Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 –6131
Vol. 26(6): 751–764(2022)
www.ejabf.journals.ekb.eg


# Seasonal Abundance and Molecular Identification of Aquatic Larvae of *Culex pipiens* L. and *Culex antennatus* Becker in Fayoum Governorate, Egypt

**Ahmed Z.I. Shehata*, Mohamed A.M. El-Tabakh, Hassan O. Waheeb, Deyaa E.M. Emam, Mostafa M. Mokhtar**

Department of Zoology, Faculty of Science, Al-Azhar University, Nasr City, Cairo 11651, Egypt

*** Corresponding author: ahmed.ibrahem84@azhar.edu.eg**

**ARTICLE INFO**

**Article History:**
Received: Nov. 13, 2022
Accepted: Dec. 8, 2022
Online: Dec. 21, 2022

**Keywords:**
*Culex pipiens*,
*Culex antennatus*,
Mosquito,
Larvae

**ABSTRACT**

The present study was designed to investigate the seasonal abundance of *Culex pipiens* and *Culex antennatus* aquatic larvae in Fayoum Governorate, Egypt. The study was carried out in the En Nazla region, Ibshway during winter, spring, summer and autumn of 2021. In addition, the physico-chemical parameters of breeding water habitat were evaluated during different seasons, and relationships between measured parameters and mosquito abundance were addressed. PCR and sequencing confirmed the genetic identification of both *Culex* species. Generally, *C. pipiens* was more abundant than *C. antennatus* in the study area. Summer recorded the highest abundance for both *C. pipiens* and *C. antennatus* (about 683 and 400 larvae), superior to what has been recorded in other seasons with a significant margin ($P< 0.05$), while winter recorded the lowest abundance of both mosquito species. The highest correlation between seasons for *C. pipiens* calculated an abundance between winter and autumn (0.83), while the lowest was recorded between winter and summer (0.22). Data assessed revealed that, the TDS is the most reliable factor that mosquito abundance depends on, followed by the pH value for *C. pipiens* and temperature for *C. antennatus*. On the other hand, *C. pipiens* showed a clear relationship with species identified from Portugal and Kenya, with an evolutionary distance of approx. 0.001164. Moreover, *Cx antennatus* displayed a close relationship to a species identified from Madagascar and Kenya, with an evolutionary distance of approx. 0.00734, respectively.

## INTRODUCTION

*Culex pipiens* Linnaeus (Diptera: Culicidae) is an omnipresent mosquito originated in Africa and distributed through human activity to tropical and temperate climate zones worldwide **(Sayed *et al.*, 2018; El-Mehdawy *et al.*, 2021)**. It is a major vector of various pathogens causing some serious and deadly diseases to humans and animals, such as the West Nile (W.N.) encephalitis, Rift Valley Fever (RVF), St. Louis, Japanese, Venezuelan and eastern equine encephalitis **(Amraoui *et al.*, 2012; Abouzied, 2017; Kassem *et al.*, 2018; Baz *et al.*, 2022)**. For Egypt, *C. pipiens* is the primary vector of the nematode *Wuchereria bancrofti*, the causative agent of lymphatic filariasis, as well as RVF and W.N. viruses **(Elhawary *et al.*, 2021; El-Mehdawy *et al.*, 2022)**. Another important Culicine, *Culex antennatus* Becker, is also a primary vector of the viruses (RVFV), (WNV), Sindbis (SINV), Acado (ACDV) and Perinet (PERV) **(Harbach *et al.*, 1988; Fang *et al.*, 2022; WRBU, 2022)**.

In addition, *C. pipiens* and *C. antennatus* are pervasive mosquito species in most urban, suburban and rural areas of Egypt **(Harbach *et al.*, 1988)**. One of the best ways to control dangerous and epidemic diseases is vector control, and sometimes it is the only way to control diseases with no effective cure, such as the West Nile fever and Dengue fever **(Hassan *et al.*, 2014)**. Developing a good knowledge of vectors' biology, ecology, distribution, and bionomics are crucial for establishing effective control programs.

Historically, the identification of mosquito species depended on physical traits although these qualities may be difficult to understand without specific taxonomic knowledge, and the distinction between species is further hampered if similar characteristics are destroyed **(Verna & Munstermann, 2011)**. For these reasons, a clear, trustworthy, and user-friendly identification solution is in constant demand **(Laurito *et al.*, 2013)**. Recently, DNA barcoding has been reliable for identifying several species including mosquitoes **(Wang *et al.*, 2012)**. Furthermore, molecular assays are crucial to discriminate between *C. pipiens* and *C. antennatus* **(Shahhosseini *et al.*, 2019)**.

Based on the afore- mentioned points, the present study focused on providing an updated information about the seasonal abundance of both *C. pipiens* and *C. antennatus* in En Nazla region, Ibshway, Fayoum Governorate, Egypt and study the relationship between different physico-chemical parameters of breeding water and the abundance of both mosquito species which are responsible for previously mentioned diseases.

## MATERIALS AND METHODS

### 1. Study area and collection of mosquitos' larvae

Larvae of *Culex pipiens* and *Culex antennatus* were collected from four localities of En Nazla region (29°18'58.3" N, 30°39'08.8" E), Fayoum Governorate, Egypt by netting during winter, spring, summer and autumn 2021 (Fig. 1). Collected larvae were put in a white pan filled with clean water. For larval identification, ethanol (70%) was used to kill larvae. Larvae of both *C. pipiens* and *C. antennatus* were morphologically identified according to the key of **Harbach (1985)**. Identified larvae were isolated for molecular identification.

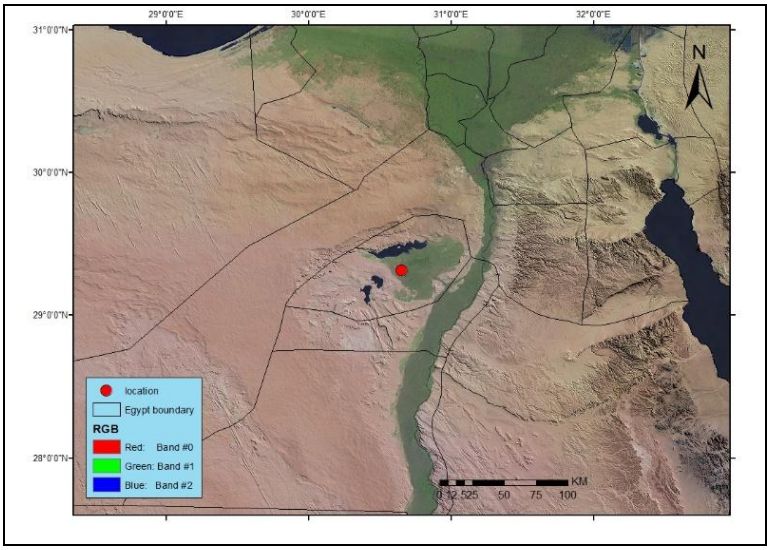


**Fig. 1.** The study area; En Nazla region, Ibshway, Fayoum Governorate, Egypt.

## 2. Physico-chemical parameters of breeding water

Water temperature and total dissolved solids were determined using ADwa (AD31) apparatus (Waterproof Conductivity-TDS-Temp Pocket Testers with the replaceable electrode). Potential hydrogen (pH) was determined using ADwa (AD11) apparatus (Waterproof pH-TEMP Pocket Tester with the replaceable electrode). Meanwhile, the dissolved oxygen (D.O.) was estimated using the method of **Labasque *et al.* (2004)**.

