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The Mosquitoes of Morelos, Mexico: DNA Barcodes, Distribution, Ecology and the Resurrection of the Name *Culiseta dugesi* Dyar and Knab (Diptera: Culicidae) †

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Abstract: To update the record of mosquito diversity in the state of Morelos, Mexico, mosquito collections were conducted in both physiographical regions of Morelos: the Neo-volcanic Axis and the South Mountain Range. Immature stages were collected from aquatic habitats, while adult mosquitoes were collected using CDC light traps, Shannon traps and approaching/landing on the collecting personnel. All specimens were identified using taxonomic keys and the mitochondrial cytochrome *C oxidase* subunit 1 (*COI*). Following Mexico State, this is the second study of mosquito taxonomy of a complete Mexican state using *COI* barcoding. In Morelos, a total of 58 species of mosquito occurs, of which we report 12 for first time (*Aedes vexans*, *Ae. euplocamus*, *Ae. vargasi*, *Psorophora cilipes*, *Ps. lineata*, *Culex restrictor*, *Cx. interrogator*, *Cx. nigripalpus*, *Cx. tarsalis*, *Cx. sandrae*, *Cx. lactator* and *Toxorhynchites moctezuma*). Morelos now ranks sixth in mosquito diversity among Mexican states. Phylogenetic trees were constructed for 32 species, including *Ae. shannoni*, which is a new record for GenBank databases. Additionally, we report *COI* barcode sequences of *Culiseta dugesi* Dyar and Knab, which is resurrected from the synonymy of *Cs. particeps* (Adams). Fourth-instar larvae and adult females of *Cs. dugesi* were redescribed. This brings the number of mosquito species in Mexico to 248. Ecological and distributional notes for the new records, the medical importance of mosquito species in Morelos, and an identification key for larvae and females of the known *Culiseta* species occurring in Mexico are also included.

Keywords: mosquitoes; DNA barcode; Morelos; Mexico; resurrection of *Culiseta dugesi*

1. Introduction

The family Culicidae currently includes approximately 3720 described species [1]. Several species are biting pests and also play an important role as vectors of pathogens of humans and livestock [2]. These include some viral diseases such as dengue, chikungunya, Japanese encephalitis, yellow fever, West Nile virus, Rift Valley fever and Zika, as well as several nematodes and protozoans such as *Dirofilaria* spp. and *Plasmodium* spp. [2,3]. As a result, mosquitoes are one of the principal target groups of surveillance and control programs worldwide [4]. In addition, mosquitoes may serve as key indicators of landscape degradation [5–7].

Current approaches to species identification still rely heavily upon morphology-based procedures, which typically require substantial training and may not always provide an accurate resolution on specimen identity due to homogeneity between life stages of different species and the presence of complex species [8–11]. To overcome this impediment, a small portion (658 bp) of the mitochondrial cytochrome *C oxidase* unit I (*COI*) gene was proposed as a standardized DNA marker in support of species identification for animal barcodes, in a process commonly referred to as DNA barcoding [12,13].

Prior to this study, 247 species of Culicidae mosquitoes belonging to 21 genera have been reported in Mexico [14]. Of the 10 Mexican states that have been studied in terms of taxonomy and distribution of mosquito species, the largest numbers have been found in Veracruz (*n* = 139 spp.) [14–17], Tabasco (*n* = 105 spp.) [14,18–21], Quintana Roo (*n* = 85 spp.) [14,22–29] and Tamaulipas (*n* = 80 spp.) [14,30], whereas the states with the least numbers of species are Tlaxcala (*n* = 26 spp.) [31], Mexico City (*n* = 28 spp.) [32,33], Querétaro (*n* = 50 spp.) [34], Mexico State (*n* = 51 spp.) [35], Hidalgo (*n* = 57 spp.) [36,37] and Nuevo León (*n* = 68 spp.) [14,38–40].

Prior to this study, 45 mosquito species were reported in Morelos. However, most records were based on collections made in urban and sub-urban regions, neglecting collections from conserved forest and jungle regions, mainly in the south of the state. In this study, the two physiographical regions of Morelos (Neo-Volcanic Axis and South Mountain Range) were sampled during the dry and rainy seasons, with special emphasis on conserved forest regions and other sylvan areas. In the present study, we apply the *COI* DNA barcoding approach in support of the identification of mosquitoes in Morelos. In addition, we assessed the DNA barcode variability using Bayesian phylogenetic methods to detect cryptic diversity across the taxa we analyzed.

Morphological and molecular taxonomic evidence suggest that, in Morelos, 58 mosquito species occur. In addition, the use of barcodes, used in parallel with techniques of classical taxonomy, was useful for the re-discovery and description of *Culiseta dugesi* Dyar and Knab, which had been synonymized under the name *Cs. particeps* (Adams). Systematic and ecological information, and morphological descriptions of fourth-instar larvae and adult females of *Cs. dugesi*, are provided in this study. Additionally, biological information for each state's species records, the northernmost and southernmost distributions of some species and information on species originally discovered in Morelos, as well as the medical importance of each species, are provided.

2. Material and Methods

2.1. Study Area

Morelos is located in south-central Mexico (19°7′54″ and 18°19′56″ N; 98°37′58″ and 99°29′39″ W). Morelos has an area of 4950 Km^2, the second smallest state in Mexico. Morelos is bordered to the north by Mexico City and Mexico State, to the south by Puebla and Guerrero, to the west by Guerrero and Mexico State, and to the east by Puebla. Morelos is divided into two physiographical regions and three subregions (shown in parenthesis) (Figure 1): Neo-volcanic Axis with two sub-regions (Lakes and Volcanoes of Anahuac and Mountain Ranges of Southern Puebla) and the South Mountain Range with one sub-region (Mountain Ranges and Valleys of Guerrero) [41]. The regions and sub-regions of Morelos including the municipalities sampled are depicted in Table 1.

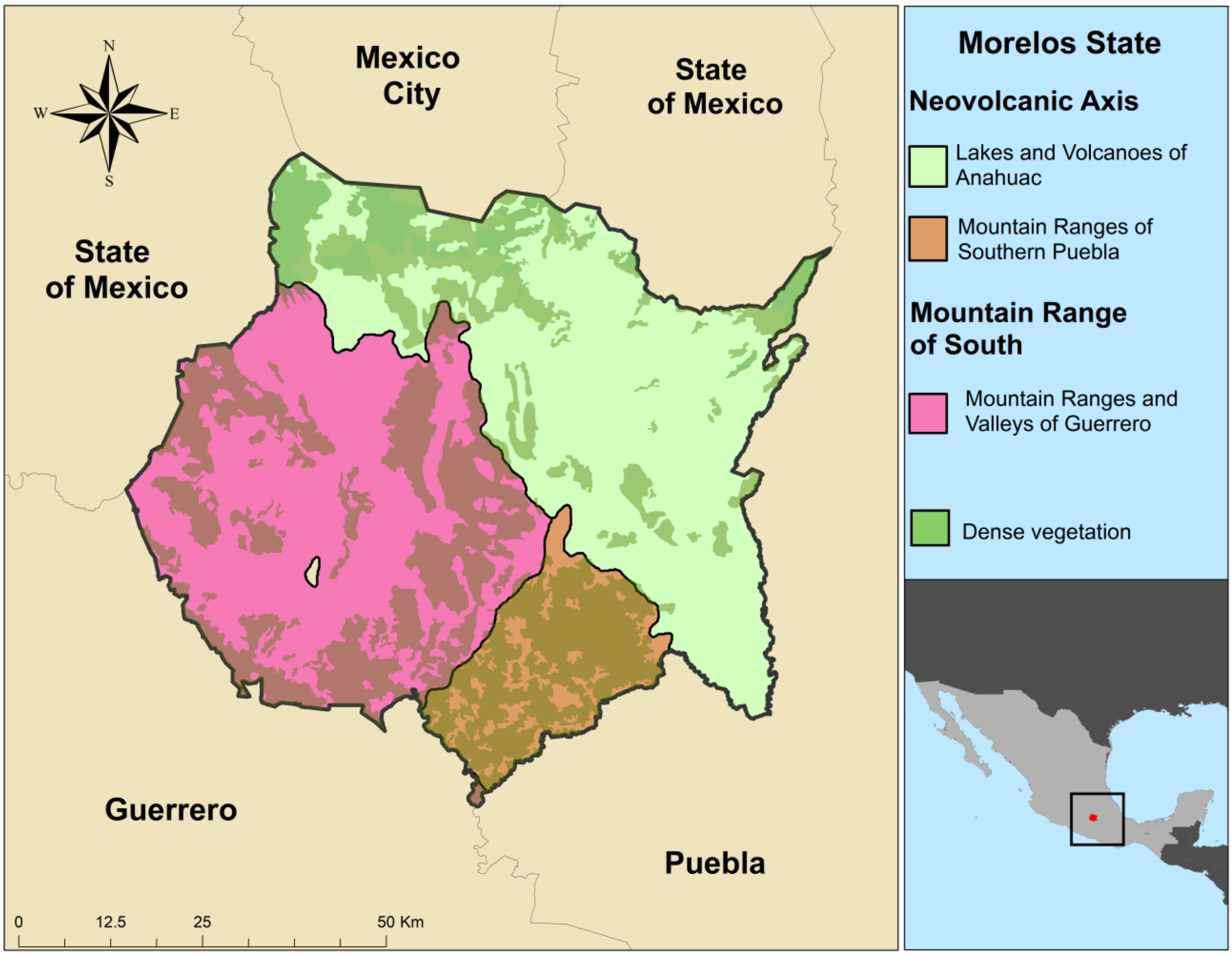

Figure 1. Physiography of Morelos state. Map originally elaborated for this article.

