identified in *Cs. particeps* (2.51%), with two BINs (BOLD:ADM1783, ADF3447). This is reflected by slight division in the NJ analyses. Similar observations were reported in another *Culiseta* species, such as *Cs. litorea* (Shute) from Spain and United Kingdom, which was found to have a 5.35% intraspecific genetic divergence (Hernández-Triana et al., 2019). Similarly, two BINs (BOLD:AAC9486, BOLD:ADP6375) were assigned to the sequences of *Ae. trivittaus* generated in the current study, however no divergence was observed in the NJ tree.

Although there was a slight separation between *Cx. pseudostigmatosoma* and *Cx. nigripalpus*, both species were closely grouped on the NJ tree. In addition, interspecific distances between the two species is less than the proposed 2% (Hebert et al., 2003), which is probably due to both taxa belonging to the same subgenus *Culex.* Chan-Chable et al. (2019) reported similar results between *Cx. interrogator* and *Cx. nigripalpus*, where the *COI* marker did not separate these two species. This occurrence is especially common among *Culex* mosquitoes as reported elsewhere (Laurito et al., 2013; Wang et al., 2012) and it is indicative that the *COI* genetic marker sometimes does not carry sufficient information to completely distinguish many *Culex* species in the Neotropical Region. This occurrence could also be a result of incomplete lineage sorting or introgression events (Beebe, 2018; Chan-Chable et al., 2019). It is important, therefore, to continue developing the BOLD database with sequences of extant species which could resolve these incongruencies. In the meantime, the combination of *COI* DNA barcode with nuclear markers such as ITS2 or the use of microsatellites has been suggested (Hernández-Triana et al., 2019). In general, all morpho-species identified in this study formed groups with high bootstrap values. Congeneric species mostly grouped together; however, *Ae. kompi* grouped closely with *Cx. restrictor.* The reason is quite unclear, but *Ae. kompi* belongs to the sub-genus *Protomacleaya* which is a polyphyletic assemblage of species, comprising of individuals whose relationships with other generic-level taxa cannot be determined (Reinert et al., 2008, 2006; Zavortink, 1972).

5. Conclusions

Overall, *Aedes* and *Culex* genera were the most abundant and widely dispersed from the Mexico State, with the three most common species collected being *Aedes epactius* (30%), *Culiseta particeps* (21.5%), and *Cx. thriambus* (20.7%). In general, 17 species (33.3%) are of potential medical importance. *Ae. aegypti* and *Ae. albopictus* are undoubtedly of the greatest medical importance because of their role in transmission of pathogens such as ZIKV, DENV, CHIKV, and Yellow fever (YF). *An. aztecus* and *An. pseudopunctipennis* Theobald are also important vectors of *Plasmodium* causing malaria.

This study provides evidence supporting the use of *COI* DNA barcoding in combination with ecological and morphological traits as a suitable approach for cataloguing the mosquito species fauna in Mexico State. In general, our study provides information for 35 new records of species for the state and provide evidence for the presence on cryptic diversity in *An. pseudopunctipennis* as well as highlighting taxonomic issues within barcode sequences in *Cs. particeps*, *Cx. pseudostigmatosoma* and *Cx. nigripalpus*. The information provided in this study will further support the development of a DNA barcode reference library for the mosquito fauna in Mexico.

Author's contributions

The study was designed by AAA, LMHT, AIOM, JAGH, HHJ, MAPR. Specimens were collected by AAA, AIOM, JAGH, JCR, and sequences and metadata generated by AAA, LMHT, NIN, RJCC, RVM. The data were analysed and figures produced by AAA, LMHT, RJCC. The manuscript was first drafted by AAA and LMHT, and then improved by AIOM, JAGH, JCR, RJCC, RFV, HHJ, NIN, ARF and MAPR. Funding was obtained by ARF, MAPR, and AIOM. All authors agree to the findings in this publication and hereby declare no conflict of interest. Funders

of this study had no role in the design, collection, analysis or interpretation of the results, nor in the writing of this report or the decision to publish it.

Credit author Statement

Adebiyi A. Adeniran, Luis M. Hernández-Triana, Aldo I. Ortega-Morales, Javier A. Garza-Hernández, Mario A. Pérez-Rodríguez: Contribution to the study conception and design. **Adebiyi A. Adeniran, Javier A. Garza-Hernández, Aldo I. Ortega-Morales, Josué de la Cruz-Ramos, Herón Huerta-Jiménez:** Material preparation, specimen's collection and morphological identification of specimens, interpretation for the work. **Adebiyi A. Adeniran**, **Luis M. Hernández-Triana, Ramos Rahuel J. Chan-Chable, Rafael Vázquez-Marroquín:** Molecular identification and analysis of sequences. **Luis M. Hernández-Triana, Anthony R. Fooks, Mario A. Pérez-Rodríguez**: Funding acquisition. **Adebiyi A. Adeniran, Luis M. Hernández-Triana, Aldo I. Ortega-Morales Javier, A. Garza-Hernández, Josué de la Cruz-Ramos, Rahuel J. Chan-Chable, Rafael Vázquez-Marroquín, Herón Huerta-Jiménez, Nadya I. Nikolova, Anthony R. Fooks, Mario A. Pérez-Rodríguez:** Drafting the manuscript or revising it critically for important intellectual content (

Conflict of Interest Statement

None

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Table 1. Description of the physiographic characteristics of collection sites, in Mexico State, Mexico.