## *3.* Genetic identification of mosquito larvae

### *3.1. Extraction of DNA from mosquito larvae*

Using a scalpel under sterile conditions, the mosquito larvae were cut into small pieces and placed in 1.5 L Eppendorf tubes. The PureLink® Genomic DNA kits were used to extract the mosquito DNA (Invitrogen, Waltham, Massachusetts, USA). Briefly, each sample was mixed with tissue lysis buffer (between 180 and 250µL depending on the size of the larvae), treated with proteinase K (10µL for each 180µL of tissue lysis buffer), and incubated for 4 hours at 56°C. The supernatant was then transferred to a new tube in accordance with the manufacturer's (Invitrogen, Waltham, Massachusetts, USA) instructions. The lysate received 200µl of ethanol and 200µl of Lysis/Binding Buffer before being vortexed. The mixture was then put into a spin column and centrifuged for one minute at 10,000xg. Following two times of washings with wash buffers, DNA was eluted in 50µl of elution buffer and kept at -20°C until use.

### *3.2. Polymerase chain reaction (PCR)*

To identify mosquito species, specific primers amplifying the cytochrome oxidase C.O. subunit I (COI) of mosquito' mitochondrial DNA LCO1490:5′-GGTCAACAAATCATAAAGATATTGG-3′ as a forward primer and HCO2198:5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ as a reverse primer were used **(Folmer *et al.*, 1994)**. The PCR amplification was performed in a final reaction volume 2X (50 µl), containing 25µl of 2X master mix solution (*i*-Taq, *iNt*RON, Seongnam, Korea), 0.2µM (2µl) of each primer, 4µl of template DNA, and 0.2mg/ ml of BSA and 14.5µl of nuclease-free water. The thermal cycling program of ticks consisted of an initial denaturation at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 1min, annealing at 46°C for 1min and extension at 72°C for 1min. A final extension was carried out for 10min at 72°C. PCR amplicon was run on a 1% agarose gel, stained with ethidium bromide to check the quality and yield of the PCR product using the transilluminator (U.V. transilluminator, Spectroline, Westbury, USA).

### *3.3. Sequence analysis*

The PCR products were purified using a Macrogen reagent (Seoul, Korea). Single-strand DNA sequencing was performed, after which nucleotide sequences of mosquito larvae *COI* were aligned.

### *3.4. Bioinformatics*

The obtained sequences were assembled using Chromas Pro 1.5 beta (Technelysium Pty., Tewantin, QLD, Australia). The newly COI sequences *Culex pipiens* (accession number: MK052921.1) and *Culex antennatus* (accession number: OP714215.1) were compared to those available in GenBank, using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Muscle alignment was used to align the sequences using MEGA 11.0 software. Kimura's two-parameter as used to calculate

the sequence divergences (K2P) **(Kimura, 1980)**. To illustrate the patterns of species divergence, N.J. trees use the Tamura 3-parameter method **(Tamura, 1992)**. Bootstrapping was performed in MEGA 11.0 **(Kumar *et al*., 2004)** with 1000 replications. Visualization enhancement was done using ITOl software **(Letunic & Bork, 2021)**. The minimum spanning network for haplotype divergence was evaluated using inPHAP v.1.1, HapFlow v.1.1.2, and PopArt v.3.0.

**4. Statistics**

Data were analyzed using variance analysis (ANOVA) according to **Bailey (1981)**. Data were coded and entered using the statistical package SPSS V.22. In addition, data were statistically described in terms of mean, median, standard deviation and standard error for quantitative variables, in addition to frequency for categorical variables. A probability value (*P*- value) less than 0.05 was considered statistically significant. The correlation coefficient between parameters was done in all the surveyed stations during the study period using MINITAB V.14 computer program. Data visualization becomes available using R-studio V.4.1.3.

## RESULTS

**1. The abundance of *C. pipiens and C. antennatus* larvae**

As shown in Table (1) and Figs. (2, 3), a significant variation ($P< 0.05$) was detected between both investigated species in their calculated abundance throughout four seasons except for winter, where there was no significant variation ($P> 0.05$) in the abundance recorded for both species. While, summer showed the highest abundance for both *C. pipiens* and *C. antennatus* (about 683 and 400 larvae), superior to what was recorded in other seasons with a significant margin ($P< 0.05$). In the same manner, winter was significantly variant ($P<0.05$), compared to other seasons due to the low abundance recorded.

**Table 1.** Seasonal abundance of *C. pipiens* and *C. antennatus* larvae in En Nazla region, Ibshway, Fayoum Governorate, Egypt

| Mosquito Species | Winter | Spring | Summer | Autumn |
|---|---|---|---|---|
| ***Clex pipiens*** | 112±15 | 286±10 | 683±49 | 243±16 |
| ***Culex antennatus*** | 109±8 | 163±12 | 400±16 | 186±10 |