Table 1. Description of the physiography of Morelos and list of municipalities sampled [41].

Region (Sub-Region)	Municipalities Sampled	Description of Sub-Regions
Neo-volcanic Axis (Lakes and Volcanoes of Anahuac)	Axochiapan, Ayala, Cuautla, Cuernavaca, Huitzilac, Jantetelco, Jonacatepec, Temoac, Tepalcingo, Tepoztlán, Tetela del Volcán, Tlanepantla, Tlayacapan, Yecapixtla, Zacualpan	This sub-region has a relief with hills and mountains with elevations above 3000 m., the climate is temperate humid, cool in summer. Extensive regions of pine and oak forest, as well as shrub lands and grasslands.
Neo-volcanic Axis (Mountain Ranges of Southern Puebla)	Amacuzac, Jojutla, Tepalcingo, Tlaquiltenango	This sub-region is composed of volcanic rocks and hills, some mountains with elevations above 1500 m, the climate is temperate humid in winter and warm humid in summer. Extensive areas of sub-tropical forest and grasslands.
South Mountain Range (Mountain Ranges and Valleys of Guerrero)	Mazatepec, Miacatlán, Puente de Ixtla, Xoxocotla, Yautepec,	This sub-region is made up of small canyons and lower mountains, the climate is warm and humid, with rains during the summer. The vegetation is composed of grasslands, shrubs, copal and amate forests

2.2. Mosquito Collection and Identification

Mosquitoes were collected in all life stages (except eggs), in specific sites in the two physiographic regions of Morelos (Table 1). The collections were conducted in both the dry and rainy seasons from 2009–2018. Immature stages were collected from any available water body, and some environmental parameters were recorded such as pH, salinity, and temperature which were measured using a portable Hanna® (mod. HI98129) tester. Larvae and pupae were placed in cups with water from the original aquatic habitat and transported alive to the Laboratory of Parasitology of the Autonomous Agrarian University Antonio Narro Laguna Unit (LBM-UAAAN-UL). A portion of fourth-instar larvae from each collection was mounted on microscope slides using Euparal as the mounting medium. The other larvae were placed into individual emergence tubes to obtain the adult stages and the larval and pupal exuviae. Male genitalia were dissected to assist the taxonomic identification when it was required. Adults were collected using CDC light traps baited with cotton balls containing drops of octenol, which were placed 20:00–9:00 h. and checked daily. Shannon traps baited with humans were used during daylight hours, and resting mosquitoes were aspirated directly from caves and vegetation. All adult mosquitoes were killed using triethylamine vapors and later mounted on insect pins. Mosquitoes mounted on insect pins were identified using specific identification keys and a stereomicroscope Discovery V8 (Zeiss, Jena, Germany), while immature stages and exuviae were identified using a Primostar microscope (Zeiss). The terminology proposed by Harbach and Knight [42] for mosquito anatomy and the classification system for the family Culicidae proposed by Wilkerson et al. [43] are used in this study.

2.3. Genomic DNA Extraction

We employed a modified Hotshot technique [44] for the DNA extraction. In this case, 1–2 legs from each mosquito voucher were placed directly into 50 µL of alkaline lysis buffer (25 mM NaOH, 0.2 mM Na_2EDTA) in a 96-well plate, which was then sonicated (Grant Ultrasonic 3 L Bath) in a water bath for 15 min. The plate was subsequently incubated in a thermocycler for 30 min at 94 °C, cooled for 5 min at 4 °C, and then centrifuged for 3 min at 3000 rpm, after which 50 µL of the neutralizing buffer (40 mMTri-HCL) was added to each sample. The plate was then stored at −80 °C until analysis.

2.4. Polymerase Chain Reaction and DNA Sequencing

PCR amplification of the full-length *COI* barcode region [44,45] was performed using Folmer primers (LCO1490 and HCO2198) and a Quiagen PCR system with the following reaction mix, final volume 50 µL: 2 µL of DNA template, 25 µL H_2O, 5 µL NH_4, 5 µL of dNTPs (2 mM/µL), 2.5 µL of $MgCl_2$ (25 mM/µL), 0.1 µL Bioline Taq Polymerase (Bioline Reagents Ltd., London, UK), 5 µL of each primer (each at 10 pmol/µL), and 0.38 µL of bovine serum albumin (20 mg/mL) [39–41]. The thermal profile consisted of the following: an initial denaturation step at 94 °C for 1 min., 5 cycles of preamplification of 94 °C for 1 min., 45 °C for 1.5 min., 72 °C for 1.5 min., followed by 35 cycles of amplification of 94 °C for 1 min., 57 °C for 1.5 min., and 72 °C for 1 min., followed by a final elongation step of 72 °C for 5 min [4,46,47]. Products showing the correct band size were sequencing in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) at the Sequencing Unit, Animal and Plant Health Agency, United Kingdom. Paired bi-directional sequence traces were combined to produce a single consensus sequence (the full-length 658 bp barcode sequence). To achieve this, individual forward and reverse traces were oriented, edited, and aligned using the Sequencer (v.4.5; Genes Codes Corporation, Ann Harbour, MI, USA), GenDoc (v. 2.6.02) and ClustalX sequence analysis programs [4]. Full details for each specimen and sequence information can be found at the Barcode of Life Database (BOLD) within the "Human Pathogens and Zoonoses Initiative", Working Group 1.4.

2.5. Genetic Data Analysis

For molecular species identification using the *COI* DNA barcoding region, the protocols of Hernández-Triana et al. [46,47] and Hebert et al. [12,13] were followed. Amplicons were obtained using a Qiagen PCR system following the reaction mix of Hernández-Triana et al. [4]. The dataset was analyzed in MEGA v.6 [48]. The Bayesian inference was performed in BEASTv.2.6.7 [49] using the TrNef substitution model (ln = −7561.4940) and estimated parameter priors with the Yule model. Three independent Markov chains of 10,000,000 replicates were run with sampling every 10,000. The sample parameters were combined with LogCombiner and basic parameters were checked in Tracer v.1.7.1 [50]. The sampled trees were combined with LogCombiner and then summarized with TreeAnnotator. The posterior probability was calculated for each node in a maximum clade credibility tree with a burn-in of 10%. For barcode sequences larger than 500 bp, a Barcode Index Number (BIN) was assigned and each BIN was mapped according to species. The taxonomic discordance in the dataset [4] was analyzed using BOLD as detailed in Ratnasingham and Hebert [51].

2.6. Ethical Consideration

All specimens studied during this research were collected by official personnel of the Mexican public health and vector control programs, and personnel from the Mexican network of Entomological and Bioassay Research Units (UIEBs). The different UIEBs located in most states of Mexico serve as reference laboratories for taxonomic identification of vectors collected on the field, and design specific strategies for control of vector-borne diseases. This study was approved and supported by the National Center of Preventive Programs and Disease Control (CENAPRECE).

3. Results

3.1. Mosquitoes and New Records

A total of 3515 specimens from 200 collection samples were studied. Among the specimens were 1284 fourth-instar larvae, 468 larval exuviae, 846 pupal exuviae, 32 pupae, 487 adult females, 392 adult males, and 6 dissected male genitalia. The mosquito fauna of Morelos state consists of 58 species representing the subfamilies Anophelinae and Culicinae, 6 tribes of the subfamily Culicinae, 10 genera, and 23 subgenera (Table 2). Four subgenera (*Aedimorphus*, *Psorophora*, *Anoedioporpa* and *Micraedes*) and twelve species [*Aedes vexans* (Meigen), *Ae. euplocamus* Dyar and Knab, *Ae. vargasi* Schick, *Psorophora cilipes* (Fabricius), *Ps. lineata* (von Humboldt), *Culex restrictor* Dyar and Knab, *Cx. interrogator* Dyar and Knab, *Cx. nigripalpus* Theobald, *Cx. tarsalis* Coquillett, *Cx. sandrae* Berlin, *Cx. lactator* Dyar and Knab and *Toxorhynchites moctezuma* (Dyar and Knab)] are recorded in Morelos for the first time (Table 2). One species (*Cs. dugesi*) was re-discovered, described and resurrected from the synonymy of *Cs. particeps* herein. The species accumulation curve of the 43 mosquito species collected is shown in Figure 2.

