EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
ENTOMOLOGY

A

ISSN
1687-8809

WWW.EAJBS.EG.NET

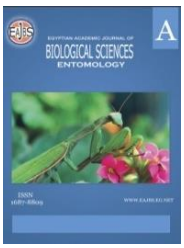
Vol. 17 No. 4 (2024)

Citation: *Egypt. Acad. J. Biolog. Sci. (A. Entomology) Vol. 17(4) pp.1-16 (2024)*
DOI: **10.21608/EAJBSA.2024.384484**

Egypt. Acad. J. Biolog. Sci., 17(4):1-16 (2024)

Egyptian Academic Journal of Biological Sciences
A. Entomology

ISSN 1687- 8809
http://eajbsa.journals.ekb.eg/



Molecular Identification and DNA Barcoding of some Megophthalminae Species (Hemiptera: Cicadellidae) from Egypt

Rabab M. El-Hady[1] and Ahmed F. El-Sayed[2]

[1]Survey &Taxonomy Department, Plant Protection Research Institute, Agriculture Research Centre, Giza, Egypt.

[2] Microbial Genetics Department, Biotechnology Research Institute, National Research Centre, Giza, Egypt.

***E-mail:** elhady_rabab@yahoo.com-ahmedfikry.nrc@gmail.com

ARTICLE INFO

Article History
Received:24/8/2024
Accepted:7/10/2024
Available:11/10/2024

Keywords:
Auchenorrhyncha, phylogenetics, Taxonomy, morphology, mitochondrial COX gene, 28S rDNA gene, leafhoppers.

ABSTRACT

Two species of leafhoppers, *Austroagallia sinua*ta (Mulsant & Rey, 1835) and *Anaceratagallia laevis* (Ribaut, 1935) are described, illustrated, and Molecularly Identified. There are also comprehensive descriptions of intraspecific variation in male genitalia and images of the two species. Material is deposited in the Reference Egyptian Museum of Insects at the Plant Protection Research Institute, Agriculture Research Centre. Using specific primers from the investigated Megophthalminae species, the 28S rDNA and mt COX genes were amplified. The nucleotide sequences of the genes EGY-ARC-3and EGY-ARC-15 were entered into the National Centre for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) and contrasted with those stored in the GenBank DNA database. Based on Multiple Sequence Alignment (MSA) using the Clustal W2 algorithm with MEGA default parameters, the findings showed the homology percentage between the partial sequences of the 28S rRNA gene from each species and related species. The data revealed more similarities between species belonging to the Megophthalminae subfamily. This work verified the morphological identities of the two leafhopper species through molecular characterization and the sequencing of 28rDNA and COX genes. They were identified as *Austroagallia sinuata* EGY-ARC3 and *Anaceratagallia laevis* EGY-ARC15. Additionally, their sequencing of the COX and 28 srDNA genes was submitted to GenBank, where it was assigned accession numbers (LC775124.1 and LC775136.1) for the COX gene and (LC670606.1, and LC670615) for the 28 srDNA, respectively.

INTRODUCTION

Austroagallia sinuata (Mulsant & Rey, 1855) and *Anaceratagallia laevis* (Ribaut, 1935) belong to the tribe Agalliini which is a large tribe in the subfamily Megophthalminae (Hemiptera: Cicadellidae). This tribe comprises 37 genera and 650 species worldwide (Oman 1949; Viraktamath 2005; Oman *et al.*, 1990; Viraktamath 2011). About 14 species of this tribe are known for transmitting phytoplasmas and plant pathogenic viruses (Nielson

1979; Weintraub & Beanland 2006). The species of this tribe attack a wide range of economic crops. *Au. sinuata* is a cosmopolitan species reported from the Afrotropical, Western Oriental, Neotropical, and Southern Palaearctic regions. (Viraktamath, 2011).