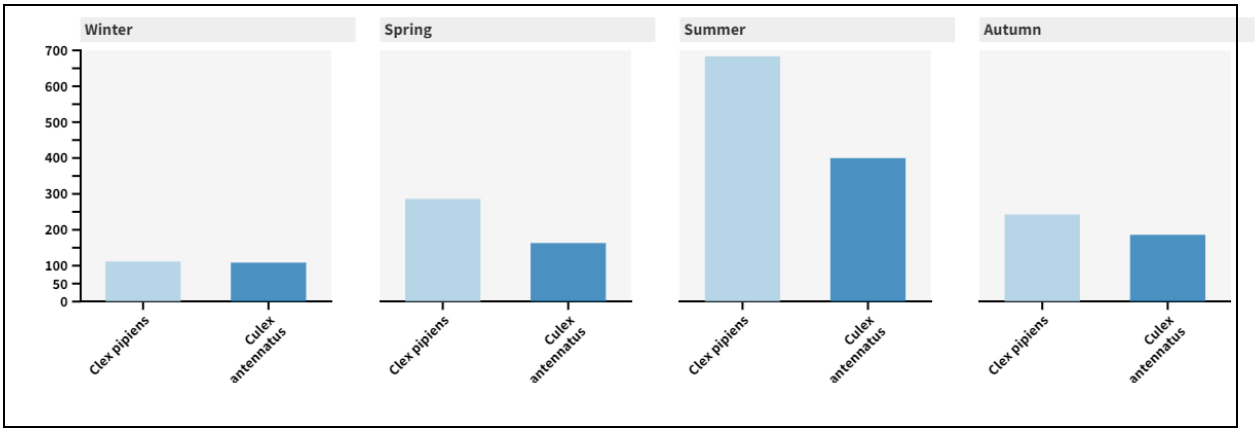


**Fig. 2.** Gradient column chart representing the average abundance of *Clex pipiens* and *Culex antennatus* larvae

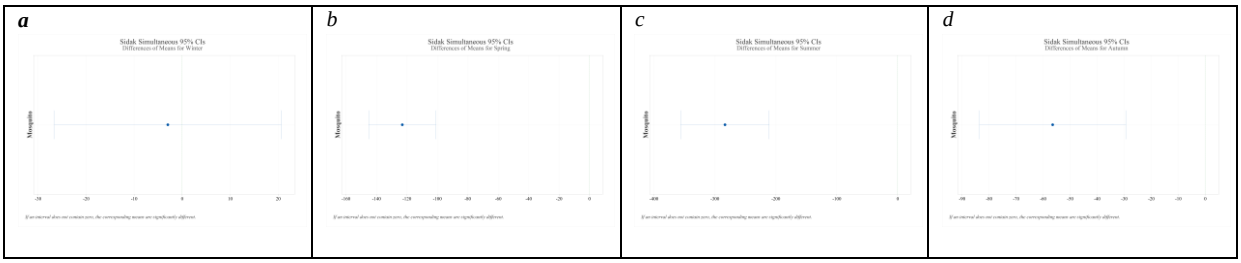


**Fig. 3.** Sidak simultaneous 95% C showing differences of the mean for (*a*) Winter, (*b*) Spring, (*c*) Summer, and (*d*) Autumn

In addition, the heatmap map represented a comparison abundance between both investigated species throughout the study. As the color grade turns red, the abundance increases; at the same time, the color grade gets blue as the abundance of *C. pipiens* and *C. antennatus* declines (Fig. 4).

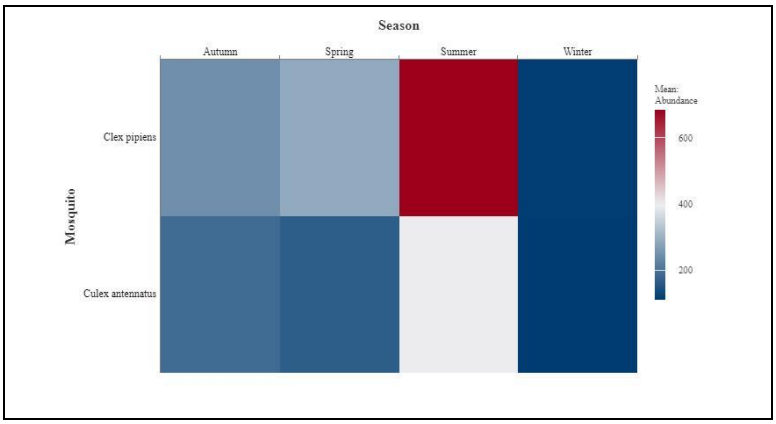


**Fig. 4.** Heatmap representing the abundance of seasons for both investigated species

In addition, the highest correlation between seasons for *C. pipiens* calculated abundance was found between Winter and Autumn (0.83), while the lowest was recorded between winter and summer (0.22). Only one weak negative correlation was recorded between spring and summer (-0.34). Similarly, the correlogram revealed a powerful negative correlation between Spring and Autumn (-0.71) (Figure 5).

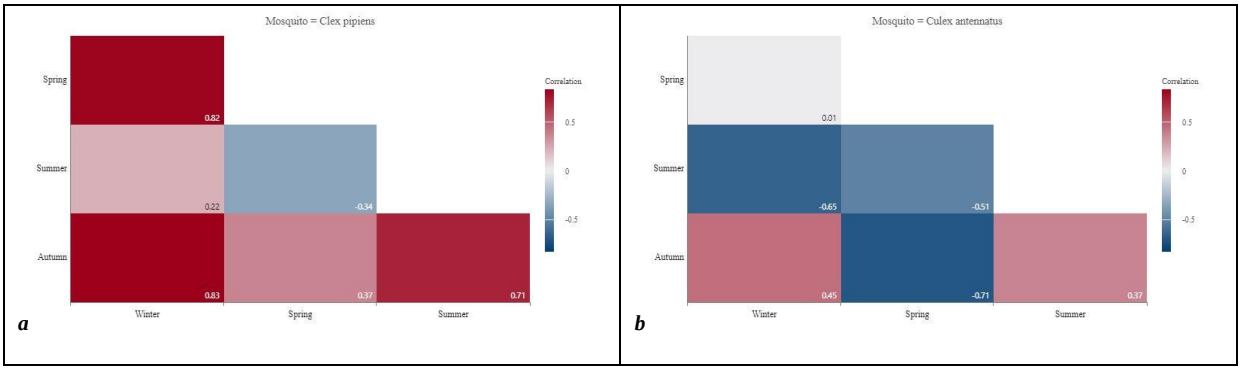


**Fig. 5.** correlogram of seasons abundance representing correlation relationship for **(*a*)** *Clex pipiens* and **(*b*)** *Culex antennatus*

## 2. *Physico-chemical parameters of breeding water:*

Temperature tends to follow semi bell shape trend; all season's temperatures were significantly varied (P< 0.05), except between spring and autumn, there was no significant variation (P> 0.05). The same trend was also observed for TDS, except the only significant variation (P< 0.05) was recorded between winter and summer. The pH values follow an uneven trend with a high peak recorded in autumn, but no significant differentiation (P> 0.05) was recorded among all seasons in their calculated pH value. Finally, a reverse semi-bell shape trend was observed for D.O., with only significant variation (P< 0.05) observed between winter and summer (Table 2 and Figures 6 & 7).