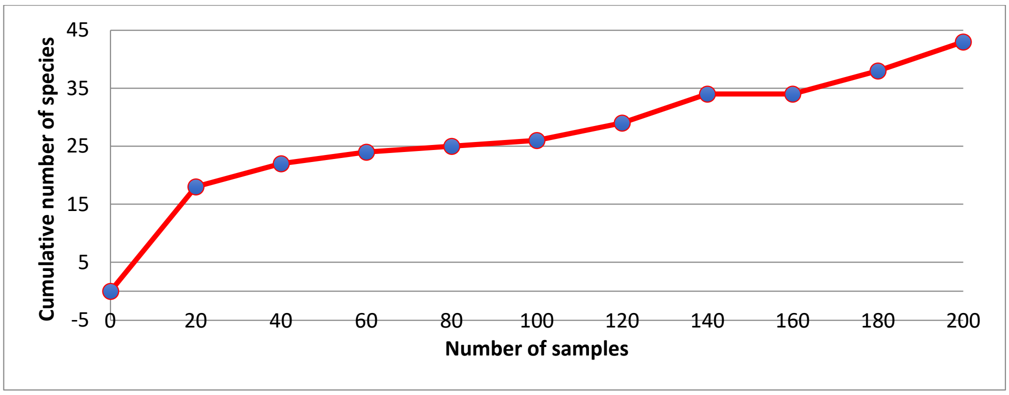

Figure 2. Species accumulation curve for the 43 mosquito species (200 collections) collected in Morelos during 2009–2018.

Table 2. Checklist of the mosquito species of Morelos. Ma: Martini [52]; VD: Vargas and Downs [53]; VM: Vargas and Martínez-Palacios [54]; Va: Vargas [55]; DV: Díaz-Nájera and Vargas [56]; Sc: Schick [57]; Za: Zavortink [58]; HB: Heinemann and Belkin [59]; IM: Ibáñez-Bernal and Martínez-Campos [60]; Vi: Villegas-Trejo et al. [61]; Or: Ortega-Morales et al. [62]; He: Hernández-Guevara et al. [63]; F.R.: First recorded; NSR: New state record; NS: New species. New records for Morelos are indicated in bold.

Taxa	F.R.	Taxa	F.R.
Anopheles (*Anopheles*)		***Culex* (*Anoedioporpa*)**	
1. *An. eiseni* Coquillett	VM	30. ***Cx. restrictor* Dyar and Knab**	**NSR**
2. *An. parapunctipennis* Martini	VM	*Culex* (*Culex*)	
3. *An. pseudopunctipennis* Theobald	VM	31. *Cx. bidens* Dyar and Knab	IM
***Aedes*(*Aedimorphus*)**		32. *Cx. coronator* Dyar and Knab	Va
4. ***Ae. vexans* (Meigen)**	**NSR**	33. *Cx. declarator* Dyar and Knab	DV
Aedes (*Aztecaedes*)		34. ***Cx. interrogator* Dyar and Knab**	**NSR**
5. *Ae. ramirezi* Vargas and Downs	VD	35. ***Cx. nigripalpus* Theobald**	**NSR**
Aedes (*Georgecriagius*)		36. *Cx. quinquefasciatus* Say	Ma
6. *Ae. epactius* Dyar and Knab	DV	37. *Cx. restuans* Theobald	Vi
Aedes (*Howardina*)		38. *Cx. salinarius* Coquillett	Va
7. *Ae. allotecnon* Kumm, Komp, and Ruiz	DV	39. *Cx. stigmatosoma* Dyar	Ma
8. *Ae. guerrero* Berlin	HB	40. ***Cx. tarsalis* Coquillett**	**NSR**
9. *Ae. quadrivittatus* (Coquillett)	Va	41. *Cx. thriambus* Dyar	Va
10. *Ae. sexlineatus* (Theobald)	DV	*Culex* (*Melanoconion*)	
Aedes (*Ochlerotatus*)		42. *Cx. conspirator* Dyar and Knab	Va
11. *Ae. angustivittatus* Dyar and Knab	Va	43. *Cx. erraticus* (Dyar and Knab)	Va
12. *Ae. shannoni* Vargas and Downs	VD	44. *Cx. iolambdis* Dyar	Va
13. ***Ae. euplocamus* Dyar and Knab**	**NSR**	45. *Cx. limacifer* Komp	Va
14. *Ae. taeniorhynchus* (Wiedemann)	DV	46. *Cx. pilosus* (Dyar and Knab)	Va
15. *Ae. trivittatus* (Coquillett)	HB	*Culex* (*Micraedes*)	
Aedes (*Protomacleaya*)		47. ***Cx. sandrae* Berlin**	**NSR**
16. *Ae. chionotum* Zavortink	Za	*Culex* (*Neoculex*)	
17. *Ae. gabriel* Schick	Sc	48. *Cx. apicalis* Adams	Va
18. *Ae. idanus* Schick	HB	49. *Cx. arizonensis* Bohart	Va
19. *Ae. kompi* Vargas and Downs	VD	*Culex* (*Phenacomyia*)	
20. *Ae. vargasi* Schick	**NSR**	50. *Cx. corniger* Theobald	Vi
Aedes (*Stegomyia*)		51. ***Cx. lactator* Dyar and Knab**	**NSR**
21. *Ae. aegypti* (Linneaus)	Va	*Lutzia* (*Lutzia*)	
22. *Ae. albopictus* (Skuse)	Vi	52. *Lt. bigoti* (Bellardi)	Va
Haemagogus (*Haemagogus*)		*Culiseta* (*Culiseta*)	
23. *Hg. anastasionis* Dyar	DV	53. ***Cs. dugesi* Dyar and Knab**	**NSR**
24. *Hg. equinus* Theobald	Or	54. *Cs. particeps* (Adams)	DV
25. *Hg. mesodentatus* Komp and Kumm	DV	*Mansonia* (*Mansonia*)	
Psorophora (*Grabhamia*)		55. *Ma. dyari* Belkin, Heinemann, and Page	He
26. *Ps. columbiae* (Dyar and Knab)	DV	*Toxorhynchites* (*Lynchiella*)	
Psorophora (*Janthinosoma*)		56. ***Tx. moctezuma* (Dyar and Knab)**	**NSR**
27. *Ps. ferox* (von Humboldt)	HB	*Uranotaenia* (*Uranotaenia*)	
***Psorophora* (*Psorophora*)**		57. *Ur. coatzacoalcos* Dyar and Knab	Va
28. ***Ps. cilipes* (Fabricius)**	**NSR**	58. *Ur. sapphirina* (Osten Sacken)	Va
29. ***Ps. lineata* (von Humboldt)**	**NSR**		

3.2. Species Originally Described in Morelos

This species-group is composed of endemic Mexican mosquito species, and although all these species were originally discovered in Morelos, they have currently also been reported in other states of Mexico. *Aedes ramirezi*, *Ae. shannoni*, *Ae. chionotum*, *Ae. gabriel*, and *Ae. kompi* were originally discovered in Morelos.

On 29 June 1947, immature stages of *Ae. kompi* were first collected by W. G. Downs from tree holes and rock holes in Tepoztlán. In this study, immature stages of *Ae. kompi* were collected from one tree hole with colored water at 2 m. from ground level in Tepoztlán in association with *Ae. gabriel* and *Ae. ageypti.* In Huitzilac, females of *Ae. kompi* were collected approaching humans during the day with partial shade in association with *Cs. dugesi. Aedes kompi* has been found only in Mexico State and Morelos.

On 10 June 1948, immature stages of *Ae. shannoni* were first collected by W. G. Downs in Cuernavaca county from temporal ground pools; since then, very few records of the presence of *Ae. shannoni* have been published, it being one of the most uncommon mosquito species in Mexico. In this study, immature stages of *Ae. shannoni* were collected from temporal ground pools with clean water and emergent vegetation with partial shade and from volcanic rock holes with clean water and partial shade in Tlaquiltenango, Puente de Ixtla and Tlayacapan counties. Females of *Ae. shannoni* were also collected biting humans diurnally; this species is a persistent biter, that kept biting even when the host was moving; associated species in this collection were *Ae. epactius*, *Hg. mesodentatus*, and *Ae. gabriel*. *Aedes shannoni* has been found in the states of Guerrero, Mexico State, Michoacán, Morelos, and Querétaro.