Region	Collection sites	Description
Neo-Volcanic Axis	Municipalities of Apulco, Jilotepec, Chapa de Mota,	Covers over 60% of the land of the state and extends from the Gulf of Mexico in

	Tonanitla, Villa de Carbon, Villa de Nicolas Romero, Tlazala de Favela, Otzolotepec, Xonaratlan, Toluca, Metepec, Temascaltepec, Coatepec, Villa Guerrero, Tenango de Valle, San Mateo Texalyacac, Santiago Tianguistenco de Galeana, Ocuilan, Tenancingo, Tejupilco, Valle de Bravo, Donato Guerra, San Juan del Rincón, and El Oro de Hidalgo	the East to the Pacific Ocean. It includes a chain of volcanoes with elevations of 2,000 masl, most of which are covered by perpetual snows and includes pine, fir and oak forests, temperate plateaus and plains, grasslands and shrublands. It is an area of rough terrain where rounded hills predominate, some reaching elevations of 2,400 masl; the weather is dry and semi-warm, with warm temperatures during the summer, and cold during the winter. It has sub-regions with relief with hills and mountains with elevations above the 3,000 masl; temperate humid climate that is cool in the summer.
Sierra Madre del Sur	Municipalities of Texcaltitlan, Ocuilan, Malinalco, Zumpahuacan, Coatepec, Almoloya Alquisiras, Zacualpan, Sultepec, Ixtapan de la Sal, Tlatlaya, Tejupilco, Zacazonapan, and Temascaltepec	Runs along the Pacific coast through Oaxaca and Guerrero and is separated from the central highlands by the deep valley of Rio Balsas. This crystalline mountain often achieves elevation of 2,100-2,400 masl.

Table 2. List of mosquito species, country of collection, number of specimens with DNA barcodes and Barcode Index Number (BIN) from Mexico State, Mexico. Mean (%) intraspecific values of sequence divergence using the Kimura two-parameter distance (K2P) are shown with missing entries indicating that less than two specimens were analysed.

Species	F.R.	P.S.	O.C.	(n)	(d)	BINs
***Anopheles* (*Anopheles*)**						
1. *aztecus* Hoffmann	Ho*	X-L	Mexico	0	N/A	
2. ***franciscanus* McCracken**	**NSR**	✓	USA	9	0.45	
3. *pseudopunctipennis* Theobald	Ho**	✓	Mexico, USA	23	3.95	BOLD:AAF5940
***Aedes* (*Aztecaedes*)**						
4. ***ramirezi* Vargas and Downs**	**NSR**	✓	Mexico	4	0.24	BOLD:ADR1037
***Aedes* (*Georgecraigius*)**						
5. *epactius* Dyar and Knab	OM	✓	Mexico	6	0.27	BOLD:ADM2281
***Aedes* (*Howardina*)**						
6. ***allotecnon* Kumm, Komp and Ruiz**	**NSR**	✓	Mexico	0	N/A	
7. ***guerrero* Berlin**	**NSR**	✓	Mexico	1	N/A	BOLD:ADM1245
8. ***lorraineae* Berlin**	**NSR**	✓	Mexico	0	N/A	
9. ***quadrivittatus* (Coquillett)**	**NSR**	✓	Mexico	7	0.18	BOLD:ADM1683
***Aedes* (*Lewnielsenius*)**						
10. *muelleri* Dyar	MA	X-L	USA	1	N/A	
***Aedes* (*Ochlerotatus*)**						
11. ***scapularis* (Rondani)**	**NSR**	X-C	French Guiana	2	0.15	
12. ***shannoni* Vargas and Downs**	**NSR**	✓	Mexico	0	N/A	
13. *trivittatus* (Coquillett)	MA	✓	Canada, Mexico	9	2.01	BOLD:AAC9486, BOLD:ADP6375
***Aedes* (*Protomacleaya*)**						
14. ***chionotum* Zavortink**	**NSR**	✓	Mexico	7	1.16	BOLD:ADL9968
15. ***gabriel* Schick**	**NSR**	✓	Mexico	10	0.49	BOLD:ADM0866
16. ***idanus* Schick**	**NSR**	✓	Mexico	0		
17. ***kompi* Vargas and Downs**	**NSR**	✓	Mexico	7	0.00	BOLD:ADT4232
18. ***vargasi* Schick**	**NSR**	✓	Mexico	8	0.5	BOLD:ADM0829
19. ***zoosophus* Dyar and Knab**	**NSR**	X-C	USA	1	N/A	

