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Barcoding of mosquito species considered potential vectors of *Yellow fever virus* in epidemiological risk areas of northeastern Argentina

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Código de barras de especies de mosquitos consideradas potenciales vectores del virus de la fiebre amarilla en zonas de riesgo epidemiológico del noreste argentino

RESUMEN. El gen mitocondrial Citocromo C Oxidasa Subunidad I, también llamado ADN barcode o códigos de barra, constituye una herramienta útil que complementa la identificación morfológica de las especies. Dado que las secuencias de nucleótidos de Culicidae de Argentina son limitadas, resulta difícil realizar comparaciones con datos provenientes de otros países publicados en las bases de datos debido a inconsistencias o grandes distancias genéticas que suelen observarse. En este trabajo, cinco especies de mosquitos sospechados de ser vectores del virus de la fiebre amarilla fueron identificados por su morfología y posteriormente confirmados por ADN barcoding y registrados en el GenBank. Las secuencias COI de *Haemagogus leucocelaenus* (Dyar & Shannon) son las primeras obtenidas a partir de ejemplares colectados en la Argentina. Este estudio proporciona nuevas secuencias de la fauna de mosquitos de la región, que pueden ser utilizadas como un recurso adicional en la identificación taxonómica de los culícidos.

PALABRAS CLAVE. Código de barras. Identificación. Mosquitos. Nordeste de Argentina. Vectores.

ABSTRACT. The mitochondrial Cytochrome C Oxidase Subunit I (COI) gene, also called DNA barcoding, is a useful tool that complements morphological identification of species. Since nucleotide sequences from Culicidae of Argentina are limited, it is difficult to make comparisons with data from other countries present in databases due to inconsistencies or large genetic distances that are usually observed. In this work, five species of mosquitoes suspected to be vectors of *Yellow fever virus* were morphologically identified, confirmed by DNA barcoding and registered to the GenBank. *Haemagogus leucocelaenus* (Dyar & Shannon) COI sequences are the first obtained from specimens collected in Argentina. This study provides new sequences of the mosquito fauna in the region, which can be used as an additional resource in the taxonomic identification of Culicidae.

KEYWORDS. Barcode sequence. Identification. Mosquitoes. Northeastern Argentina. Vectors.

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Since humans began studying mosquitoes and their medical importance, there have been difficulties in morphological species diagnosis due to the limited number of experts dedicated to this field of research (Díaz-Nieto et al., 2016). In addition, for morphological identification the insects must be in almost perfect conditions and for this reason, the identity often ends up being unknown. In the case of adult specimens, the females of some species are almost morphologically identical to each other, making it necessary to observe the male genitalia to carry out the taxonomic identification, but in epidemiological studies the captured specimens are in general females.

The analysis of DNA sequences has been used as a complement to morphological identification (Díaz-Nieto et al., 2016). The most useful sequence is a fragment of mitochondrial Cytochrome C Oxidase Subunit I (COI) gene, also called DNA barcoding (Hebert et al., 2003). Unlike morphological diagnosis, DNA barcoding could be used in the instances where mosquito specimens are damaged, when the stage or larval instar is not appropriate for been identified using morphological keys. This methodology has limitations because the COI sequence of each species must be submitted in databases to make comparisons and sometimes the single use of this marker is not enough for successfully distinguishing very close related species, as occurs in the genus *Culex* Linneaus where the COI barcode does not contain sufficient information to distinguish species inside species complex of subgenus *Culex* (Laurito et al., 2013). In these cases, alternatives for molecular identification have been developed, such as PCR-based assays that use polymorphisms for the identification of members of *Cx. pipiens* L. complex (Smith & Fonseca, 2004). Moreover, genetic distance between species increases with the geographic distance (Bergsten et al., 2012), and according to Laurito et al. (2022) the disparity between morphology and barcode-based identifications could be explained by synonymy, species complexes occurrence, hybridization or incomplete lineage sorting. Likewise, phenotypic plasticity of species could result in misidentification made by morphological characters before the submission of sequences in databases.

Despite its limitations, DNA barcoding is widely used and the number of public sequences is increasing. Currently, Bold System (www.boldsystems.org) has 75,786 public records belonging to 1,516 species of Culicidae, where Brazil is in the fifth place in number of records with 2,616, and GenBank has 2,361,235 records for Culicidae.