On 19 August 1965, immature stages of *Ae. chionotum* were first collected by D. Schroeder from tree holes in Cuernavaca. Zavortink [58] described all life stages except the eggs. Additional collection records were provided by Heinemann and Belkin [59]. During this study, immature stages of *Ae. chionotum* were collected from ovitraps placed in a tree above 1.5 m. from ground level with clean water and leaves at the bottom in Huitzilac and from volcanic rock holes with clean water and abundant leaves at the bottom with partial shade in Tepoztlán. *Aedes chionotum* has been found in Mexico State, Morelos, and Oaxaca.

On 7 September 1965, immature stages of *Ae. gabriel* were first collected by D. Schroeder from a large tree hole in Cuernavaca county. Schick [57] described all life stages except the eggs and additional collection records were provided by Heinemann and Belkin [59] and Ortega-Morales et al. [37]. In this study, *Ae. gabriel* was one of the most common species collected from tree holes, being well distributed in all sub-regions of Morelos where it finds favorable conditions. Immature stages of *Ae. gabriel* were collected from several tree holes with colored water and total shade and from discarded tires in Tlaquiltenango, Jojutla and Tepoztlán counties in association with *Ae. epactius*, *Ae. chionotum*, *Ae. kompi*, *Ae. vargasi*, *Ae. aegypti*, *Ae. albopictus*, *Hg. equinus*, *Hg. mesodentatus*, *Cx. coronator*, *Cx. declarator*, *Cx. quinquefasciatus*, *Cx. stigmatosoma Cx. thriambus*, *Cx. corniger*, *Cx. lactator* and *Tx. moctezuma*. Additionally, immature stages were collected from rock holes in the Olmec ruins of Chimalactlán. In Tepoztlán, females associated with *Ae. epactius*, *Ae. guerrero*, *Ae. shannoni* and *Hg. mesodentatus* were collected biting humans during daylight hours. *Aedes gabriel* has been found in the states of Hidalgo, Jalisco, Mexico State, Morelos, and Zacatecas.

In 29 June 1969, immature stages of *Ae. ramirezi* were first collected by W.G. Downs from volcanic rock holes in Cuernavaca county. Zavortink [58] re-described fourth-instar larvae, pupae, and the male and female of this species, and additional collection records were provided by Heinemann and Belkin [59]. In Morelos, immature stages of *Ae. ramirezi* are frequently found in volcanic rock holes, which presumably are the typical aquatic habitat for this species. During this study, immature stages of *Ae. ramirezi* were collected from rock holes in the Olmec ruins of Chimalactlán in association with *Ae. gabriel* and *Cx. coronator*; in this same location, adults were collected resting from dry rock holes, while females were collected biting humans diurnally in Huautla; additionally, immature stages were collected from discarded tires with colored water and abundant leaves at the bottom and total shade in Huitzilac associated with *Ae. epactius*, *Cx. coronator*, *Cx. quinquefasciatus*, *Cx. stigmatosoma* and *Cs. particeps*. *Aedes ramirezi* has been found in the states of Jalisco, Morelos, Sinaloa, Sonora, and Veracruz.

3.3. Biological and Ecological Notes for the New State Records

Biological and ecological notes for each species-group are reported here. Specific notes including the collection sites, date of collection, larval habitat, aquatic parameters, and associated species are shown in Table 3.

3.3.1. Genus *Aedes*

Of the 19 species of *Aedes* known from Morelos, three are reported for the first time in the state: *Ae. vexans, Ae. euplocamus* and *Ae. vargasi*. Females of *Ae. vexans* were collected approaching humans at a single location in one single site in Miacatlán, while immature stages of *Ae. euplocamus* were collected from a temporal ground pool during the rainy season. Immature stages of *Aedes vargasi* were commonly found in tree holes and artificial containers in the conserved regions of the state.

3.3.2. Genus *Psorophora*

The subgenus *Psorophora* of genus *Psorophora* is recorded for the first time in Morelos: *Ps. cilipes* and *Ps. lieanata* were both collected from a single site each; adults of *Ps. cilipes* were collected resting intra-domiciliarily, while immature stages of *Ps. lineata* were collected from an artificial container.

3.3.3. Genus *Culex*

The genus *Culex* is the most diverse group of mosquitoes in Morelos; of the twenty-one species of *Culex* known, six are reported for the first time in the state, and the subgenera *Anoedioporpa* and *Micraedes* are first reported here. Immature stages of *Cx. restrictor* were collected from a tree hole with colored water in single site, while immature stages of *Cx. interrogator* were commonly found in temporal ground pools and adult stages were collected resting in vegetation, it being a common species in Morelos. Immature stages of *Cx. nigripalpus* were collected once from a temporal ground pool; adults of *Cx. tarsalis* were collected resting in vegetation with total shade; while immature stages of *Cx. sandrae* were collected from bromeliad axils 2–3 m above the ground in a forest region of the state, and immature stages of *Cx. lactator* were collected once from a tree hole in a conserved area of Morelos.

3.3.4. Genus *Toxorhynchites*

The genus *Toxorhynchites*, subgenus *Lynchiella* had already been previously reported in Morelos with *Tx. theobaldi* (Dyar and Knab) [61]. This record was misidentified with *Tx. moctezuma*, of which immature stages were collected from artificial containers and tree holes in one conserved forest of Morelos.

3.4. Molecular Analysis, COI-Barcoding Identification and Cryptic Diversity

In total, we analyzed 112 DNA barcode sequences of 32 species, which represents 56% of all species occurring in Morelos. Three sequences were obtained from the BOLD database from specimens collected in Querétaro, Mexico (*Cs. particeps* BOLD:ADQ4018) and were added to the dataset. One single sequence of *Ae. shannoni* (BOLD:ADP9644) was obtained; this species is a new sequence record for the *COI* GenBank database. Phylogenetic relationships were reconstructed by Bayesian inference (Figure 3). We did not obtain DNA barcodes for six species, as they were collected as immatures and previously mounted on microscope slides for identification, as follows: *An. eiseni*, *An. pseudopunctipennis*, *Ps. lineata*, *Cx. declarator*, *Cx. nigripalpus* and *Cx. pilosus*. A further seven species were processed, but failed to amplify DNA: *Ae. vexans*, *Hg. mesodentatus*, *Ps. cilipes*, *Cx. tarsalis*, *Cx. sandrae*, *Cx. lactator* and *Ma. dyari*. In general, all specimens of the same species clustered together, although there was a deep split in *Cx. stigmatosoma* (BOLD:ABY6041 and BOLD:AAA7661) and *Cs. particeps* (BOLD:ADQ4018 and BOLD:ADF3447) with two BINS for each species.

Table 3. Collection records of the newly reported species found in Morelos, Mexico. The type of aquatic habitat of the immature stages or environmental condition where adults were collected (pH, temperature in Celsius degrees scale, and dissolved salts in scale of parts per million; the location and county within Morelos where species were collected; the geographic coordinates in scale of degrees, minutes, and seconds; elevation (meters above sea level); date of collection; and associated species of each collection.

Species	Habitat/Parameters	Environment	Location, County	Coordinate/Elevation	Collection Date	Associated spp.
Aedes vexans	Human-biting	Shrub region	Miacatlán	18°46′44″ N–99°21′50″ W, 1004 m.	12 September 2017	No associated spp.
Aedes euplocamus	Temporal ground pool with clear water (pH 7.9, 23 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′52.61″ N–99°0′44.5″ W, 1098 m.	7 June 2017	*Ae. shannoni*, *Ae. trivittatus*, *Cx. stigmatosoma*
Aedes vargasi	Tree hole, colored water, (pH 9.8, >2000 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′16″ N–99°0′50.94″ W, 1118 m.	8 June 2017	*Ae. gabriel*, *Tx. moctezuma*
	Tree hole (*Licania arborea* Seeman), colored water, (pH 8.5, 470 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′26.44″ N–99°1′5.75″ W, 1125 m.	8 June 2017	*Hg. equinus*
	Tree hole (*Ruprechtia fusca* Meyer), colored water, (pH 8.9, 950 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′26.44″ N–99°1′5.75″ W, 1125 m.	8 June 2017	*Ae. gabriel*, *Hg. equinus*
	Tree hole (*Bursera lancifolia* Schltdl.,), colored water, (pH 8.10, 418 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′26.44″ N–99°1′5.75″ W, 1125 m.	8 June 2017	*Ae. gabriel*, *Hg. mesodentatus*, *Cx. lactator*
	Tree hole (*Licania arborea* Seeman), colored water, (pH 8.17, 218 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′52.23″ N–99°0′50.04″ W, 1121 m.	8 June 2017	*Ae. gabriel*, *Cx. stigmatosoma*, *Cx. corniger*
	Discarded tire, clean water, (pH 8, 145 PPM, 29 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′32.33″ N–99°1′7.97″ W, 1089 m.	7 June 2017	*Ae. gabriel*
	Plastic bottle, colored water, (pH 7.18, 3.75 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′39.82″ N–99°0′53.12″ W, 1087 m.	8 June 2017	*Ae. albopictus*
	Adults resting intra-domiciliary	Mountain region	Quilamula, Tlaquiltenango	18°30′40.8″ N–99°1′10.47″ W, 1087 m.	7 June 2017	*Hg. mesodentatus*
Psorophora cilipes	Adults resting intra-domiciliary	Shrub region	Miacatlán	18°46′44″ N–99°21′50″ W, 1004 m.	12 September 2017	No associated spp.
Psorophora lineata	Artificial pool with clean water without vegetation	Urban area	Tepoztlán	18°58.908′ N–99°6.66″ W, 1793 m.	12 July 2009	No associated spp.
Culex restrictor	Tree hole, colored water with abundant leaves	Mountain region	San Andrés de la Cal, Tepoztlán	18°57′21″ N–99°6′46″ W, 1487 m.	2 July 2017	*Ae. albopictus*