Molecular data analyses are now an effective tool in phylogenetic research, and they have been applied to leafhopper studies (Fang *et al*., 1993; Dietrich *et al.*, 1997, 1998, 2002). This study aims to confirm the identification and examine the phylogenetic relationships among the two species using mitochondrial COX I and 28S rDNA gene sequences combined with morphological data.

MATERIALS AND METHODS

1-Sampling and Identification:

In the current study, Megophthalmine specimens were collected from different plants between 2018 and 2023 from several Egyptian localities: Alexandria, Qena, Giza and Toshka using a sweep net, aspirator, and light trap. Some specimens were then conserved in 70% ethanol and kept at −20 °C until the DNA was extracted. Others were used for preparing the male genitalia. The genitalia were examined under a microscope after being cleaned with water and glycerol. Digital images were captured using an Olympus EP 50 camera (5 MP) with an Olympus stereomicroscope. Current taxonomic keys and related literature were used to identify specimens, such as (Habib *et al.*, 1975a, 1975b, 1976; Knight 1983; Oman *et al.*, 1990; Gnaneswaran *et al.*, 2010; Zahniser and Dietrich 2010, Viraktamath, 2011). Morphological terminology is based on Dietrich (2005). The investigated specimens were deposited in the Reference Egyptian Museum of Insects, PPRI, ARC.

2-Molecular Identification Using 28srDNA and Mitochondrial COX1 Genes:

2.1- Extraction of DNA:

Three individual leafhoppers were immersed in liquid nitrogen and crushed using a mill and pestle. DNA was extracted using the Gene JET Genomic DNA Purification Kit (Thermo Scientific, USA). Using a pestle and mortar and liquid N2, each insect species was crushed into a fine powder and then placed into a 1.5 ml microcentrifuge tube. Then, 1 ml of extraction buffer (100 mM Tris-Cl, 50 mM EDTA, 50 mM NaCl, 20% SDS) was mixed to the powdered sample. The homogenate was then kept in a microcentrifuge tube in a hot water bath at 65 °C for 30 minutes. After that, the tubes were taken out of the water bath and the supernatant was transferred to a fresh microcentrifuge tube. Then, equal quantities of isoamyl alcohol, chloroform, and phenol (25:24:1) were added, and everything was thoroughly mixed. Then, the samples were centrifuged at 10,000 rpm for 15 minutes. After carefully pouring the supernatant into a fresh microcentrifuge tube, 100% cooled ethanol was added in double the amount, and the tube was refrigerated at 4 °C for the whole night. The DNA was pelletized for 10 minutes at 10,000 rpm using centrifugation. The DNA pellet was then cleaned using cold 70% ethanol. The recovered DNA pellet was allowed to air-dry before being dissolved in 50 µl of 1× TE buffer that included 1 mM EDTA and 10 mM Tris (pH 8).

2.2- Molecular Identification by 28srDNA Barcoding:

28srDNA gene was amplified using the primers 28SF1 and 28R (Table 1). The DreamTaq kit (Thermo Scientific, USA) was used to perform the PCR. PCR programs were performed at 95°C for 3 min, 35 cycles of 95°C for 0.5 min, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 10 min (Applied Biosystems, Foster City, CA, US).

2.3- Molecular Identification by COX Barcoding:

Using the HCO and LCO primers, a 550 bp segment of the (COX) gene was amplified. PCRs were performed in 50 µl reaction volumes with 25 µl DreamTaq kit (Thermo Scientific, USA), 0.5µl of primer, and 4 µl of DNA template. PCR programs were

performed for three minutes at 95°C, 35 cycles of 95°C for one minute, 55°C for thirty seconds, and 72°C for one minute, and a final extension step of 72°C for ten minutes. PCR products were quantitatively evaluated using 2% agarose gel electrophoresis.