***Aedes* (*Stegomyia*)**						
20. *aegypti* (Linnaeus)	DV	✓	Mexico	8	1.04	BOLD:AAA4210
21. ***albopictus* (Skuse)**	**NSR**	✓	USA, Thailand	9	0.33	
***Haemagogus* (*Haemagogus*)**						
22. ***equinus* Theobald**	**NSR**	✓	Mexico	5	0.93	BOLD:ADP5251
23. ***mesodentatus* Komp and Kumm**	**NSR**	✓	Mexico	2	1.39	BOLD:ADM0318
***Psorophora* (*Janthinosoma*)**						
24. ***ferox* (von Humboldt)**	**NSR**	✓	Mexico, USA	4	1.69	BOLD:ADQ2015
***Culex* (*Anoediopora*)**						
25. ***restrictor* Dyar and Knab**	**NSR**	✓	Mexico	7	0.66	BOLD:ADU1036
Culex (Culex)						
26. ***bidens* Dyar and Knab**	**NSR**	X-C	Argentina	3	0.1	
27. ***coronator* Dyar and Knab**	**NSR**	✓	French Guiana, Mexico, USA	19	0.78	BOLD:AAN3636
28. *erythrothorax* Dyar	MA	X-L	USA	1	N/A	

Table 2. Continuation.

Species	F.R.	P.S.	O.C.	(n)	(d)	BINs
29. ***nigripalpus* Theobald**	NSR	✓	Colombia, Dominican Republic, Puerto Rico, USA	12	0.24	
30. ***pseudostigmatosoma* Strickman**	NSR	✓	Mexico	15	0.27	BOLD:AAF1735
31. *quinquefasciatus* Say	MP	✓	Mexico	4	0.15	BOLD:AAA4751
32. *restuans* Theobald	MP	✓	Canada, Mexico, USA	15	0.23	BOLD:AAA7661
33. ***salinarius* Coquillett**	NSR	✓	Canada, Mexico, USA	12	0.84	BOLD:ABZ7941
34. *stigmatosoma* Dyar	MA	✓	Mexico	7	0.62	BOLD:ACX7140
35. *tarsalis* Coquillett	MP	✓	Canada, Mexico, USA	19	0.85	BOLD:ABY6040
36. *thriambus* Dyar	MP	✓	Mexico	3	0.62	BOLD:ABY6041
Culex (Microculex)						
37. ***rejector* Dyar and Knab**	NSR	✓	Mexico	3	0.10	BOLD:ADP5264
Culex (Neoculex)						

38.	*apicalis* Adams	MA	X-L	USA	2	0.0	
39.	*arizonensis* Bohart	MP	✓	Mexico	3	0.31	BOLD:ADM0469
	Culex (Phenacomyia)						
40.	***corniger* Theobald**	NSR	✓	Colombia	4	0.38	
41.	***lactator* Dyar and Knab**	NSR	X-C	Colombia	3	0.00	
	Lutzia (Lutzia)						
42.	***bigoti* (Bellardi)**	NSR	✓	Mexico	4	0.18	BOLD:ADQ2012
	Culiseta (Culiseta)						
43.	***incidens* (Thomson)**	NSR	✓	USA	2	0.61	
44.	*inornata* (Williston)	MA	✓	Canada, Mexico, USA	38	0.37	BOLD:AAC9132
45.	*particeps* (Adams)	MA	✓	Mexico	4	2.51	BOLD:ADM1783, BOLD:ADF3447
	Limatus						
46.	***durhamii* Theobald**	NSR	X-C	French Guiana	5	0.12	
	Wyeomyia (Wyeomyia)						
47.	***mitchellii* (Theobald)**	NSR	✓	Mexico, USA	14	0.67	BOLD:ADM1845
	Toxorhynchites (Lynchiella)						
48.	***moctezuma* (Dyar and Knab)**	NSR	✓	Mexico	1	N/A	BOLD:ADS6264
	Uranotaenia (Uranotaenia)						
49.	***geometrica* Theobald**	NSR	✓	Mexico	2	0.00	BOLD:ADQ6930
50.	***lowii* Theobald**	NSR	X-C	USA	7	0.27	
51.	***sapphirina* (Osten Sacken)**	NSR	X-C	USA	11	0.52	

F.R.=First Record; Ho**: Hoffmann, 1932; Ri: Ripstein, 1934; Ma: Martini, 1935; Ho*: Hoffmann, 1935; MP: Martínez-Palacios, 1952; VM: Vargas and Martínez-Palacios, 1956; DV: Díaz-Nájera and Vargas, 1973; HB: Heinemann and Belkin, 1977; OM: Ortega-Morales et al., 2018c; NSR: New State record, in bold; (✓) = Collected in present study; P.S.= Present Study; X-C: Record obtained from CAIM; X-L: Record obtained from literature; O.C. = Originating country of sequences analysed; (n): Number of sequences; (d): Average intraspecific divergence (%); (N/A): Not applicable. In bold face all new records for Mexico State.