Table 3. *Cont.*

Species	Habitat/Parameters	Environment	Location, County	Coordinate/Elevation	Collection Date	Associated spp.
Culex interrogator	Temporal ground pool with clean water and abundant emergent vegetation (pH 9.38, 798 PPM)	Hills region	Tehuixtla, Jojutla	18°33′55″ N–99°16′7″ W, 884 m.	13 June 2017	*Ae. epactius*, *Cx. coronator*
	Temporal ground pool with colored water (pH 9.96, 187 PPM)	Hills region	Puente de Ixtla	18°36′47″ N–99°18′45″ W, 898 m.	14 June 2017	*Ae. epactius*, *Cx. coronator*, *Cx. stigmatosoma*
	Temporal ground pool with clean water and abundant vegetation (pH 9.53, 246 PPM)	Hills region	Puente de Ixtla	18°36′47″ N–99°18′45″ W, 898 m.	14 June 2017	*Cx. coronator*, *Cx. stigmatosoma*, *Cx. quinquefasciatus*
	Temporal ground pool with colored water and abundant vegetation (pH 9.53, 246 PPM)	Hills region	Puente de Ixtla	18°37′20″ N–99°19′7″ W, 902 m.	14 June 2017	*Ae. epactius*, *Ae. shannoni*, *Cx. coronator*
	Temporal ground pool with clean water and abundant vegetation	Mountain region	Mazatepec	18°43′21.78″ N–99°21′24.03″ W, 968 m.	14 June 2017	*Cx. coronator*, *Cx. quinquefasciatus*, *Cx. stigmatosoma*
	Temporal ground pool with clean water and abundant vegetation	Hills region	Xoxocotla, Puente de Ixtla	18°39′42″ N–99°13′33″ W, 940 m.	14 June 2017	*Cx. coronator*, *Cx. stigmatosoma*
	Adults resting in vegetation, total shade	Hills region	Jojutla	18°36′27″ N–99°11′1″ W, 880 m.	14 June 2017	*Cx. coronator*
Culex nigripalpus	Temporal ground pool with clean water, abundant vegetation and green algae (pH 8.1, 266 PPM, 29 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′52.61″ N–99°0′44.5″ W, 1098 m.	7 June 2017	*An. pseudopunctipennis*, *Ae. shannoni*, *Cx. coronator*, *Cx. declarator*
Culex tarsalis	Adults resting in vegetation, total shade	Hills region	Jojutla	18°36′27″ N–99°11′1″ W, 880 m.	14 June 2017	No associated spp.
Culex sandrae	Axils of bromeliads with clean water, 2 mts. from ground level	Mountain region	Los Venados, Tetela del Volcán	18°53′59″ N–98°42′26″ W, 2182 m.	30 June 2017	*Ae. quadrivittatus*
	Axils of bromeliads with clean water, 3 mts. from ground level	Mountain region	Tetela del Volcán	18°54′33.01″ N–98°42′9.46″ W, 2314 m.	1 July 2017	No associated spp.
Culex lactator	Tree hole (*Bursera lancifolia* Schltdl.,), colored water, (pH 8.10, 418 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′26.44″ N–99°1′5.75″ W, 1125 m.	8 June 2017	*Ae. gabriel*, *Ae. vargasi*, *Hg. mesodentatus*
Culiseta dugesi	Adults approaching humans	Mountain region	CEDIF, Huitzilac	19°1′58.11′ N″–99°15′18.21″ W, 2600 m.	15 June 2017	*Ae. kompi*, *Cx.* sp.
	Discarded tire with clean water and total shade (pH 7.2, 110 PPM, 20.4 °C)	Mountain region	La Encantada, Huitzilac	19°0′59′ N″–99°13′13″ W, 2450 m.	18 September 2017	*Cx. arizonensis, Cs. particeps*
	Adults resting inside artificial container	Mountain region	La Encantada, Huitzilac	19°0′59′ N″–99°13′13″ W, 2450 m.	18 September 2017	No associated spp.

Table 3. *Cont.*

Species	Habitat/Parameters	Environment	Location, County	Coordinate/Elevation	Collection Date	Associated spp.
Toxorhynchites moctezuma	Tree hole, colored water, (pH 9.8, >2000 PPM, 27 °C)	Mountain region	Quilamula, Tlaquiltenango	18°30′16″ N–99°0′50.94″ W, 1118 m.	8 June 2017	*Ae. gabriel*, *Ae. vargasi*
	Discarded tire with clean water and total shade	Hills region	Yautepec	18°54′13″ N–99°3′51″ W, 1223 m.	27 June 2017	No associated spp.

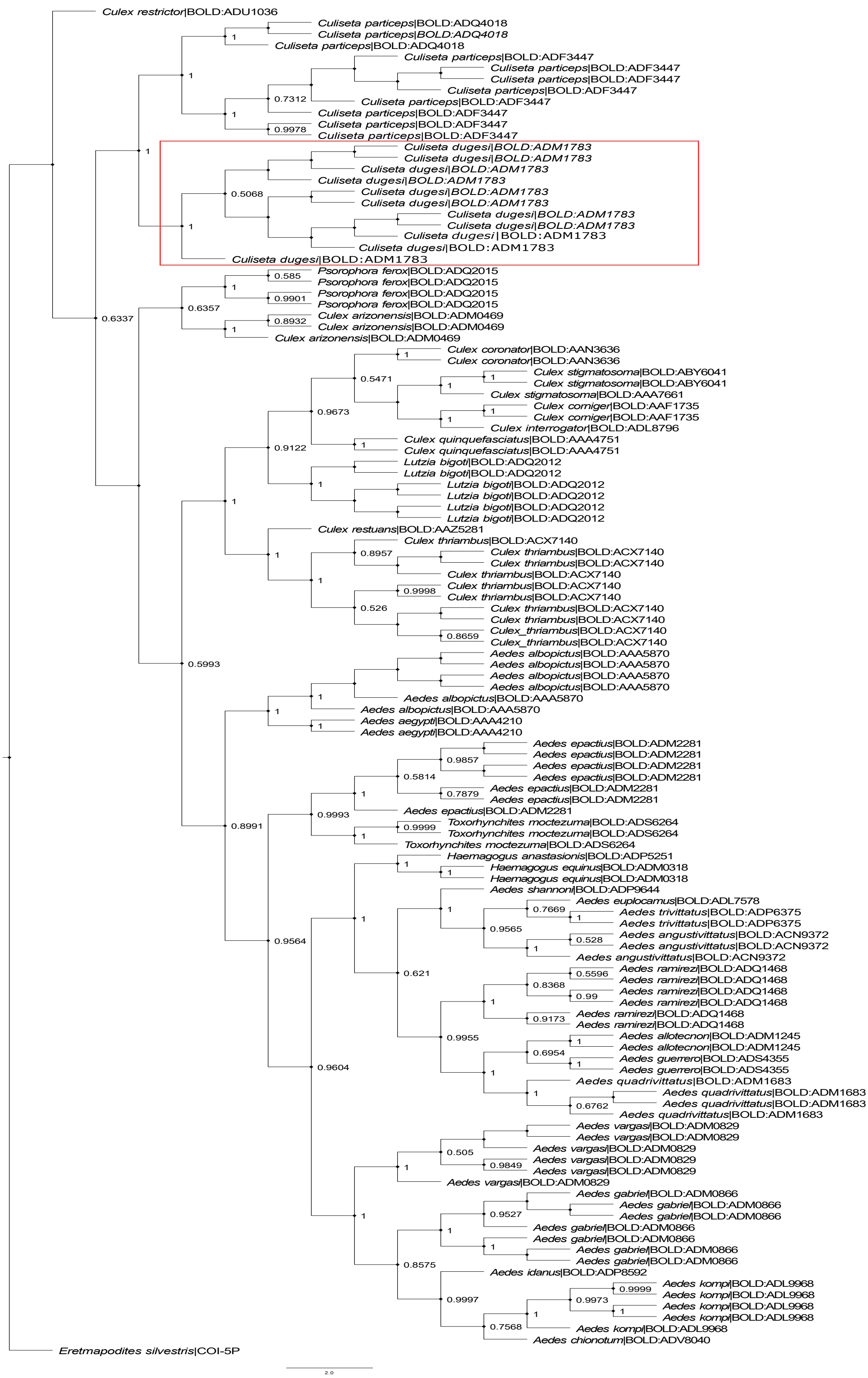

Figure 3. Bayesian phylogenetic tree of COI DNA barcode of the mosquitoes of Morelos. Red box shows the *Culiseta dugesi* sequences.