2.4- Sequence Analysis and Phylogenetic Analysis:

28srDNA and COX amplicon were purified using a Gel Extraction Kit (Thermo Fisher, USA) and directly sequenced in both directions using an automatic DNA sequencer (Macrogen, Korea). The software Bio Edit was used to edit and trim the chromatograms. Edited sequences were aligned using Clustal X of Clustal W packages (Thompson *et al*., 1994) and submitted to GenBank. For the pairwise genetic distance (PWG) method, In the tree-based analysis, neighbor-joining (NJ) trees were constructed in MEGA11 (Tamura *et al.*, 2021).

Table 1: Specific primer of 28S-rDNA and COX markers used in this study:

Gene	Primer	Orientation	Primer sequence (5=3)	References
28S	28S	**F**	5´-AGAGAGAGTTCAAGAGTACGTG-3´	(Hancock *et al.*,1988 ; Campbell *et al.*, 1993)
		R	5´-TTGGTCCGTGTTTCAAGACGGG-3´	
COX	HCO	**F**	5'-TTTTCTACHAAYCATAAAGATATTGC-3'	Linares *et al*., 1991
	LCO	**R**	5'-TATAAACYTCDGGATGNCCAAAAAA-3'	

RESULTS AND DISCUSSION

Key to the Genera of the Tribe Agalliini (Cicadellidae: Megophthalminae) from Egypt:

- Pronotum pale without distinct markings, with two posterior round black spots, scutellum clear, (aedeagus asymmetrical)…….. …….*Austroagallia* Evans
- Pronotum dark brown with distinct markings, with two anterior black blotches (patches), scutellum dark with two distal black triangles (aedeagus symmetrical)…… ……*Anaceratagallia* Zachvatkin

Genus I: *Austroagallia* Evans

Austroagallia Evans, 1935: 70.

Type species: *Austroagallia torrida* Evans, by monotypy.

Diagnosis (Fig. 1. A-F):

Head: Pale yellow with two dark black circular spots on vertex, and a posterior larger pair posteriorly on pronotum. Hind margin of vertex sinuate behind eyes. Ocelli closer together than to the adjacent eye margin. Face (including eyes) longer than wide.

Thorax: Pronotum has thin border on either side, frequently punctured and arcuate (Fig.1. E.). Pale yellow forewing claval vein without cross vein. Fronto-clypeus small. Clypellus long and slender. Gena long and thin (Fig.1. F**.).** Three anteapical cells on forewings, inner anteapical cell opened basally.

Abdomen: Black; occasionally whitish between apical terga.

Notes: The significantly asymmetrical aedeagus, the well-developed anal collar processes, and the sinuate hind border of head behind the eyes are good characteristics to identify this genus.

***Austroagallia sinuata* (Mulsant & Rey, 1835)**:

Bythoscopus sinuatus Mulsant and Rey, 1835: 67, by original designation.

Agallia quadrisignata Flor, 1861: 557, synonymy by Fieber 1868: 462.

Agallia homeyeri Kirschbaum, 1868:32, synonymy by Fieber 1872: 32.

Agallia fieberi Vismara, 1878: 41, synonymy by Löw 1885: 346.

Austroagallia afganistanensis Kameswara Rao, Ramakrishnan and Ghai 1979: 655–656.

Austroagallia sinuata (Mulsant & Rey): Viraktamath 2011: 34

Worldwide Distribution: Afro-tropical Region, East Palearctic, Europe, Near East, and North Africa. In Egypt. Lower and Upper Nile Valley, Alexandria, Qena, Giza and Toshka

(Sources: Mohamad, 1996, El-Hady *et al.*, 2020).

Diagnosis:

Body pale yellow, vertex thin, with two large black circles in center and two weak brown lines running parallel to each other. ocelli arranged more closely together than to the adjacent eye margin. Pronotum with two round black dots. Face yellowish, with brown spots at base of antennae and below it, with Y-shaped stripe on upper section, with a stripe along the outer edge of the frontoclypesus, and with lines mark the facial sutures. scutellum yellow-colored, with two black spots protruding from base. Pale yellow-brown forewings with darker veins. Clavus with pale veins and deeper brown stripes in between (Fig. 1.A-D).