Table 3. Biological notes of the new records of mosquito species found in Mexico State, Mexico.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
Anopheles franciscanus	Santa María de la Asunción, Zumpahuacán (18º48'33''N-99º33'26''W), 1,882	Swamp with abundant emerging vegetation, and brown algae	*An. pseudopunctipennis, Cx. pseudostigmatosoma*	7.0	223	23
Aedes ramirezi	Cemetery of San Pedro Guadalupe, Zumpahuacán (18º48'47''N-99º32'13''W), 1,946	Flower vase	*Ae. epactius*	9.0	324	22
		Rock hole	*Ae. epactius*	6.5	115	22.3
		Rock hole	*Ae. epactius*	6.5	78	22.4
		Rock hole	*Ae. epactius*	6.5	84	22
Aedes allotecnon	El Peñón, Temazcaltepec (19º2'46.2''N-100º7'20.1''W)	Bromeliad axil	*Cx. rejector*	4.5	30	22.7
Aedes guerrero	Carretera a Coatepec, Coatepec (18º54'41''N-99º47'50''W), 2,104	Human biting at day	---	---	---	---
Aedes lorraineae	Xonacatlán (19º24'54''N-99º30'14''W), 2,586	Unused water tank	*Cx. arizonensis*	7.5	106	21.9
	Ocuilán (18º58'53.7''N-99º18'41.3''W), 2,180	Human biting at day	*Ae. chionotum*	---	---	---
Aedes quadrivittatus	Chiltepec de Hidalgo, Coatepec (18º54'35''N-99º49'53''W), 2,394	Bromeliad axil	*Cx. rejector*	6.5	33	17
	Carretera a Coatepec (18º54'41''N-99º47'50''W), 2,104	Bromeliad axil	---	7.12	37	23
	Ocuilán (18º58'44''N-99125'27''W), 2,337	Human biting at day	---	---	---	---
	Ocuilán (18º58'44''N-99125'27''W), 2,337	Bromeliad axil	*Cx. rejector*	6	18	21
	Carretera a Ocuilán (18º59'5''N-99º20'7''N), 2,110	Human biting at day	*Ae. chionotum*	---	---	---
Aedes scapularis	Bejucos, Tejupilco (18º53'52.5''N-100º8'35.7''N)	Pond	---	---	---	---
	Tatlaya, San Francisco de Asis (18º37'35.1''N-100º16'42.5''N)	Pond	---	---	---	---
Aedes shannoni	Puente Alameda, Malinalco (18º50'32''N-99º27'28''W), 1,300	Human biting at day	*Ae. albopictus*	---	---	---
Ae. chionotum	El Ocotol, Tonanitla (19º41'50''N-99º4'2''W), 2,640	Approaching to humans	*Cx. arizonensis*	---	---	---

Table 3. Continuation.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
	Unknown location (19º44'12.5''N-99º28'51.7''W)	Discarded tire	*Cs. particeps*	6.9	446	18
	Carretera Ocuilán-Cuernavaca, Ocuilán (18º59'5''N-99º20'7''W), 2,110	Human biting at day	*Ae. quadrivittatus*	---	---	---
	Carretera Ocuilán-Cuernavaca, Ocuilán (18º59'5''N-99º20'7''W), 2,110	Tree hole	*Cx. restrictor*	6.9	1005	23.3
	Carretera Ocuilán-Cuernavaca, Ocuilán (18º59'5''N-99º20'7''W), 2,110	Tree hole	*Cx. restrictor*	7.5	621	22.5
	Carretera Mexicapa-Cuernavaca (18º58'53.7''N-99º18'41.3''W), 2,180	Tree hole	*Cs. particeps*	6.27	125	20
	Carretera Mexicapa-Cuernavaca (18º58'53.7''N-99º18'41.3''W), 2,180	Human biting at day	*Ae. lorreineae*	---	---	---
Aedes gabriel	Chalma, Malinalco (18º55'31''N-99º26'29''W), 1,600	Human biting at day	*Ae. epactius, Ae. trivittatus, Ps. ferox, Wy. mitchellii*	---	---	---
	(18º55'27''N-99º29'20''W), 1,576	Human biting at day		---	---	---
	Coquillo, Sultepec (18º43'19.5''N-99º57'7.4''W)	Tree hole	*Cx. restrictor*	6.8	331	25.7
	Coquillo, Sultepec (18º43'19.5''N-99º57'7.4''W)	Tree hole	*Cx. restrictor*	6.65	221	25
	Jalpan, Sultepec (18º39'14.6''N-99º59'10''W)	Discarded tire	*Ae. vargasi, Cx. restrictor, Lt. bigoti, Tx. moctezuma*	6.7	193	27.5
	San Miguel Totalmaloya, Sultepec (18º38'13''N-99º58'52''W), 1,031	Artificial container	*Ae. aegypti*	6.8	95	26
	Tenanguillo de las Peñas, Sultepec (18º37'8.2''W-100º3'3.1''W)	Human biting at day	*Hg. equinus*	---	---	---
	El Puerto, Tlaflaya (18º32'39''N-100º12'55''N), 1,870	Discarded tire	*Cx. restrictor, Tx. moctezuma*	7.1	57	37.8
	Lampazos, Temazcaltepec (19º3'24.7''N-100º13'30.9''W)	Discarded tire	---	7.8	2000	25
Aedes idanus	Carretera Diego de Sáchez-Sultepec, Sultepec (18º53'16''N-100º3'46''W), 2,270	Tree hole	*Cx. restrictor*	7	99	23
Aedes kompi	Ahuatenco, Ocuilán (18º56'45.4''N-99º20'13.9''W), 1,899	Tree hole	---	7.84	276	20