**Table 2.** Physico-chemical parameters of *C. pipiens* and *C. antennatus* larval breeding water in En Nazla region, Ibshway, Fayoum Governorate, Egypt.

| Parameters | Winter | Spring | Summer | Autumn |
|---|---|---|---|---|
| **Temperature** | 19.65±0.87 | 26±0.46 | 28.03±0.19 | 25.30±0.31 |
| **pH** | 7.40±0.25 | 7.58±0.19 | 7.45±0.17 | 7.63±0.15 |
| **TDS** | 984±8.51 | 1000±8.97 | 1016.75±6.18 | 1000±5.52 |
| **D.O.** | 3.28±0.15 | 3.03±0.14 | 2.83±0.15 | 3.05±0.05 |

pH: Potential Hydrogen, TDS: Total Dissolved Solids, D.O.: Dissolved Oxygen.

The environmental-abundance relationship was studied using pairwise regression, as shown in Figures (8 a & b); TDS is considered the most reliable factor that mosquito abundance depends on, followed by pH value for *C. pipiens* and temperature for *C. antennatus.* The model summary for both species was presented with $R^2$= 99.96 and 99.93%, respectively. The regression equation to predict *C. pipiens is* illustrated as *C. pipiens* abundance = -12780 - 726.8 pH + 18.567 TDS. Meanwhile, the regression equation to predict *C. antennatus* is illustrated as: *C. antennatus* abundance = -17813 - 39.53 Temp + 19.002 TDS.

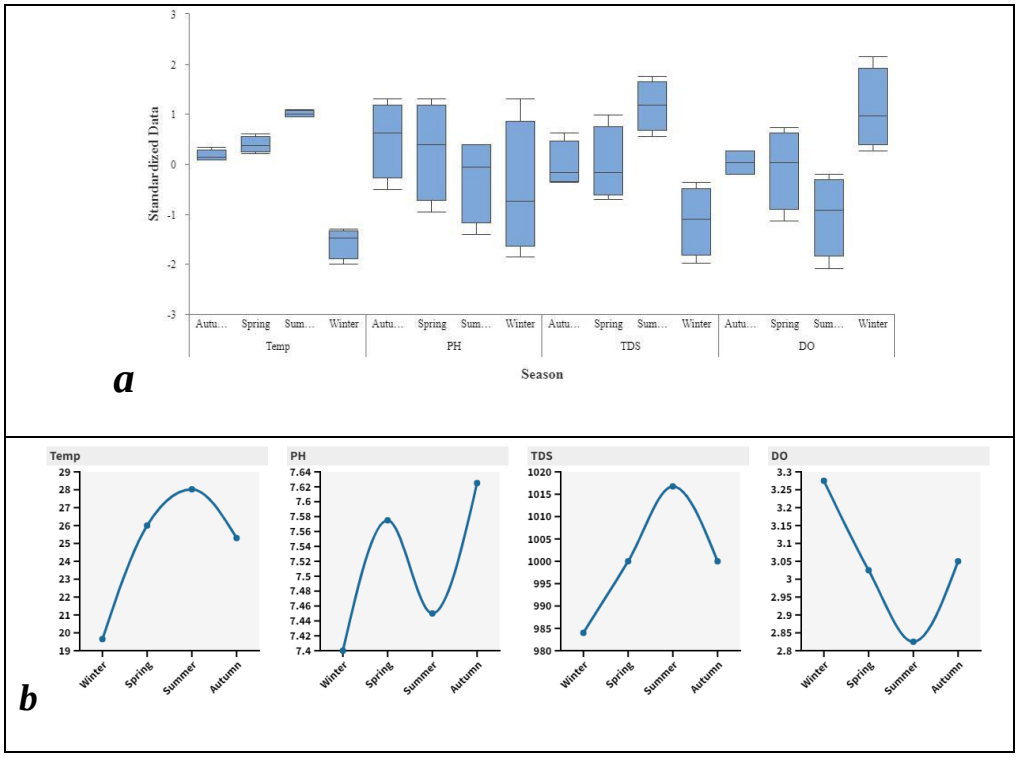


**Fig. 6. (*a*)** box plot and **(*b*)** Gradient line chart of analyzed physical parameters. Sum: Summer; Autu: Autumn.

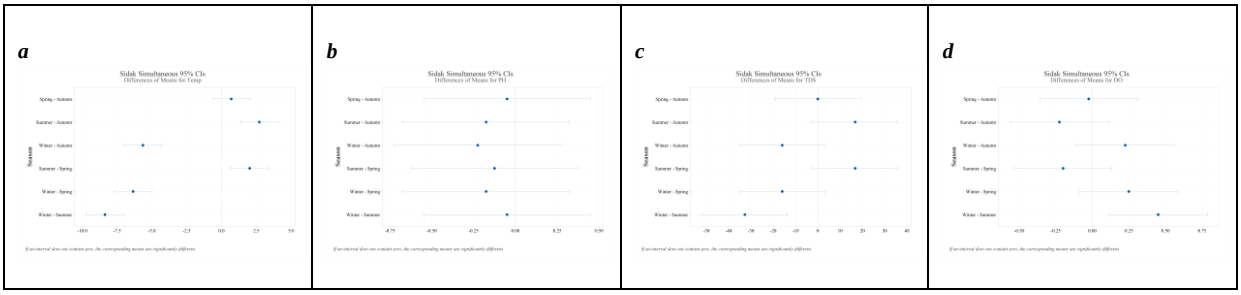


**Fig. 7.** Sidak simultaneous 95% Cis differences of the mean for **(*a*)** Temp, **(*b*)** pH, **(*c*)** TDS, and **(*d*)** DO