Male Genitalia (Fig. 1. G.)**:** Male mesal pygofer lobe bent caudally and situated close to hind end. Triangular subgenital plate with few setae. Styles with inner branches slightly longer than outer ones, proximal style bent and narrow. Anal collar process hook-like, with robust dentical and long slender twisted hook at base. Connective wide with rounded lobe-like at anterolateral angles. Aedeagus asymmetrical and sinuate at the tip, with laminate processes and with well-developed dorsal apodeme. Gonopore apical.

Measurements: Body length: Male length 3.56-3.75 mm. Female length 3.86-4.35 mm. Forewing length 2.85-3.0 mm., vertex width with eyes 1.0-1.2 mm; vertex length 0.1-0.13mm; pronotum width, 0.9-1.1 mm; pronotum length, 0.46-0.49 mm; scutellum width, 0.60-0.75 mm; scutellum length, 0.31-0.36 mm.

Material Examined:

Assiut, 3. IV. 1917 (4); Dakhala Oasis, 20.III. 1932 (1); Helwan, 20.XII. 1933 (1); Helwan, 13.VI. 1934 (1), (The Reference Egyptian Museum of Insects), PPRI, ARC). Dishna, 30.VI.2018 (2); Al Qlamina, 14. V11.2018 (1); Al Qlamina,15. VIII.2018(2); Qus, 30.VI.2018 (2); Qus, 30.VII.2018 (2); Qus, 1.IX.2018 (2), Borg el Arab, 15.XI.2018 (2); Borg el Arab, 15. V.2018 (1); Saft, 5.IV.2019 (7); Toshka,15. V.2019 (100); Toshka, 30.VI.2019 (220); Toshka, 15.VII.2019 (109); Toshka, 15. VIII.2019 (205); Saft, 6.II.2020 (3); Saft, 12.VI.2022 (2), (The author collection).