Table 3. Continuation.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
Aedes vargasi	Coquillo, Sultepec (18º43'19.5''N-99º57'7.4''W)	Tree hole	*Cx. restrictor*	6.5	354	28
	Jalpan, Sultepec (18º39'14.6''N-99º59'10''W)	Discarded tire	*Ae. gabriel, Cx. restrictor, Lt. bigoti, Tx. moctezuma*	6.7	193	27.5
	San Miguel Totalmaloya, Sultepec (18º38'13''N-99º58'52''W), 1,031	Flower vase	*Ae. epactius, Cx. coronator*	7	94	26.7
	Tenanguillo de las Peñas, Sultepec (18º37'8.2''N-100º0'3.1''W)	Tree hole	*Cx. restrictor, Tx. moctezuma*	6.1	126	25.6
Aedes zoosophus	Tatlaya, San Francisco Limon (18º23'11.5''N-100º17'8.8''N)	Discarded tire	---	---	---	---
Aedes albopictus	Palo Dulce, Malinalco (18º51'4''N-99º27'20''W), 1,405	Discarded tire	*Ae. epactius*	7.4	42	28
	Puente Alameda (18º50'32''N-99º27'28''W), 1,300	Artificial container	---	7.1	7	29
	Puente Alameda (18º50'32''N-99º27'28''W), 1,300	Human biting at day	*Ae. shannoni*	---	---	---
Haemagogus equinus	Tenanguillo de las Peñas, Sultepec (18º37'8.2''N-100º0'3.1''W)	Human biting at day	*Ae. gabriel*	---	---	---
	San Pedro Limón, Tlatlaya	Ovitraps	---	---	---	---
Haemagogus mesodentatus	Coquillo, Sultepec (18º43'19.5''N-99º57'7.4''W)	Human biting at dusk	---	---	---	---
Psorophora ferox	Chalma, Malinalco (18º55'31''N-99º26'29''W), 1,600	Human biting at day	*Ae. epactius, Ae. trivittatus, Ae. gabriel, Wy. mitchellii*	---	---	---
Culex restrictor	La Rampa, Malinalco (18º55'27''N-99º29'20''W), 1,576	Discarded tire	*Ae. epactius*	7	116	26.5
	Carretera Diego Sánchez-Sultepec, Sultepec (18º53'16''N-100º3'46''W), 2,270	Tree hole	*Ae. idanus*	7	99	23
	Coquillo, Sultepec (18º43'19.5''N-99º57'7.4''W)	Tree hole,Tree hole Tree hole Tree hole	*Ae. vargasi* *Ae. gabriel* *Ae. gabriel* ---	6.5 6.8 6.6 6.6	354 331 221 131	28 25.7 25 26.5
	Jalpan, Sultepec (18º39'14.6''N-99º59'10''W)	Discarded tire	*Ae. gabriel, Ae. vargasi, Lt. bigoti, Tx. moctezuma*	6.7	193	27.5
	Tenanguillo de las Peñas, Sultepec (18º37'8.2''N-100º0'3.1''W)	Tree hole	*Ae. vargasi, Tx. moctezuma*	6.1	126	25.6

Table 3. Continuation.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
Culex restrictor	Tenanguillo de las Peñas, Sultepec (18º37'8.2''N-100º0'3.1''W)	Tree hole	*Ae. vargasi*, *Tx. moctezuma*	6.1	126	25.6
	Calotenango, Sultepec (18º36'36.1''N-100º0'1''W)	Discarded tire	---	7.2	597	22.9
	El Puerto, Tlaflaya (18º32'39''N-100º12'55''W), 1,870	Discarded tire	*Ae. gabriel*, *Tx. moctezuma*	7.1	57	37.8
	Carretera Ocuilán-Cuernavaca, Ocuilán (18º59'5''N-99º20'7''W), 2,110	Tree hole Tree hole Adults resting into tree hole	*Ae. chionotum* *Ae. chionotum* ---	6.9 7.5 ---	1005 621 ---	23.3 22.5 ---
Culex bidens	Tlatlaya, San Francisco Limon (19º46'55''N-99º10'40''W)	Discarded tyre	---	---	---	---
	Teoloyucan (18º36'1''N-100º18'12''W)	Pond				
		Unused water tank				
Culex coronator	Puente caporal, Malinalco (18º54'8''N-99º27'21''W), 1,502	Discarded tire	*Ae. epactius*, *Cx. quinquefasciatus*	6.75	74	27.4
	Llano Grande, Coatepec (18º49'31''N-99º46'33''W), 1,846	Discarded tire	*Ae. epactius*, *Cx. quinquefasciatus*	7	25	25.6
	Totoltepec de la Paz, Almoloya Alquisiras (18º51'4''N-99º50'40''W), 1,850	Discarded tire Artificial container Artificial container	*Ae. epactius*, *Lt. bigoti* *Ae. epactius* *Ae. epactius*, *Cx. quinquefasciatus*	7 8 7.3	29 55 81	26.5 24.5 24
	San Miguel Totalmaloya, Sultepec (18º38'13''N-99º58'52''W), 1,031	Flower vase Flower vase Flower vase	*Ae. epactius*, *Ae. vargasi* *Ae. epactius* *Ae. aegypti*	7 8 8	94 101 87	26.7 27.8 28.1
Culex nigripalpus	Arroyo zarco, Jilotepec (20º6'52''N-99º42'54''W), 2,517	Pond with abundant emerging vegetation	*Cx. thriambus*	8.3	104	20
Culex pseudostigmatosoma	Santa María de la Asunción, Zumpahuacán (18º48'33''N-99º33'26''W), 1,882	Swamp with abundant emerging vegetation, and brown algae	*An. franciscanus*, *An. pseudopunctipennis*	7	223	23
	El Peñón, Temazcaltepec (19º2'35.5''N-100º7'42.1''W)	Pond with abundant emerging vegetation	---	6.9	15	26.3
Culex salinarius	San Pedro Tultepec, Lerma (19º16'27.1''N-99º31'17.04''W)	Pond with abundant emerging vegetation	---	6.48	430	25