Argentina has more than 240 recorded species of Culicidae (Stein et al., 2018; Campos et al., 2023) and fewer than 60 species of them have been linked to sequences in the databases. Genbank (www.ncbi.nlm.nih.gov/genbank/) only have 684 sequences from Argentina, with 413 COI sequences. Bold System, instead, has 467 public sequences from Argentina and only 368 have species name, among them are the records corresponding to Mosquitoes of Argentina (MOSA) project (Laurito et al., 2022), with 190 available records of *Culex*, *Psorophora* Robineau-Desoidy, *Anopheles* Meigen, *Aedes* Meigen, *Mansonia* Blanchard, *Uranotaenia* Lynch Arribálzaga, *Coquillettidia* Dyar, *Aedeomyia* Theobald & *Haemagogus* Williston genera, also provides information about the collection as well as photographs of diagnostic characters.

Although significant contributions have been made on the topic (Díaz-Nieto et al., 2013; Laurito et al., 2013, 2017, 2022), there are still gaps in mosquito research with a molecular perspective in certain areas of Argentina. Because of this, the sequence comparisons are mainly based on data from Brazil as both countries share the distribution of many mosquito species. Furthermore, it is imperative to acquire a greater number of local sequences to enhance the morphological identification of species from Argentina.

Our study enriches the molecular reference library of the mosquito fauna in Argentina because we submitted to a public database a total of 12 COI sequences of *Haemagogus leucocelaenus* (Dyar & Shannon), *Ochlerotatus scapularis* (Rondani), *Ochlerotatus serratus* (Theobald), *Psorophora ferox* (von Humboldt) and *Sabethes albiprivus* Theobald, species considered potential vectors of *Yellow fever virus* (YFV) (Cano et al., 2022).

The entomological survey was carried out between March and April 2021 in the northeast of Corrientes Province and southeast of Misiones Province, both epidemiological risk areas in northeastern Argentina. The sampling sites were selected based on previous knowledge about presence of social groups of black-and-gold howler monkeys (*Alouatta caraya* (Humboldt)) considered yellow fever sentinels due to their high susceptibility to YFV (Holzmann et al., 2010; Kowalewski et al., 2011). Sites and GPS coordinates were: Puerto Azara, next to Chimiray stream in Misiones Province (28°7'39.37"S, 55°38'44.95"W), and Gobernador Virasoro (Las Marías establishment) (28°6'39.05"S, 56°3'16.58"W); San Carlos (27°44′31″S, 55°53′41.18″W); Colonia Liebig (27°53′35.32″S, 55°52′58.62″W); Garabí (Paraje Las Chispas) (28°12'37.88"S, 55°49'11.84"W); Garruchos (28°10′37.04″S, 55°38′31.13″W) in Corrientes Province.

The capture of adult mosquitoes was performed with manual vacuums and nets at ground level. In the laboratory (LaCVEIS, Barceló Foundation), the specimens were initially stored in pools in liquid nitrogen for transportation to CENDIE, Buenos Aires. At CENDIE, the specimens underwent morphological identification using dichotomous keys (Lane, 1953; Darsie, 1985) on a frozen surface. They were then grouped into pools and stored in a freezer at -70 °C. The best-preserved individuals from each species were selected, and a single leg was removed for DNA extraction. Voucher specimens were subsequently preserved in entomological boxes within the

Diptera collection at the La Plata Museum (*Hg. leucocelaenus*: MLPDipC10002-10004; *Oc. scapularis*: MLPDipC10009-10012; *Oc. serratus*: MLPDipC10008; *Ps. ferox*: MLPDipC10005-10007; *Sa. albiprivus*: MLPDipC10013).

Genomic DNA was isolated and purified with Puro Genomic DNA Kit of PB-L (Productos Bio-lógicos, Argentina) and Wizard Genomic DNA Purification Kit of Promega, according to the instructions of the manufacturer.