Within the genus *Aedes*, there are three strong groups of monophyletic species with boostrap values of more than 80%: (1) the subgenus *Stegomyia*: *Ae. aegypti* (BOLD:AAA4210) and

Ae. albopictus (BOLD:AAA5870); (2) the subgenera *Aztecaedes*: *Ae. ramirezi* (BOLDADQ1468), *Howardina*: *Ae. allotecnon* (BOLDADM:1245), *Ae. guerrero* (BOLD:ADS4355), and *Ae. quadrivittatus* (BOLD:ADM1683); and *Ochlerotatus*: *Ae. angustivittatus* (BOLD:ACN9372), *Ae. euplocamus* (BOLD:ADL7578), *Ae. shannoni*, and *Ae. trivittatus* (BOLD:ADP6375); and (3) the subgenus *Protomacleaya*: *Ae. chionotum* (BOLD:ADV8040), *Ae. gabriel* (BOLD:ADM0866), *Ae. idanus* (BOLD:ADP8592), *Ae. kompi* (BOLD:ADL9968), and *Ae. vargasi* (BOLD:ADM0829). Within the genus *Culex*, the subgenera *Culex*: *Cx. coronator* (BOLD:AAN3636), *Cx. interrogator* (BOLD:ADL8796), *Cx. quinquefasciatus* (BOLD:AAA4751) *Cx. stigmatosoma*; and *Phenacomyia*: *Cx. corniger* (BOLD:AAF1735) formed a monophyletic group (>0.75). Finally, all specimens identified as *Cs. dugesi* (BOLD:ADM1783) grouped closely with *Cs. particeps*, although both taxa were well separated with high bootstrap support for posterior probability (Figure 3). The BINS corresponding to *Cs. dugesi* and *Cs. particeps* formed sister clades sharing a monophyletic origin (>0.75). Although *Cs. dugesi* did not segregate and display low support values, this group is well differentiated from the *Cs. particeps* clade.

The specimens placed in the clade of the Scapularis group of *Aedes* (*Ochlerotatus*) were *Ae. angustivittatus*, *Ae. euplocamus*, *Ae. shannoni* and *Ae. trivittatus*. The voucher specimen of *Ae. euplocamus* was identified using the Arnell [64] key, and it fits with the *Ae. condolescens* Dyar and Knab description; however, since the distribution of *Ae. condolescens* is restricted to the Antillean region, from the Greater Antilles to the Lucayan Archipelago, and it has also been reported in Florida, USA, the presence of this species in Mexico is doubtful. For this reason, the identity of this taxon is kept under the name of *Ae. euplocamus* until we can obtain specimens for further morphological examination. Although the affinities of *Ae. shannoni* with other *Ochlerotatus* species are unknown, it is possible that a new species-subgroup within the Scapularis group, which contain species with rings of white scales on each segment of the hind leg, including the Mexican endemic species *Ae. amateuri* Ortega and Zavortink and *Ae. shannoni*, could be related to this group of species.

3.5. Re-Description of Culiseta dugesi Dyar and Knab

***Culiseta* (*Culiseta*) *dugesi* Dyar and Knab** type specimens: *Lectotype* assigned by Stone and Knight [65]: adult female without associated larval and pupal exuviae [USNMENT01935000, USNM, Waashington, DC, USA], Guanajuato, Mexico, 20 January 1905, col. A. Dugès (Figures 4 and 5). *Paratypes*: 4 adult female, without associated larval and pupal exuviae, same data as lectotype; unspecific number of adults collected from Mexico city, Mexico, 26 October 1900, col. S. Arara. Synonymized with *Cs. particps* by Stone [66].

Fourth-instar larvae. Head: Setae 4-C double, 5,6-C with fewer branches and usually somewhat longer than 5-C (Figure 6A). Saddle with coarse dorsoposterior aciculae. Thorax: Integument without spicules. Abdomen: Sometimes dark spots on dorsal segments. Segment VIII: Comb scales moderate in size, evenly fringed, with sub-equal spinules. Siphon with row of single spicules distal to pecten (Figure 6B).

Pupa. Unknown.

Female. Head: Occiput and vertex covered with narrow decumbent pale scales, inter-ocular setae dark and large, pedicel orange with few pale scales. Antenna about 0.50–0.75 fore-femur length. Clypeus bare, dark-brown. Maxillary palpus about 0.25 proboscis length, three-segmented, with some pale scales. Proboscis long as fore femur, sometimes slightly longer, with pale and dark scales intermixed. Thorax: Integument of scutum yellowish or light-brown, covered with yellow bristles and yellow narrow decumbent scales, achorstichal setae absent (Figure 7). Prescutellar area dark, with dark bristles. Scutellum trilobed, with patch of creamy scales on each lobe. Integument of mesokatepisternum and mesanepimeron yellowish, mostly covered with large patch of white scales. Wing: Approximately 1.50–1.80 mm, scales on veins all narrow and dark, cross veins with scales. Halter: Dark-brown scaled. All femora speckled with pale scales, sub-apical spot of white scales, tibia intermixed with dark and pale scales, tarsomeres of each leg with basal rings of white scales on each segment. Abdomen: All tergum covered with dark scales but with some white scales, conspicuous basal bands of white scales on all terga, sternum with white scales (Figure 8).

Figure 4. *Culiseta dugesi* lectotype (**A**) General aspect of adult female; (**B**) Dorsal view of head and occiput; (**C**) Lateral view of proboscis; (**D**) Original labels.

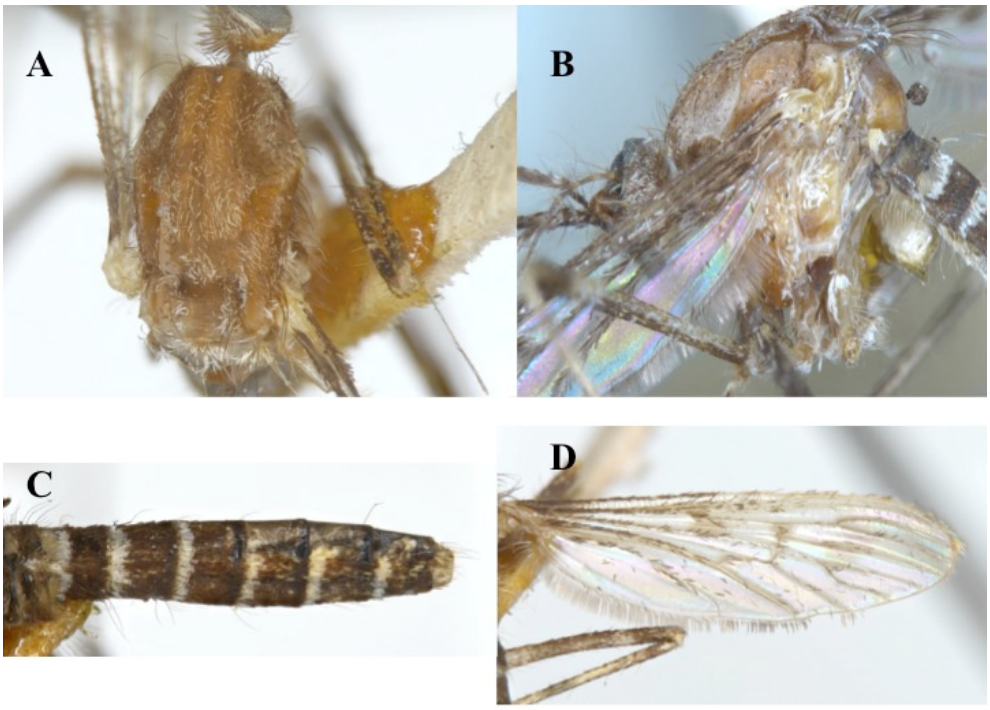

Figure 5. *Culiseta dugesi* lectotype (**A**) Dorsal view of scutum; (**B**) Lateral view of thorax; (**C**) Dorsal view of abdomen; (**D**) Wing.