Table 3. Continuation.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
Culex rejector	Chiltepec de Hidalgo, Coatepec (18º54'35''N-99º49'53''W), 2,394	Bromeliad axil	*Ae. quadrivittatus*	6.5	33	17
	Totolmajac, Villa Guerrero (18º56'0'N-99º41'45''W), 2,138	Bromeliad axil	---	6	8	21
	Santa María, Ocuilán (18º58'44''N-99º25'27''W), 2,337	Adults resting in bromeliad bracts Bromeliad axils	--- *Ae. quadrivittatus*	--- 6	--- 18	--- 21
	El Peñón, Temazcaltepec (19º2'46.2''N-100º7'20.1''W)	Bromeliad axil Bromeliad axil	--- *Ae. allotecnon*	5.8 4.5	10 30	25.7 22.7
Culex corniger	La Rampa, Malinalco (18º55'27''N-99º29'20''W), 1,576	Approaching to humans	---	---	---	---
Culex lactator	Tejupilco, Luviano (18º55'18.8''N-100º17'53.3''W)	Unused water tank	---	---	---	---
Lutzia bigoti	Chiltepec de Hidalgo, Coatepec (18º54'35''N-99º49'53''W), 2,394	Artificial container	*Ae. epactius, Cx. thriambus*	6.2	16	17.7
	Carretera a Coatepec, Coatepec (18º54'41''N-99º47'50''W), 2,104	Discarded tire	*Cx. arizonensis, Cs. particpes*	6.4	25	20
	San Pedro, Tenancingo (18º58'3''N-99º31'45''W), 2,083	Discarded tire	*Cx. quinquefasciatus, Cx. thriambus, Cs. particeps*	6.9	90	24
	Totoltepec de la Paz, Almoloya de Alquisiras (18º51'4''N-99º50'40''W), 1,850	Discarded tire	*Ae. epactius, Cx. coronator*	7	29	26
	Tepextitla, Zacualpan (18º47'47''N-99º44'40''W), 1,829	Artificial container	*Aedes epactius*	7.3	73	21
	Diego Sánchez, Sultepec (18º51'25''N-99º57'57''W), 2,270	Discarded tire	*Cx. thriambus*	7.2	82	21.3
	Jalpan, Sultepec (18º39'14.6''N-99159'10''W)	Discarded tire	*Ae. gabriel, Ae. vargasi, Cx. restrictor, Tx. moctezuma*	6.7	193	27.5
Culiseta incidens	Laguna Chignahuapán, San Mateo Texcalcayac (19º9'6''N-99º31'9''W), 2,580	Swamp with abundant aquatic vegetation and Brown algae	*Cx. quinquefasciatus, Cx. tarsalis*	6.9	150	18
	Laguna Chignahuapán, San Mateo Texcalcayac (19º9'6''N-99º31'9''W), 2,580	Water channel with abundant emerging vegetation	*Cx. restuans, Cx. tarsalis, Cx. thriambus*	6.7	201	18

Table 3. Continuation.

Species	Collection site, Coordinate, Elevation (m.a.s.l.)	Habitat	Species associated	pH	Salts (PPM)	Temp (°C)
Limatus durhamii	Tejupilco, Luvianos (19º1'30.3''N-100º18'8.7''W)	Unused water tank	---	---	---	---
Wyeomyia mitchellii	Chalma, Malinalco (18º55'31''N-99º26'29''W), 1,600	Human biting at day	*Ae. epactius, Ae. trivittatus, Ae. gabriel, Ps. ferox*	---	---	---
Toxorhynchites moctezuma	Zumpahuacán (18º50'13''N-99º35'14''W), 1.650	Discarded tire	---	7	256	24
	Jalpan, Sultepec (18º39'14.6''N-99159'10''W)	Discarded tire	*Ae. gabriel, Ae. vargasi, Cx. restrictor, Lt. bigoti*	6.7	193	27.5
	Tenanguillo de las Peñas, Sultepec (18º37'8.2''N-100º0'3.1''W)	Tree hole	*Ae. vargasi, Cx. restrictor*	6.1	126	25.6
	El Puerto, Tlaflaya (18º32'39''N-100º12'55''N), 1.870	Discarded tire	*Ae. gabriel, Cx. restrictor*	7.1	57	37.8
	El Bejuco, Tejupilco (19º5'26''N-99º52'22''W), 1,360	Flower vase	*Ae. aegypti*	7.4	110	27.5
Uraenotaenia geometrica	Lampazos, Temazcaltepec (19º3'24.7''N-100º13'30.9''W)	Unused water tank	*Ae. epactius*	7.2	106	23
Uranotaenia lowii	Otzoloapan, San Martín Otzoloapan (19º3'15.4''N-100º18'22.2''W)	Discarded tyre	---	---	---	---
Uranotaenia sapphirina	Puerta de Golpe, Tejupilco (18º59'11.9''N-100º17'19.5''W)	Unused water tank	---	---	---	---