For the amplification of a fragment of COI gene we used the primers C1-J-1718 and C1-N-2191 (Simon et al., 1994), LCO1490 and HCO2198 (Folmer et al., 1994), and combinations of them. Optimised conditions for PCR amplification and sequencing methods for each species analysed are shown in Table I. In Sanger method, the sequences were generated in both directions and edited manually using the BioEdit sequence alignment Editor version 7.2.5 (Hall, 1999), finally the consensus sequence was generated using Clustal Omega software (Sievers & Higgins, 2018). For Ilumina sequencing we used the computational package wa 0.7.17-r1188, ivar 1.3 (Grubaugh et al., 2019) as assembly method. We compared the sequences obtained with the NCBI NR database (non-redundant) using the BLAST tool and a neighbour-joining (NJ) tree of K2P distances (Kimura 1980; Saitou & Nei, 1987) was created for a graphic visualisation of clustering pattern with the most similar COI sequences of these species available in GenBank. The statistical support was estimated using bootstrap support values (BSV) (1000 replicates). Likewise, we calculated the values of pairwise nucleotide sequence divergences and estimated the intraspecific and interspecific distances using K2P distance (Kimura, 1980) implemented in MEGA v5 (Tamura et al., 2011).

We collected 498 mosquitoes: *Hg. leucocelaenus* (n= 107), *Oc. scapularis* (n= 226), *Oc. serratus* (n= 5), *Ps. ferox* (n= 36) and *Sa. albiprivus* (n= 124). One to four sequences of each species were obtained and 12 sequences were submitted to the GenBank with the specimen identification and field data (Table I). The length ranged between 461-467 bp.

Table I. Details of species, Accession Number, PCR conditions, primers and sequencing technology.

Species, accession number and sampling sites	PCR type and cycle conditions	Primers	Sequencing technology
Haemagogus leucocelaenus OR420020 (San Carlos) OR420670 (Garruchos) OR421565 (Puerto Azara)	PCR conventional : 2 cycles 94º---- 3 min 94º ---- 30 sec **40º** ---- 40 sec 72º ---- 40 sec — 6 cycles 94º ---- 30 sec **47º** ---- 40 sec 72º ---- 40 sec 72º ----- 3 min — 45 cycles	C1-J-1718 C1-N-2191	Sanger method
Ochlerotatus scapularis OR469301 (Garabí) OR469302 (Col. Liebig) OR469303 (Puerto Azara) OR469304 (Las Marías)	PCR conventional (*) 94º---------- 3 min 94º --------- 30 sec **45º-47º**---- 40 sec 72º --------- 40 sec 72º --------- 3 min — 45 cycles	C1-J-1718 C1-N-2191	Illumina (new generation sequencing)
Ochlerotatus serratus OR447417 (Garabí)	PCR conventional(*): Tm **47ºC**	C1-J-1718 C1-N-2191	Illumina (new generation sequencing)
Psorophora ferox OR441317 (Garabí) OR441318 (San Carlos) OR441319 (Garruchos)			
Sabethes albiprivus OR412359 (Garabí)	Nested PCR 1º- 94º---- 5 min 94º ---- 45 sec **40º** ---- 30 sec 72º ---- 1 min 72º ----- 3 min — 35 cycles	LCO1490 C1-N-2191	Sanger method
	2º- 94º---- 1 min 94º ---- 15 sec **45º** ---- 10 sec 72º ---- 35 sec 72º ----- 3 min — 35 cycles	C1-J-1718 HCO2198	

Of the 54 records associated with *Hg. leucocelaenus* in GenBank, only 13 correspond to specific sequences and none from Argentina. Thus, we submitted the first public sequences from Argentinean specimens (OR421565, OR420670, OR420020). The comparison revealed that the most genetically similar were MG242532 from Neotropical Region (Soghigian et al., 2017) and MT987602 from Trinidad and Tobago, with 98.8% and 98.3% of identity, respectively. This is also corroborated in the NJ tree (Fig. 1), where our sequences are grouped together and did not group with any of the others analysed sequences. The K2P intraspecific distance in sequences of *Hg. leucocelaenus* ranged from 0-2.2 (97.8%) (Table II).

Genbank has 148 COI sequences identified as *Oc. scapularis* from different countries, but only two (MW363437 and MW363438) are from Argentina. We obtained four sequences (OR469301 to OR469304) from different sampling sites, in NJ tree (Fig. 1) our sequence OR469303 was clustered with MW363438 and they shared 99% of homology, as with MW363437 and OM630623 (Table II). The mean intraspecific K2P distance was of 99.03%, where the minimum was 0.002 (99.8%) and maximum were 0.023 (97.7%) (Table II). It was interesting to discover that the sequence OM630623 from French Guiana was genetically most similar to the sequences from Argentina with genetic distances ranging between 0 to 0.015 (Table II).