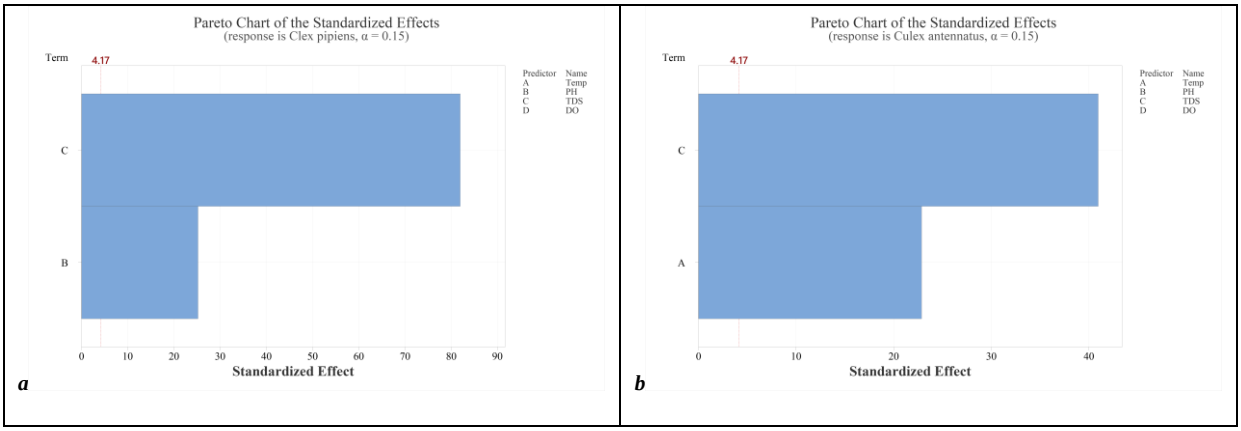


**Fig. 8.** Pareto chart of standardized effects for **(*a*)** *Clex pipiens* and **(*b*)** *Culex antennatus*

Also, the correlogram revealed the most correlated physical parameters throughout the four seasons of the study, showing that in winter, the highest correlated parameters were

TDS and temperature. The temperature was positively correlated with pH (0.60 and 0.73, respectively) during autumn and spring. Counter, pH was powerfully negatively correlated with temperature during summer (-0.98), while TDS was the highest negatively correlated with a value of -1.0 and -0.99 with D.O. during spring and winter, respectively, where pH recorded the highest negatively correlated value (-0.85) with D.O. (Figs. 9 a, b, c & d).

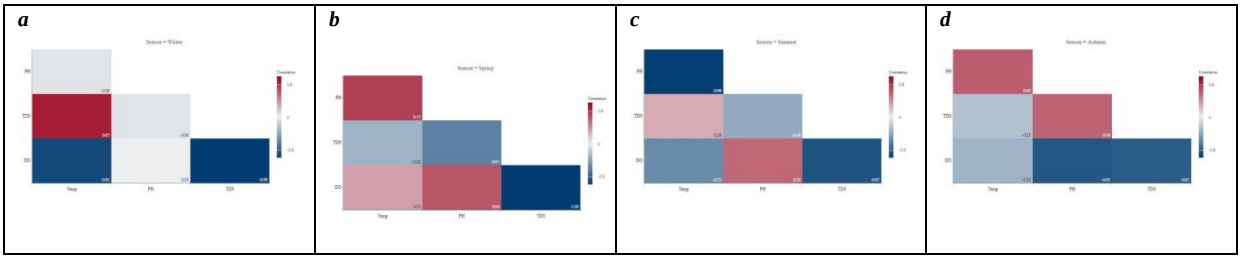


**Fig. 9.** Correlogram of seasons physical parameters representing their correlation relationship for **(*a*)** Winter, **(*b*)** Spring, **(*c*)** Summer, and **(*d*)** Autumn

### 3. *Genetic identification of collected mosquito species:*

The PCR amplification of mosquito larvae's DNA revealed positive amplification using primers that amplify the COI region. The amplicon size of both mosquito species was ~610 pb (Figure 10).

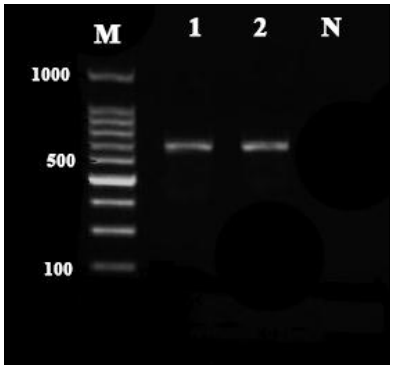


**Fig. 10.** Agarose gel electrophoresis (2 %agarose, stained with ethidium bromide): analysis of PCR amplification for detection of *Cx pipiens* and *Cx antennatus*. M: 100 bp DNA marker; N: negative control; 1&2) (*C. pipiens*, 3) *C. antennatus* 610 bp.

The Neighbor-Joining method inferred the evolutionary history **(Saitou and Nei, 1987).** The optimal tree is shown (Figure 11). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown as branches color graded according to bootstrap value **(Felsenstein 1985).** The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method **(Tamura 1992)** and are in the units of the number of base substitutions per site. This analysis involved 23 nucleotide sequences (21 donor sequences were evaluated from NCBI, including one sequence that acted as an outgroup (KR152335.1). Codon positions included were $1^{st}$ +$2^{nd}$ +$3^{rd}$ +Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 428 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 **(Tamura *et al*., 2021),** followed by visualization enhancement using ITOl software **(Letunic and Bork, 2021).**

Also, *C. pipiens* MK052921.1 (nucleotide sequences from the current study) showed a clear relationship (Figure 11) with species identified from Portugal (*C. pipiens* LC102133.1 and *C. pipiens* LC102132.1) and Kenya (*C. pipiens* KU187083.1) with an evolutionary distance of approx. 0.001164. Moreover, *C. antennatus* OP714215.1 (nucleotide sequences from the current study) displayed a close relationship to a species identified from Madagascar (*C. antennatus* MK033248.1) and Kenya (*C. antennatus* KU187037.1, KU187050.1, KU187048.1, and KU187038.1) with an evolutionary distance of approx. 0.00734.