Table 4. Medical importance and pathogens of mosquito vector species collected in the State of Mexico, Mexico.

Species	VEEV	EEEV	WEEV	WNV	CHKV	JEV	DENV	YF	ZIKV	Mal	SLE	WB	DI	CE	TV
Anopheles aztecus										✓					
Anopheles pseudopunctipennis										✓					
Aedes scapularis	✓							✓				✓			
Aedes trivittatus													✓		✓
Aedes aegypti							✓	✓	✓						
Aedes albopictus		✓	✓	✓	✓	✓	✓	✓	✓				✓		
Haemagogus equinus								✓							
Haemagogus mesodentatus								✓							
Psorophora ferox	✓														
Culex bidens	✓														
Culex nigripalpus		✓	✓								✓				
Culex quinquefasciatus			✓								✓	✓	✓		
Culex restuans			✓								✓				
Culex tarsalis			✓	✓							✓			✓	
Culiseta inornata			✓	✓											

VEEV= Venezuelan Equine Encephalitis virus; Eastern equine encephalitis virus = EEEV; western equine encephalitis virus = WEEV; West Nile Virus = WNV; Chikungunya virus =CHKV; Japanese Encephalitis Virus= JEV; dengue virus = DENV; Yellow fever virus = YF, Zika virus = ZIKV; St Louis encephalitis virus = SLE; *Wuchereria bancrofti* = WB; *Dirofilaria immitis* = DI; California encephalitis virus = CE; Trivittatus virus =TV; Malaria = Mal

Figure Captions

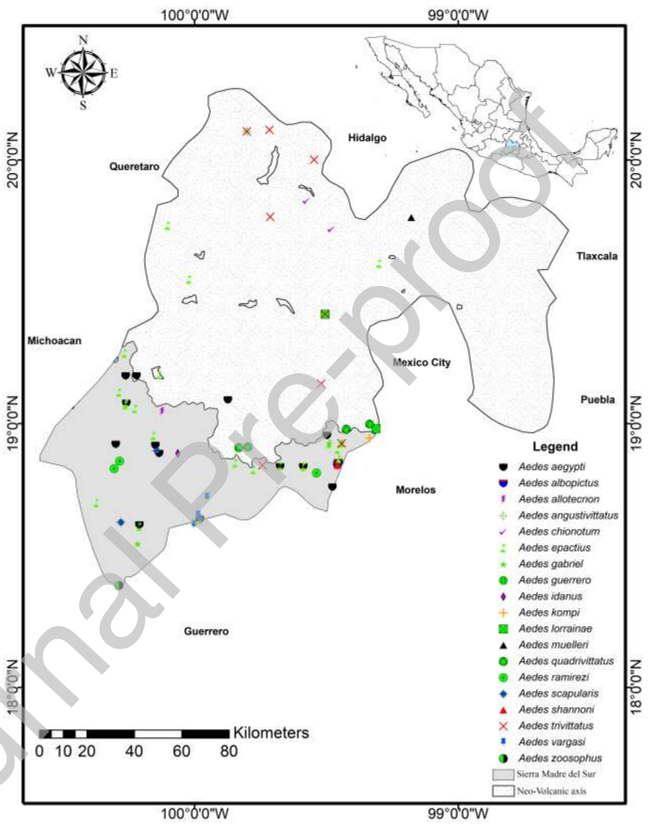

Fig. 1. Geographic distribution of species of the genus *Aedes* species in Mexico State.