From 49 records in GenBank, only three sequences are from Argentina (MW363439 to MW363441) and were submitted as *Ochlerotatus serratus* sp. group, but in BOLD these sequences are identified as *Oc. hastatus*. The identity percentage between these and our sequence (OR447418) was relatively low with values ranging from 96.2 - 98%. In the NJ tree (Fig. 1), our sequence was grouped with the sequence MN997493 from Colombia and showed an intraspecific K2P distance of 2% between them, being the lowest value observed for this species.

For *Ps. ferox*, 75 sequences are registered in GenBank and only four from Argentina (MW363459, MW363458, GU992945 and GU992946). Our sequences OR441317 and OR441318 are genetically most similar to MN997518 from Colombia with 99.3% of identity (Table II), nevertheless in NJ tree, OR441317 was established as independent branch (Fig. 1). Finally, our sequence OR441319 shared 100 % similarity with MW363458 from Argentina and are grouped together in the tree of distances (Fig. 1).

GenBank has only five sequences of *Sa. albiprivus* (GU992947 to GU992951) from Argentina. Our sequence OR412359 share 100% similarity with GU992947, GU992950, GU992951 while 99.3% and 99.8% with GU992949 and GU992948, respectively (Table II). This is reflected in the NJ tree (Fig. 1).

The NJ tree showed that all mosquitoes are grouped according to the taxonomic identification made based on morphological characters for *Hg. leucocelaenus, Oc. scapularis, Oc. serratus, Ps. ferox* and *Sa. albiprivus* (Fig. 1). Additionally, each species was supported by 100% BSV, even when it included sequences generated from representatives of Brazil, Colombia, French Guiana, Dominican Republic and Trinidad and Tobago, except for *Oc. serratus* whose support was 99%.

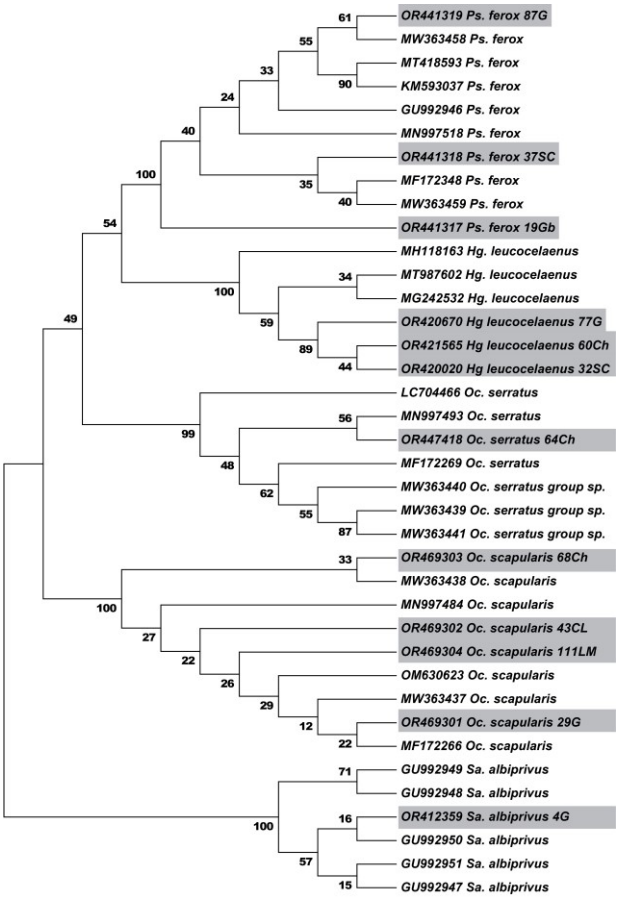


Fig. 1. Neighbor-joining tree based on Cytochrome C Oxidase Subunit I (COI) for sequences of *Psorophora ferox, Haemagogus leucocelaenus, Sabethes albiprivus, Ochlerotatus scapularis* and *Ochlerotatus serratus.* Bootstrap values (1,000 replicates) are given on the branches. In bold the sequences obtained in this work.

The pairwise distance analysis showed low values (0-2.3 %) of intraspecific genetic divergence in *Hg. leucocelaenus, Oc. scapularis, Ps. ferox* and *Sa. albiprivus*. The K2P intraspecific distance of *Oc. serratus* ranged from 2-3.8 %, because the comparison was made with *Oc. hastatus*, another species of *Ochlerotatus serratus* sp. group (MW363439-MW363441) or sequences from other countries.