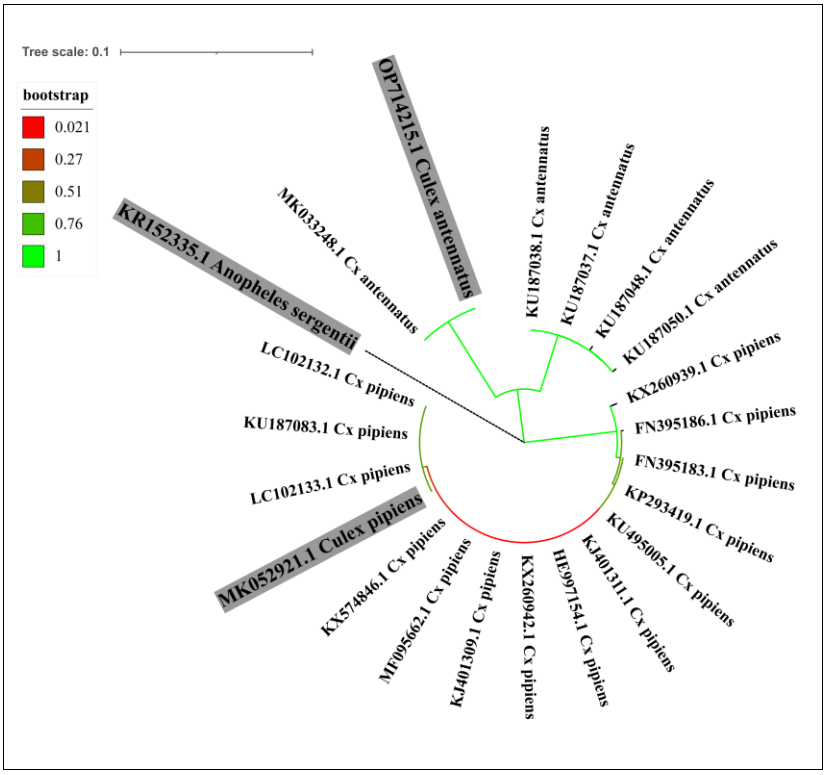


**Fig. 11.** Neighbor-Joining phylogenetic evolutionary tree.

In addition, the minimum haplotype spanning network represented the haplotype and geographical location of the 23 nucleotide sequences evaluated in the present work. Revealing the haplotype diversity and change throughout geographical differences. Nucleotide diversity shows a pi-value of 0.096; the number of segregating sites was 80, while 53 parsimony-informative sites were observed. The Tajima's D statistic was calculated to be -2.6005 with P (D >= -2.6005) = 0.999968 (Figure 12).

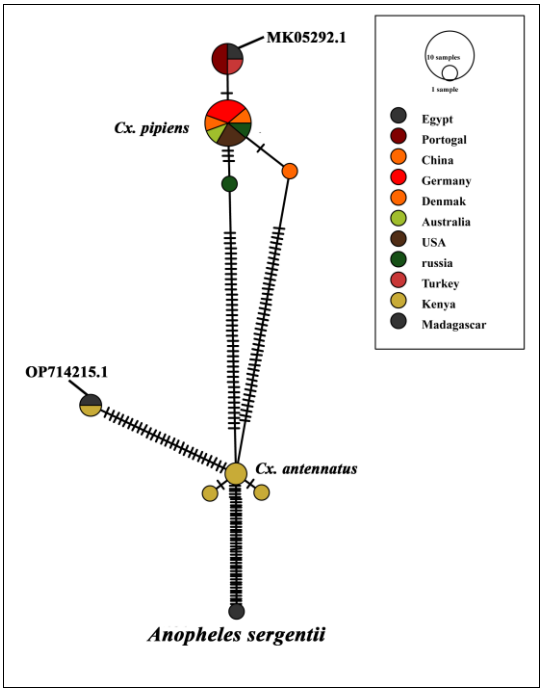


**Fig. 12.** Minimum haplotype spanning network.

## DISCUSSION

As shown from obtained results, *Culex pipiens* and *Culex antennatus* reported a prevalence in Fayoum Governorate, Egypt, along different seasons; however, *C. pipiens* was more abundant than *C. antennatus* in the study area. The prevalence of the *Culicine* mosquito in Egypt was recorded by **Kenawy *et al.* (2013), Hassan *et al.* (2014), Elhawary *et al.* (2021), and El-Mehdawy *et al.* (2021 & 2022).** Summer recorded the highest abundance for mosquito species, superior to that recorded in other seasons, while winter was found to be had the lowest abundance. Such results can be attributed to the increase in water temperature during summer (about 28.03°C, respectively) than winter (about 19.65°C, respectively). In agreement with the recorded results, **White (1974)** recorded that the temperature is an important factor affecting the development and growth of different mosquito larvae, **Kenawy *et al.* (2013)** reported a temperature range of 17-30°C for *C. pipiens* in Cairo Governorate and **Elhawary *et al.*, (2020),** who recorded a temperature ranged between 21 and 32°C for *C. pipiens*, and *Culiesta longiareolata* in different breeding sites in Egypt. Also, water breeding habitats in the study area were slightly alkaline, preferable to mosquito larvae **(Pelizza *et al.*, 2007 & Oyewole *et al.*, 2009).** The alkalinity of breeding water may be due to the nature of these habitats.

In addition, obtained results revealed that TDS is the most reliable factor affecting both mosquito species' abundance. The highest TDS (1016.75 ppm) was recorded in summer, and the lowest (984 ppm) was recorded in winter. In agreement with **Williams (2001),** we can say that increasing TDS values in summer than in other seasons can be due to Anthropogenic activities during summer. In **1995, Chavasse *et al.*** reported that culicine mosquitoes prefer breeding sites with turbid water (polluted environments such as septic tanks and blocked drains) where they can successfully breed. This explains the highest

abundance of *C. pipiens* and *C. antennatus* during summer, which have the highest TDS values compared with other seasons, indicating the tolerance of both species in highly turbid water. The TDS content recorded a powerful negative correlation with D.O. during different seasons. These results are consistent with the previously recorded by **Sehgal & Pillai (1970)** and **Tadesse *et al.* (2011).**

On the other hand, COI sequences obtained from *C. pipiens* in the present study were found to be identical by 100.0% to sequences from *C. pipiens* recorded in Portugal **(Mixão *et al.*, 2016)** in the GenBank, while sequences obtained from *C. antennatus* were identical 100.0% to sequences related to *C. antennatus* from Madagascar **(Jeffries *et al.*, 2018).** Also, *C. pipiens* had a greater variation in COI than *C. antennatus*, which is consistent with **(Werblow *et al.*, 2013 & 2014).** However, we found no indication of additional sub-structuring or taxonomic differentiation within *C. pipiens*, despite earlier research indicating morphological variation within the species **(Fedorova and Shaikevich, 2007).** Currently, biotypes pipiens and antennatus are considered separate monophyletic evolutionary units experiencing incipient ecological speciation; therefore, they may be distinct phylogenetic entities **(Fonseca *et al.*, 2004 and Yurchenko *et al.*, 2020 ).** Different mating practices of the two biotypes were regarded as the first cause of sympatric speciation **(Gomes *et al.*, 2009)**. The limited level of hybridization is unidirectional, with male-mediated introgression from the biotypes of both species dominating **(Gomes *et al.*, 2009).** The identical sequences may be caused by the similarity in the environment and climatic conditions found in Madagascar and Portugal to those in Egypt.

## CONCLUSION:

It is deduced from the obtained data that *Culex pipiens* and *C. antennatus* had a remarkable abundance in Fayoum Governorate, Egypt, throughout different seasons of the year, depending on the nature of breeding habitats. Hence, its role in disease transmission in Fayoum Governorate needs more investigations.