Figure 6. Immature stages of *Culiseta dugesi* (**A**) Arrow point to the Setae 4-C double; (**B**) siphon with row of single spicules distal to pecten.

Figure 7. Lateral view of adult female of *Culiseta dugesi*. Arrows point to the integument of scutum, mesokatepisternum and mesanepimeton which is yellowish and light-brown.

Figure 8. Dorsal view of adult female of *Culiseta dugesi*. Arrow point to the abdomen with tergum covered with dark scales and some white scales, conspicuous basal bands of white scales on all segments.

Male. Unknown.

Systematics. Females of *Cs. dugesi* are distinguished from all five species within the genus *Culiseta* in Mexico, *Cs. melanura* (Coquillett), *Cs. impatiens* (Walker), *Cs. incidens* (Thompson), *Cs. inornata* (Williston) and *Cs. particeps*, by having distinct basal pale bands on their abdominal terga, hindtarsomeres with broad pale bands, cross veins of the wing with scales, femora with narrow sub-apical pale-scaled bands, and the integument of scutum and mesokatepisetrnum being yellowish colored. Larvae are distinguished by a siphon with spiniforms along the midventral line, antenna shorter than the head, a saddle with coarse dorsoposterior aciculae, and the setae 4-C double, rarely single.

Bionomics. *Culiseta dugesi* have been collected in four Mexican states: three of these were Guanajuato (lectotype), Mexico City, and Huitzilac municipality, Morelos, where adult females were collected approaching humans diurnally; associated species in this site were *Ae. kompi* and *Cx.* sp., while immature stages were collected from an abandoned plastic bucket filled with rain water, in conditions of total shade and with some leaves at the bottom; associated species were *Cx. arizonensis* and *Cs. particeps*; some adults of *Cs. dugesi* were collected resting inside the same bucket. The fourth state was Querétaro, where this species was previously reported as *Culiseta* n. sp. in association with *Cx. thriambus* and *Tx. moctezuma* [34]. Immature stages of *Cs. dugesi* could occur in natural containers such as tree holes and/or volcanic rock holes at ground level. The medical importance of *Cs. dugesi* is unknown.

Distribution. *Culiseta dugesi* has been collected in these locations: the mountains and plains of the north of Guanajuato, Guanajuato State, belonging to the Central Plateau; the northern region of the state of Morelos in Huitzilac county and Mexico City, belonging to the sub-region Lakes and Volcanoes of Anahuac, of the Neo-volcanic Axis (Figure 9); and in Pinal de Amoles county, Querétaro, belonging to the Huaesteco Carso of the Sierra Madre Oriental. *Culiseta dugesi* could occur in the forested regions of the states between

Guanajuato and Querétaro such as Mexico State, Hidalgo, and northeastern Michoacán; all these states share physiographical conditions such as mountain forest regions above 2000 m of elevation, within the regions of Central Plateau, Neo-volcanic Axis and the Sierra Madre Oriental.

Figure 9. Collection site of *Culiseta dugesi* showing the conserved vegetation of temperate forest. The site is near the "Lagoons of Zempoala" National Park in Huitzilac, Morelos, Mexico.

Etymology. This species was dedicated to Alfredo Dugès (1826–1910), Mexican zoologist and botanist of French origin.

3.6. Keys to Species of Larvae and Females of Culiseta in Mexico

LARVAE (Modified from Darsie and Ward [67]).

1. Siphon with row of 8–14 setae along mid-ventral line. . .. .*Cs. melanura*
Distr. Canada, Mexico, USA [43]; Distr. Mex.: Nuevo León [68].
-Siphon with setae otherwise distributed, no mid ventral row. 2
2(1). Setae 5,6-C similar in size and number of branches. . .. .*Cs. impatiens*
Distr. Canada, Mexico, USA [43]; Distr. Mex.: Nuevo León, Sonora [14].
-Setae 6-C with fewer branches and usually somewhat longer than 5-C. 3
3(2). Saddle with coarse dorsoposterior aciculae 4
-Saddle not aciculate dorsoposteriorly. 5
4(3). Setae 4-C branched, nearly equal in size to setae 5-6C. . .. .*Cs. particeps*
Distr. Costa Rica, El Salvador, Guatemala, Mexico, Panama, USA [43]; Distr. Mexico: Baja California, Baja California Sur, Chiapas, Coahuila, Durango, Guanajuato, Guerrero, Hidalgo, Mexico City Mexico, State, Michoacán, Morelos, Nuevo León, Oaxaca, Puebla, Sonora, Tamaulipas, Tlaxcala, Veracruz [31,56,60,69–71].
-Setae 4-C double, sometimes single. . .. .*Cs. dugesi*
Distr. Mexico; Distr. Mex: Guanajuato, Mexico City, Morelos, Querétaro.
5(3). Setae 1-X with branches equal to length of saddle or longer. . .. .*Cs. inornata*
Distr. Canada, Cuba, Mexico, USA [43]; Distr. Mex.: Aguascalientes, Baja California, Baja California Sur, Coahuila, Durango, Hidalgo, Mexico City, Mexico State, Nuevo León, Sonora, Tamaulipas, Tlaxcala [31,56,71].
-Setae 1-X with fine branches, shorter than saddle. . .. .*Cs. incidens*
Distr. Canada, Mexico, USA [43]; Distr. Mex.: Baja California, Baja California Sur, Coahuila, Mexico State, Sonora, Tlaxcala [31,35,56].

ADULT FEMALE (Modified from Darsie and Ward [67]).

1. Abdominal terga without basal pale bands. . . . .*Cs. melanura*
-Abdominal terga with distinct basal pale bands. 2
2(1). Hindtarsomeres with pale scale bands on some segments. 3
-Hindtarsomeres unbanded. 5
3(2). Hindtarsomeres with narrow pale bands, covering less than 0.1 of hind tarsomere 2; cross veins of wing without scales. . . . .*Cs. incidens*
Hindtarsomeres with broad pale bands, covering 0.25–0.33 of hind tarsomere 2; cross veins of wing with scales. 4
4(3). Integument of scutum and mesokatepisternum dark-brown. . . . .*Cs. particeps*
-Integument of scutum and mesokatepisternum yellowish or light-brown. . . . .*Cs. dugesi*
5(2). Wing with dark and pale scales intermixed on anterior veins; hind tarsomeres 1,2 with dark and pale scales. . . . .*Cs. inornata*
-Wing and hind tarsomeres all dark-scaled. . . . .*Cs. impatiens*

3.7. Medical Importance of the Mosquitoes of Morelos

Some of the species reported in Morelos are of medical and veterinary importance because they are vectors of pathogens causing diseases. In Table 4 the most important public health species that occur in Morelos are listed. Additionally, although the medical importance of some species is unknown, we include here the species that were collected biting humans diurnally with conditions of partial shade.

Table 4. Medical importance and pathogens of mosquito vector species collected in Morelos state, Mexico. Mal: Malaria. DI: *Dirofilaria immitis*. DENV: Dengue virus. ZIKV: Zika virus. CHIKV: Chikungunya virus. YF: Yellow fever virus. SLE: St. Louis encephalitis virus. WNV: West Nile virus. VEEV: Venezuelan equine encephalitis virus. EEEV: Eastern equine encephalitis virus. WEEV: Western equine encephalitis virus. LCV: La Crosse virus [43]. * Species collected by human biting/approaching humans.

Taxa	Mal	DI	DENV	ZIKV	CHKV	YF	SLE	WNV	VEEV	EEEV	WEEV	LCV
An. pseudopunctipennis *	✓											
Aedes vexans *		✓		✓			✓	✓	✓	✓	✓	
Ae. ramirezi *												
Ae. epactius *												
Ae. guerrero *												
Ae. quadrivittatus *												
Ae. angustivittatus *									✓			
Ae. shannoni *												
Ae. taeniorhynchus								✓	✓	✓		
Ae. trivittatus		✓						✓		✓	✓	✓
Ae. gabriel *												
Ae. kompi *												
Ae. aegypti *			✓	✓	✓	✓		✓	✓	✓		✓
Ae. albopictus *		✓	✓	✓	✓	✓		✓	✓	✓	✓	✓
Haemagogus equinus						✓						
Hg. mesodentatus *					✓	✓		✓	✓			
Ps. columbiae								✓	✓			
Ps. ferox *								✓	✓			
Culex nigripalpus							✓	✓	✓	✓		

Table 4. *Cont.*

Taxa	Mal	DI	DENV	ZIKV	CHKV	YF	SLE	WNV	VEEV	EEEV	WEEV	LCV
Cx. quinquefasciatus					✓		✓	✓	✓	✓		
Cx. restuans							✓	✓		✓		
Cx. salinarius							✓	✓		✓		
Cx. tarsalis								✓	✓		✓	
Cx. erraticus								✓				
Cs. dugesi *												

4. Discussion and Conclusions

4.1. Mosquito Species-Groups Reaching Their Distributional Limits in Morelos

Based on our collection records and the known distributions of the mosquito species collected in Morelos, two groups of species are recognized. Some species of each group have similar geographical distributions, reaching their northern or southern distributional limits in the state.