Nucleotide sequences for mosquitoes from Argentina are currently limited. We consider it imperative to enhance the repository of sequences from our country as an additional resource for taxonomic identification of Culicidae, particularly focusing on medically significant species. This effort aims to complement morphology-based identification techniques in order to simplify taxonomic identification.

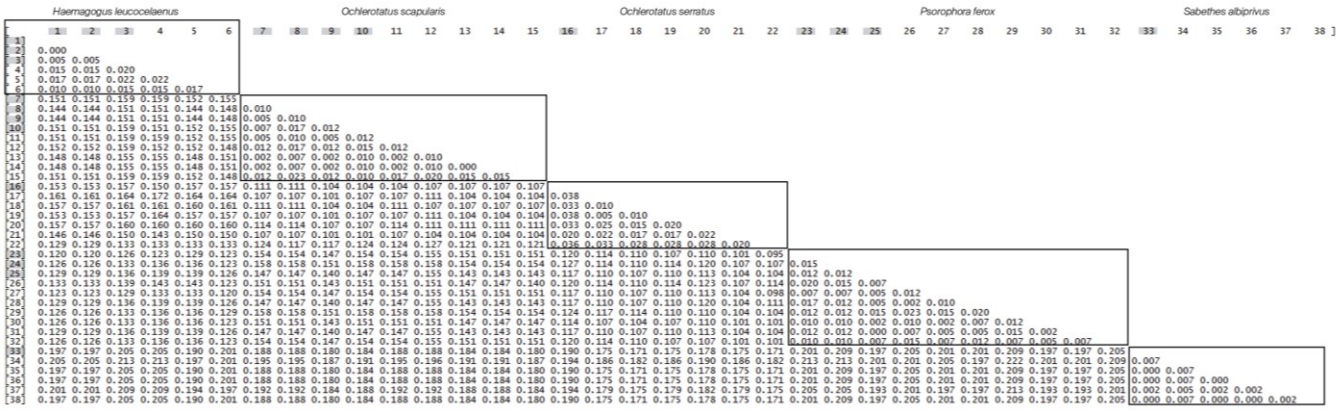


Table II. **Estimation of pairwise distance (K2P) of COI sequences.** *Haemagogus leucocelaenus* [1-**OR421565**; 2-**OR420670**; 3-**OR420020**; 4-MT987602; 5- MH118163; 6-MG242532]*, Ochlerotatus scapularis* [7- **OR469301**; 8-**OR469304**; 9-**OR469302**; 10-**OR469303**; 11-MF172266; 12-MN997484; 13-OM630623; 14- MW363437; 15-MW363438], *Ochlerotatus serratus* [16-**OR447418**; 17-MW363439; 18-MW363440; 19-MW363441; 20-MF172269; 21-MN997493; 22-LC704466] *Psorophora ferox* [23-**OR441317**; 24-**OR441318**; 25-**OR441319**; 26-MT418593; 27- MN997518; 28-KM593037 ; 29-MF172348; 30-GU992946; 31-MW363458; 32- MW363459]*, Sabethes albiprivus* [33-**OR412359**; 34-GU992949; 35-GU992951; 36- GU992950; 37-GU992948; 38-GU992947]. In bold the sequences obtained in this work.

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REFERENCES

Bergsten, J., Bilton, D.T., Fujisawa, T., Elliott, M., Monaghan, M.T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, N.G., Ribera, I., Nilsson, A.N., Barraclough, T.G., & Vogler, A.P. (2012) The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology*, **61(5)**, 851-869.

Campos, R.E., Laurito, M., & Muttis, E. (2023) Culicidae (Diptera) species from Argentina and Uruguay. https://biodar.unlp.edu.ar/culicidae (accessed December 28, 2023).

Cano, M.E., Marti, G.A., Alencar, J., Olsson, S., Silva, F., & Micieli, M.V. (2022) Categorization by score of mosquito species (Diptera, Culicidae) related to Yellow Fever epizootics in Argentina, *Journal of Medical Entomology*, **59(5)**, 1766-1777.

Darsie, R.F. (1985) Mosquitoes of Argentina. Part I. Keys for identification of adult females and fourth stage larvae in English and Spanish (Diptera, Culicidae). *Mosquito Systematics*, **17**, 153-253.