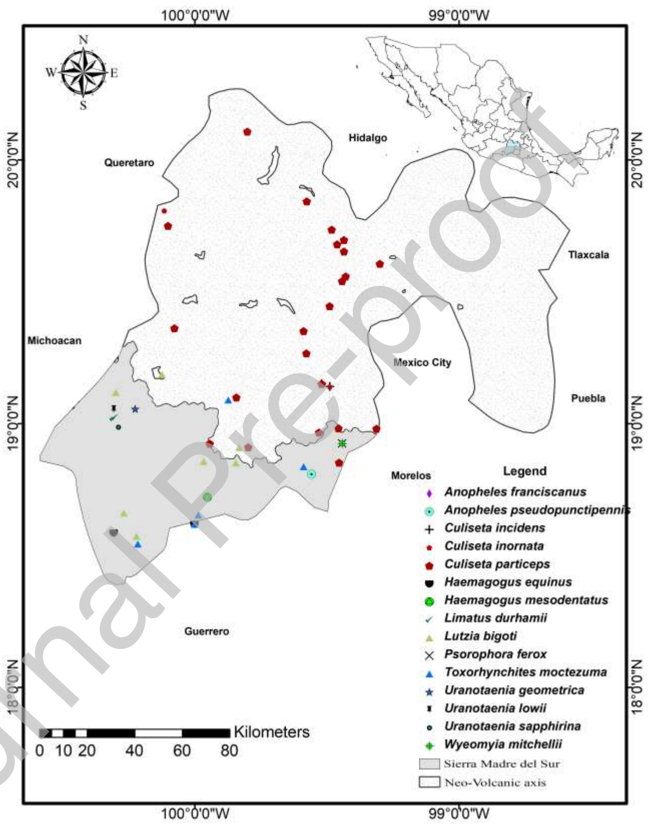

Fig. 2. Geographic distribution of species of the genus *Culex* species in Mexico State.

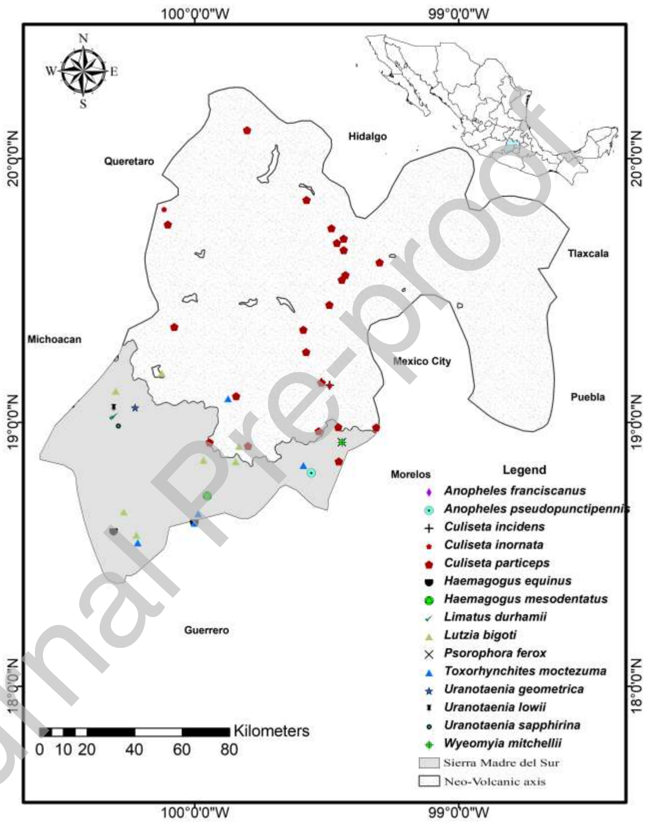

Fig. 3. Geographic distribution of other genera and mosquito species in Mexico State.

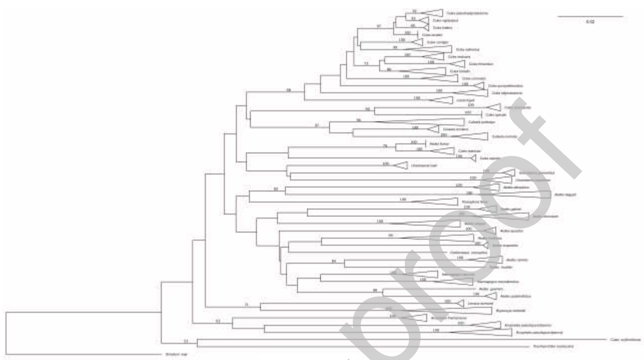

Fig. 4. Neighbour-Joining tree for mosquito species in the State of Mexico based on 658 bp sequences from the *COI* DNA barcoding region. Only bootstrap values higher than 85% are shown.

Graphical abstract

COI DNA barcoding of the mosquitoes (Diptera: Culicidae) of Mexico State, Mexico

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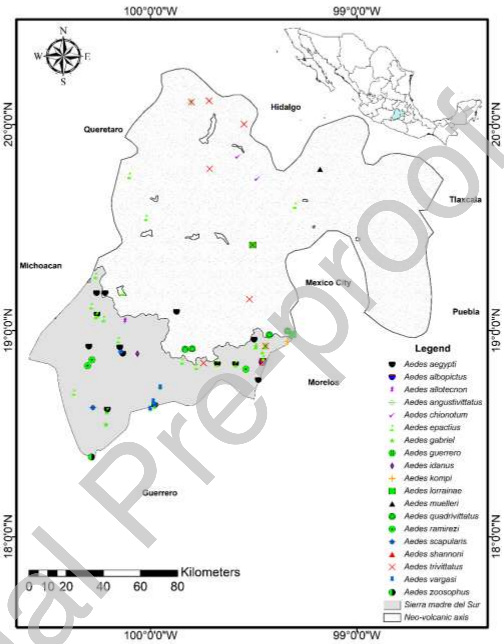

Geographic distribution of species of the genus *Aedes* species in Mexico State.