## REFERENCES

**Abouzied, E.M.** (2017). Life table analysis of *Culex pipiens* under simulated weather conditions in Egypt. J. Am. Mosq. Control Assoc., 33(1):16-24. http://dx.doi.org/10.2987/16-6608.1

**Amraoui, F.; Krida, G.; Bouattour, A.; Rhim, A. and Daaboub, J.** (2012). *Culex pipiens*, an Experimental Efficient Vector of West Nile and Rift Valley Fever Viruses in the Maghreb Region. PLOS ONE, 7(5): e36757. https://doi.org/ 10. 1371/journal.pone.0036757

**Bailey, R.A.** (1981). A unified approach to design of experiments. J. R. Stat. Soc. Ser. A. (General)., 144(2):214-223. https://www.jstor.org/stable/i349598

**Baz, M.M.; Selim, A.; Radwan, I.T.; Alkhaibari, A.M. and Khater, H.F.** (2022). Larvicidal and adulticidal effects of some Egyptian oils against *Culex pipiens*. Sci. Rep., 15:12(1):4406. https://doi.org/10.1038/s41598-022-08223-y

**Chavasse, D.C.; Lines, J.D.; Ichimori, K.; Majala, A.R.; Minjas, J.N. and Marijani, J.** (1995). Mosquito control in Dar es Salaam. II. Impact of expanded polystyrene beads

and pyriproxyfen treatment of breeding sites on *Culex quinquefasciatus* densities. Med. Vet. Entomol., 9:147-154. https://doi.org/10.1111/j.1365-2915.1995.tb00171.x

**Elhawary, E.A.; Mostafa, N.M.; Shehata، A.Z.I.; Labib, R.M. and Abdel Singab, N.B. (2021).** Comparative study of selected Rosa varieties' metabolites through UPLC-ESI-MS/MS، chemometrics and investigation of their insecticidal activity against *Culex pipiens* L. Jordan J. Pharm. Sci., 14(4): 417-433. https://journals.ju.edu.jo/JJPS/article/ view/107758

**Elhawary, N.A.; Soliman M.A.; Seif, A.I. and Meshrif, W.S.** (2020). *Culicine* Mosquitoes (Diptera: Culicidae) Communities and Their Relation to Physicochemical Characteristics in Three Breeding Sites in Egypt. Egyptian Journal of Zoology (EJZ), 74:30-42. https://dx.doi.org/10.21608/ejz.2020.40783.1039

**El-Mehdawy, A.A.; Koriem, M.; Amin, R.M.; El-Naggar، H.A. and Shehata, A.Z.I.** (2021). The photosensitizing activity of different photosensitizers irradiated with sunlight against aquatic larvae of *Culex pipiens* L. (Diptera: Culicidae). Egypt. J. Aquat. Biol. Fish., 25(5):661-670. https://dx.doi.org/10.21608/ejabf.2021.205672

**El-Mehdawy, A.A.; Koriem, M.; Amin, R.M.; Shehata, A.Z.I. and El-Naggar, H.A.** (2022). Green synthesis of silver nanoparticles using chitosan extracted from *Penaeus indicus* and its potential activity as aquatic larvicidal agent of *Culex pipens*. Egypt. J. Aquat. Biol. Fish., 26(1):425-442. https://dx.doi.org/10.21608/ejabf.2022.219887

**Fang, Y.; Khater, E.I.M.; Xue, J.-B.; Ghallab, E.H.S.; Li, Y.-Y.; Jiang, T.-G. and Li, S.-Z.** (2022). Epidemiology of Mosquito-Borne Viruses in Egypt: A Systematic Review. Viruses, 14:1577. https://doi.org/10.3390/v14071577

**Fedorova, M.V. and Shaikevich, E.V.** (2007). Morphological and molecular-genetic distinctions between adult mosquitoes *Culex torrentium* Martini and *C. pipiens* Linnaeus (Diptera, Culicidae) from Moscow Province. Èntomol. Rev., 87:127-135. https://doi.org/10.1134/S0013873807020017

**Felsenstein, J.** (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution., 39(4):783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.

**Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. and Vrijenhoek, R.** (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol., 3(5):294-299.

**Fonseca, D.M.; Keyghobadi, N.; Malcolm, C.A.; Mehmet, C.; Schaffner, F.; Mogi, M.; Fleischer, R.C. and Wilkerson, R.C.** (2004). Emerging Vectors in the *Culex pipiens* Complex. Science., 303(5663):1535-1538. https://doi.org/10.1126/science.1094247

**Gomes, B.; Sousa, C.A.; Novo, M.T.; Freitas, F.B.; Alves, R.; Côrte-Real, A.R.; Salgueiro, P.; Donnelly, M.J.; Almeida, A.P. and Pinto, J.** (2009). Asymmetric introgression between sympatric molestus and pipiens forms of *Culex pipiens* (Diptera: Culicidae) in the Comporta region, Portugal. BMC Evol. Biol., 6(9):262. https://doi.org/10.1186/1471-2148-9-262

**Harbach, R.; Harrison, B.; Gad, A.; Kenawy, M. and El-Said, S. (1988).** Records and Notes on Mosquitoes (Diptera: Culicidae) Collected in Egypt. Mosq. syst., 20: 317-342. https://apps.dtic.mil/sti/pdfs/ADA221832.pdf

**Harbach, R.E.** (1985). Pictorial keys to the genera of mosquitoes, subgenera of *Culex* and the species of *Culex (Culex)* occurring in southwestern Asia and Egypt, with a note on the subgeneric placement of *Culex deserticola* (Diptera: Culicidae). Mosq. Sys., 17:83-107.

**Hassan, M.I.; Fouda, M.A.; Hammad, K.M.; Tanani, M.A. and Shehata, A.Z.** (2014). Repellent effect of *Lagenaria siceraria* extracts against *Culex pipiens*. J. Egypt. Soc. Parasitol., 44(1):243-248. https://doi.org/10.21608/jesp.2014.90754

**Jeffries, C.L.; Tantely, L.M.; Raharimalala, F.N.; Hurn, E.; Boyer, S. and Walker, T.**

(2018). Diverse novel resident Wolbachia strains in Culicine mosquitoes from Madagascar. Sci Rep., 8(1):1-15. https://doi.org/10.1038/s41598-018-35658-z

**Kassem, Y.E.; El-Baghdady, K.Z.; Saleh, N.E.M. and Wahba, M.M.I.** (2018). Biological control of *Culex pipiens* mosquito by local bacterial isolates. African J. Biol. Sci., 14(2):21-40. https://doi.org/10.21608/ajbs.2018.205545

**Kenawy, M.A.; Ammar, S.E. and Abdel-Rahman, H.A.** (2013). Physico-chemical characteristics of the mosquito breeding water in two urban areas of Cairo Governorate, Egypt. J. Entomol. Acarol. Res., 45(3): e17. https://doi.org/10.4081/jear.2013.e17