4.1.1. Group 1

Species that occur in mountain regions in south-central Mexico and reach their southernmost distributional limit in Morelos include *Ae. gabriel* and *Cx. apicalis*. Immature stages of *Ae. gabriel* commonly develop in tree holes, although it has been found in volcanic rock holes. *Ae. gabriel* is a Mexican endemic species that has been reported in the states of Morelos, Jalisco, Zacatecas [59] and Hidalgo [36]. *Culex aplicalis* is included in this group because it occurs in the Nearctic region, extending from western USA and southern Texas [67] into Mexico throughout the sub-region of Lakes and Volcanoes of Anahuac in Morelos, where it reaches its southernmost distributional limit.

4.1.2. Group 2

Mexican endemic species that occur in mountain regions in the central–southern area of the country and reach their northernmost distributional limit in Morelos include *Ae. chionotum* and *Cx. sandrae*. Immature stages of *Ae. chionotum* develop in tree holes, and it has been reported in the states of Morelos and Oaxaca [58], while immature stages of *Cx. sandrae* develop in bromeliad axils and it has been reported in the states of Guerrero and Oaxaca [72]. Also included in this group are species that occur in the Neotropical region that extends into Mexico, and in Morelos they reach their northernmost distributional limit, including *An. parapunctipennis*, *Hg. anastasionis*, *Ps. lineata* and *Cx. limacifer*. Except for *Hg. anastasionis*, which develops in tree holes, all those species develop in ground pools. All of the pools are commonly full with summer rain, but some aquatic habitats could be permanent water bodies such as lakes and swamps.

4.2. Species Not Collected during Our Collection Trips

The 12 species of this group were not collected in the present study, but records were obtained from literature reports including those from Vargas and Martínez-Palacios [54] of *Anopheles parapunctipennis*. This species has been reported in some states of southeastern Mexico (Chiapas, Oaxaca, and Veracruz) and Guatemala, but has also been reported in central Mexico in Nearctic environments in the states of Hidalgo [73], Puebla and Veracruz [74]. Although *An. parapunctipennis* is found at elevations above 2500 m, in Morelos its presence is uncommon and it is considered a rare species.

Vargas [55] encountered *Culex salinarius*, *Cx. conspirator*, *Cx. erraticus*, *Cx. iolambdis*, *Cx. limacifer*, *Cx. apicalis*, *Ur. coatzacoalcos*, and *Ur. sapphirina*. All those species develop in temporary ponds of rainwater at ground level with emerging aquatic vegetation. This type of aquatic habitat was not found frequently during our field trips. *Culex salinarius* is a common species that has been frequently reported in coastal regions of various states of Mexico. In central states, far from the coastal regions, this species is uncommon and can be confused in its larval stage with *Cx. nigripalpus* and/or *Cx. erythrothorax*. *Culex conspirator*, *Cx. erraticus*, *Cx. iolambdis* and *Cx. limacifer* are species that are common in tropical coastal regions; these could be found in temporary pools and ponds in southern Morelos, bordering Guerrero state, where environmental conditions are favorable to their development. In Mexico, species of the subgenus *Neoculex* can be commonly found in natural and artificial containers in temperate regions, particularly in the north and middle of Mexico, where the environmental conditions are related to the Nearctic region. *Culex aplicalis* could reach its southernmost distributional rank in Morelos. Although *Ur. coatzacoalcos* was not found during our collections, this species must be common during the rainy season in the southern part of Morelos, while *Ur. sapphirina* is one of the most common species in the genus *Uranotaenia* in the Nearctic region of Mexico. This species has been reported in many states of Mexico, but, however, this was also not found during our collections.

Díaz-Nájera and Vargas [56] reported finding *Aedes sexlineatus*, *Ae. taeniorhynchus*, and *Psorophora columbiae*. In Mexico, six species within the subgenus *Howardina* of *Aedes* are found, of which four are present in Morelos. The immature stages of all Mexican species of *Howardina* typically develop in epiphytic bromeliad axils, which grow on woody trees in oak forest regions. Since species of *Howardina* were reported for the first time in Morelos, large areas of oak forest have been deforested in Morelos, especially in the north and around the capital city of Cuernavaca, causing a conversion of the habitat decreasing the population of epiphytic bromeliads that grew naturally in this region. Possibly *Ae. sexlineatus* has decreased its population due to deforestation. *Aedes taeniorhynchus* and *Ps. columbiae* are common species in the coastal regions of Mexico; those species are problematic in touristic areas because of their high numbers and persistence in biting humans. In Morelos, those species were not found and they are possibly uncommon species.

Ibáñez-Bernal and Martínez-Campos [60] found *Cx. bidens*. This tropical species has been previously reported from Mexico to Argentina. In Mexico this species has been reported in different states where apparently it seems common in tropical regions of Mexico. However, it was not found in our collections.

4.3. Species from Adjacent Regions That May Occur in Morelos

Numerous species of mosquitoes that have not yet been reported from Morelos occur in the adjacent states of Mexico City and/or Mexico State and can logically be expected to occur within Morelos. Included among these 19 species are *Anopheles aztecus* Hoffmann, *An. franciscanus* McCracken, *An. punctipennis* (Say), *Aedes lorraineae* Berlin, *Ae. muelleri* Dyar, *Ae. scapularis* (Rondani), *Ae. zoosophus* Dyar and Knab, *Psorophora cyanescens* (Coquillett), *Culex erythrothorax* Dyar, *Cx. pinarocampa* (Dyar and Knab), *Cx. pseudostigmatosoma* Strickman, *Cx. rejector* Dyar and Knab, *Culiseta incidens*, *Cs. inornata*, *Limatus durhamii* (Theobald), *Wyeomyia mitchellii* (Theobald), *Uranotaenia syntheta* Dyar and Shannon, *Ur. geometrica* Theobald and *Ur. lowii* Theobald [32,33,35].

4.4. Species Names Removed from Morelos

Some species reported in Morelos in early publications are not included in this study because their names have changed since they were originally reported. The names removed from the list of Morelos fauna and the reasons for their removal are explained here. Reasons for the removal of the following: *Cx. fatigans* Wiedemann reported by Martini [52]; *Ae. terrens* (Walker), and *Cx. virgultus* Theobald reported by Vargas [55]; *Ae. atropalpus* (Coquillett); and *Cx. peus* Speiser reported by Díaz-Nájera and Vargas [56], are explained in a previous publication on the mosquitoes of Tamaulipas by Ortega-Morales et al. [30]. Reasons for the removal of *Tx. theobaldi* (Dyar and Knab) reported by Villegas-Trejo et al. [61] are given a previous publication on the mosquitoes of Nuevo León by Ortega-Morales et al. [38].

Aedes triseriatus (Say) and *Cs. maccrackenae* Dyar and Knab were previously reported in Morelos by Vargas [55]. However, no species of the Triseriatus group of *Aedes* has been reported as far south as Morelos, with the only exception being *Ae. zoosophus* Dyar and Knab which has been previously reported in Guerrero [75]. Records of *Ae. triseriatus* in Morelos were possibly misidentified with species within the Kompi and/or Terrens groups. *Culiseta maccrackenae* was reduced to synonymy with *Cs. particeps* by Stone [66].

4.5. Mosquito Diversity in Morelos and Mexico

With the addition of the new mosquito records and the new species reported in Morelos, there are currently 58 species known in the state. Morelos ranks sixth with the greatest diversity of species of the eleven Mexican states that have been systematically inventoried for mosquito species. With the addition of *Culiseta dugesi* to the list of mosquito species in Mexico City and Mexico, there are currently 29 species and 248 known species, respectively.

4.6. DNA Barcoding

The DNA barcodes sequences of specimens we analyzed in this study grouped together in the same clade, although we detected species with two different BINs, e.g., *Cs. particeps* and *Cx. stigmatosoma*, which might be indicative of cryptic diversity. This agrees with results obtained by [4,34,35] during integrative studies to delineate the mosquito fauna in Mexico as well in other insect groups. The specimens we here identified as *Cs. dugesi* show an interspecific genetic divergence of 3.77% in comparison with *Cs. participes* and are separated with strong support values from those identified as *Cs. particeps*. This supports our hypothesis that they represent a different species. The latter finding is also supported by the different morphological traits found in the adult female general coloration and larval stage.

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