Díaz-Nieto, L.M., Maciá, A., Parisi, G., Farina, J.L., Vidal-Domínguez, M.E., Perotti, M.A., & Berón, C.M. (2013) Distribution of mosquitoes in the south east of Argentina and first report on the analysis based on 18S rDNA and COI sequences. *PLoS One*, **8(9)**, e75516.

Díaz-Nieto, L.M., Berrón, C.I., Maciá, A., & Berón, C.M. (2016) Taxonomía molecular de mosquitos. *Investigaciones sobre mosquitos de Argentina*. (ed. Berón, C.M., Campos, R.E., Gleiser, R.M., Díaz-Nieto, L.M., Salomón, O.D., & Schweigmann, N.), pp. 33-45. Universidad Nacional de Mar del Plata, Argentina.

Folmer, O., Black., M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3(5)**, 294-299.

Grubaugh, N.D., Gangavarapu, K., Quick, J., Matteson, N.L., De Jesus, J.G., Main, B.J., Tan, A.L., Paul, L.M., Brackney, D.E., Grewal, S., Gurfield, N., Van Rompay, K.K.A., Isern, S., Michael, S.F., Coffey, L.L., Loman, N.J., & Andersen, K.G. (2019) An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar. *Genome Biology*, **20(1)**, 8.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41(41)**, 95-98.

Hebert, P.D.N., Cywinska, A., Ball, S.L., & deWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings. Biological sciences / The Royal Society*, **270(1512)**, 313-321.

Holzmann, I., Agostini, I., Areta, J.I., Ferreyra, H., Beldomenico, P., & Di Bitetti, M.S. (2010) Impact of

yellow fever outbreaks on two howler monkey species (*Alouatta guariba clamitans* and *A. caraya*) in Misiones, Argentina. *American Journal of Primatology*, **72**, 475-480.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111-120.

Kowalewski, M.M., Salzer, J.S., Deutsch, J.C., Raño, M., Kuhlenschmidt, M.S., & Gillespie, T.R. (2011) Black and gold howler monkeys (*Alouatta caraya*) as sentinels of ecosystem health: patterns of zoonotic protozoa infection relative to degree of human-primate contact. *American Journal of Primatology*, **73(1)**,75-83.

Lane, J. (1953) *Neotropical Culicidae*. Vol. II. University of São Paulo, São Paulo.

Laurito, M., Oliveira, T., Almirón, W.R., & Sallum, M.A. (2013) COI barcode versus morphological identification of *Culex* (*Culex*) (Diptera, Culicidae) species: a case study using samples from Argentina and Brazil. *Memórias do Instituto Oswaldo Cruz*, **108(1)**,110-122.

Laurito, M., Ayala, A.M., Almirón, W.R., & Gardenal, C.N. (2017) Molecular identification of two *Culex* (*Culex*) species of the neotropical region (Diptera, Culicidae). *PLoS ONE*, **12(2)**, e0173052.

Laurito, M., Ayala, A.M., Arias-Builes, D.L., & Almirón, W.R. (2022) Improving the DNA Barcode library of mosquito species with new identifications and discoveries in North-Central Argentina. *Journal of Medical Entomology*, **59(1)**,173-183.

Saitou, N., & Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406-425.

Sievers, F., & Higgins, D.G. (2018) Clustal Omega for making accurate alignments of many protein sequences. *Protein Science*, **27(1)**, 135-145.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Lui, H., & Flook, P. (1994) Evolution, weighing and phylogenetic unity of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America,* **8,** 651-701.

Smith, J.L., & Fonseca, D.M. (2004) Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera, Culicidae). *American Journal of Tropical Medicine and Hygiene*, **70(4)**, 339-345.

Soghigian, J., Andreadis, T.G., & Livdahl, T.P. (2017) From ground pools to treeholes: convergent evolution of habitat and phenotype in *Aedes* mosquitoes. *BMC Evolutionary Biology*, **17(1)**, 262.

Stein, M., Alvarez, C.N., Alonso, A.C., Bangher, D.N., Willener, J.A., & Campos, R.E. (2018). New records of mosquitoes (Diptera, Culicidae) found in phytotelmata in northern Argentina. *Zootaxa*, **4399(1)**, 87-100.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28,** 2731-2739.