**Kimura, M.** (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16(2):111-120. https://doi.org/10.1007/bf01731581

**Kumar, S.; Dhingra, A. and Daniell, H.** (2004). Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol. Biol., 56(2):203-216. https://doi.org/10.1007%2Fs11103-004-2907-y

**Labasque, T.; Chaumery, C.; Aminot, A. and Kergoat, G.** (2004). Spectrophotometric Winkler determination of dissolved oxygen: re-examination of critical factors and reliability. Mar. Chem., 88(1-2):53-60. https://doi.org/10.1016/j.marchem. 2004. 03.004

**Laurito, M.; de Oliveira, T.M.; Almiron, W.R. and Sallum, M.A.M.** (2013). COI barcode versus morphological identification of *Culex (Culex)* (Diptera: Culicidae) species: a case study using samples from Argentina and Brazil. Mem. Inst. Oswaldo. Cruz., 108(1):110-122. https://doi.org/10.1590%2F0074-0276130457

**Letunic, I. and Bork, P. (2021)**. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res., 2,49(1):W293-W296. https://doi.org/10.1093/nar/gkab301

**Mixão, V.; Barriga, B;D., Parreira, R.; Novo, M.T.; Sousa, C.A.; Frontera, E.; Venter, M.; Braack, L. and Almeida, A.P.G.** (2016). Comparative morphological and molecular analysis confirms the presence of the West Nile virus mosquito vector, *Culex univittatus*, in the Iberian Peninsula. Parasit. Vectors., 9(1):1-13. https://doi.org/10.1186/s13071-016-1877-7

**Oyewole, I.O.; Momoh, O.O.; Anyasor, G.N.; Ogunnowo, A.A.; Ibidapo, C.A.; Oduola, O.A.; Obansa, J.B. and Awolola, T.S.** (2009). Physicochemical characteristics of *Anopheles* breeding sites: impact on fecundity and progeny development. Afr. J. Environ. Sci. Technol., 3(12):447-452. https://doi.org/10.4314/AJEST.V3I12.56290

**Pelizza, S.A.; Lastra, C.C.L.; Becnel, J.J.; Bisaro, V. and García, J.J.** (2007). Effects of temperature, pH and salinity on the infection of *Leptolegnia chapmanii* Seymour (Peronosporomycetes) in mosquito larvae. J, Invertebr, Pathol., 96:133-137. https://doi.org/10.1016/j.jip.2007.04.005

**Saitou, N. and Nei, M.** (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4:406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

**Sayed, R.M.; Abdalla, R.S. and El sayed T.S.** (2018). Control of *Culex pipiens* (Diptera: Culicidae), the vector of lymphatic filariasis, using irradiated and non-irradiated entomopathogenic nematode, *Steinernema scapterisci* (Rhabditida: Steinernematidae). Egypt. J. Biol. Pest Control, 28, 67. https://doi.org/10.1186 /s41938 - 018-0070-z

**Seghal, S. and Pillai, M.K.** (1970). Preliminary studies on the chemical nature of mosquito breeding waters in Delhi. Bull. WHO., 42:647-650. http://www.ncbi.nlm. nih. gov/pmc/articles/pmc2427463/

**Shahhosseini, N.; Kayedi, M.H.; Sedaghat, M.M.; Racine, T.P.; Kobinger, G. and Moosa-Kazemi, S.H.** (2019). DNA barcodes corroborating identification of mosquito species and multiplex real-time PCR differentiating *Culex pipiens* complex and *Culex torrentium* in Iran. PLoS One., 14(12): e0227018. https://doi.org/10.1371%2Fjournal.pone.0227018

**Tadesse, D.; Mekonnen, Y. and Tsehaye, A. (2011).** Characterization of Mosquito breeding sites in and in the vicinity of Tigray Microdams. Ethiop. J. Health. Sci., 21(1):57-66. https://doi.org/10.4314/ejhs.v21i1.69045

**Tamura, K.** (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol., 9(4):678-687. https://doi.org/10.1093/oxfordjournals.molbev.a040752

**Tamura, K.; Stecher, G. and Kumar, S.** (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Mol. Biol. Evol., 38(7):3022-3027. https://doi.org/10.1093/molbev/msab120.

**Verna, T.N. and Munstermann, L.E.** (2011). Morphological variants of Aedes aegypti collected from the leeward island of Antigua. J. Am. Mosq. Control Assoc., 27(3):308-311. https://doi.org/10.2987/11-6157.1

**Walter Reed Biosystematics Unit (2022):** *Culex antennatus* species page. Walter Reed Biosystematics Unit Website. http://wrbu.si.edu/vectorspecies/mosquitoes/antennatus

**Wang, G.; Li, C.; Guo, X.; Xing, D.; Dong, Y.; Wang, Z.; Zhang, Y.; Liu, M.; Zheng, Z.; Zhang, H.; Zhu, X.; Wu, Z. and Zhao, T.** (2012). Identifying the main mosquito species in China based on DNA barcoding. PLoS One., 7(10):e47051. https://doi.org/10.1371/journal.pone.0047051

**Werblow, A.; Bolius, S.; Dorresteijn, A.W.C.; Melaun, C. and Klimpel, S.** (2013). Diversity of *Culex torrentium* Martini, 1925-A potential vector of arboviruses and filaria in Europe. Parasitol. Res., 112(7):2495-2501. https://doi.org/10.1007/s00436-013-3418-z

**Werblow, A.; Klimpel, S.; Bolius, S.; Dorresteijn, A.W.C.; Sauer, J. and Melaun, C.** (2014). Population structure and distribution patterns of the sibling mosquito species *Culex pipiens* and *Culex torrentium* (Diptera: Culicidae) reveal different evolutionary paths. PLoS One., 9(7): e102158. https://doi.org/10.1371%2Fjournal.pone.0102158

**White, G.B.** (1974). *Anopheles gambiae* complex and disease transmission in Africa. Trans. R. Soc. Trop. Med. Hyg., 68:278-301. https://doi.org/10.1016/0035-9203(74)90035-2

**Williams, W.D.** (2001). Anthropogenic salinisation of inland waters. Hydrobiologia., 466:329-337. https://doi.org/10.1023/A:1014598509028

**Yurchenko, A.A.; Masri, R.A.; Khrabrova, N.V.; Sibataev, A.K.; Fritz, M.L. and Sharakhova, M.V.** (2020). Genomic differentiation and intercontinental population structure of mosquito vectors *Culex pipiens pipiens* and *Culex pipiens molestus*. Sci. Rep., 10(1):7504. https://doi.org/10.1038/s41598-020-63305-z