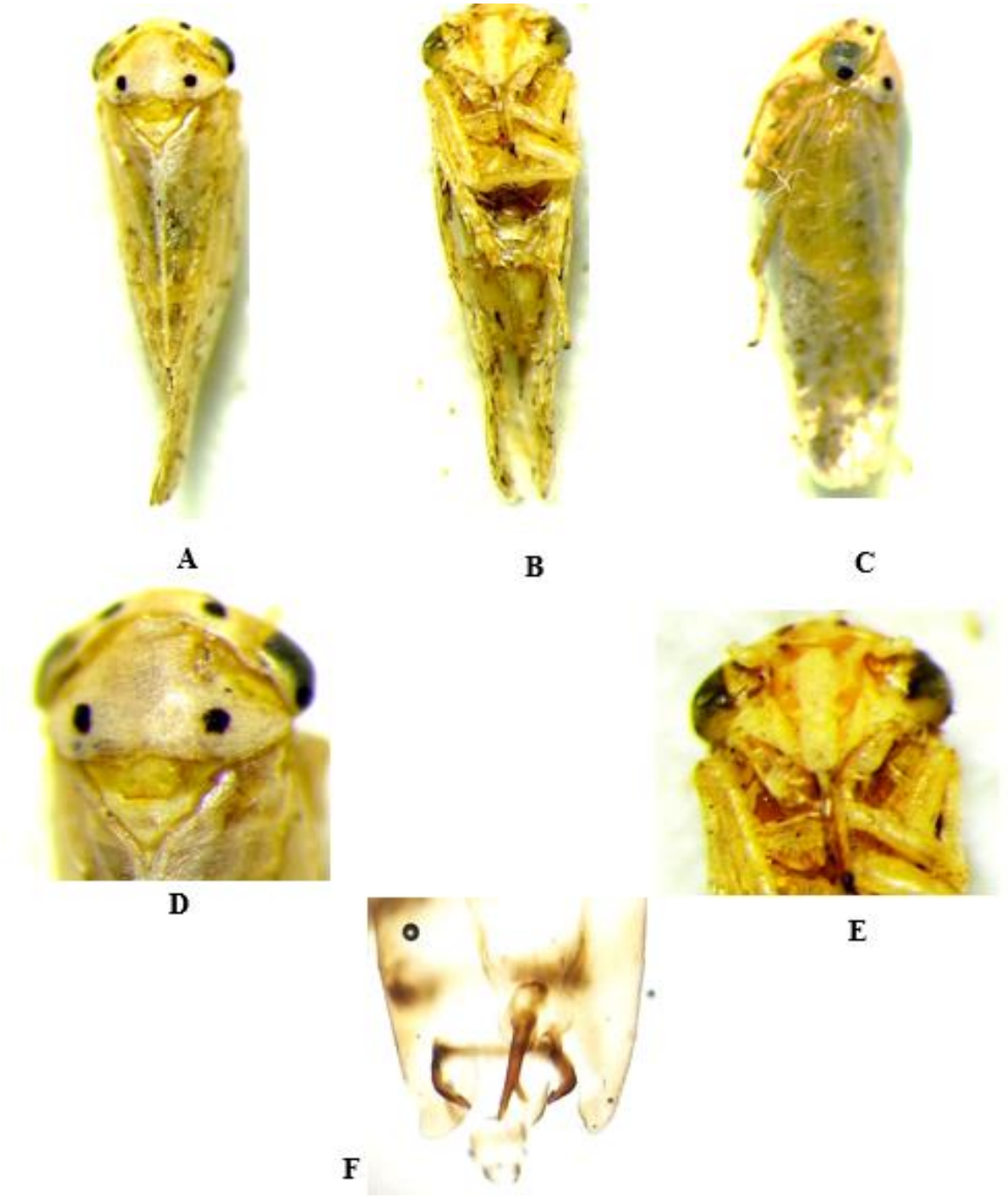


Fig. 1: *Austroagallia sinuata* (Mulsant & Rey). A –C. Habitus, A. dorsal view, B. female ventral view, C. lateral view, D. Pronotum & scutellum, E. Face, F. Male genitalia (pygofer, subgenital plate, valve, styles and connective, aedeagus).

Genus II: *Anaceratagallia* Zachvatkin, 1946:
Anaceratagallia Zachvatkin 1946: 159–161.
Type-species: *Cicada venosa* Fourcroy, 1785 by original designation.
Diagnosis:
Coloration: Dark brown leafhoppers. Face and vertex shagreened
Head: Vertex medially longer than next to eyes, with dark pattern, two spots and one median spindle shaped spot. Face convex in lateral view. Gena behind eyes sinuate.
Thorax: Pronotum transversely rugose. Scutellum with basal dark brown triangles. Forewing transparent with dark brown veins, with four apical and three anteapical cells.

Inner anteapical cell closed posteriorly by cross veins.

Remarks: The shape of the anal collar appendage and penis are the most reliable structures for species identification in this genus (Zachvatkin 1946, Viraktamath, 2012, Tishechkin, 2017).

***Anaceratagallia laevis* (Ribaut, 1935):**

Agallia laevis Ribaut, 1935: 29-36.

Worldwide Distribution: East Palaearctic, Near East, North Africa. In Egypt. Alexandria, Qena, Giza and Toshka (El-Hady *et al*., 2020).

Diagnosis: Modified after Viraktamath, 2011 and Tishechkin, 2017

Vertex narrow and pointed medially, with two adjacent big black spots. Brownish pronotum with two anterior black patches and dark markings. Face brownish. Scutellum brown, with two distal black triangles. Fore wings light brown, with darker robust brown veins. Clavus with pale veins, and darker brown streaks between them (Fig. 2. A-D).

Male Genitalia (Fig. 2. G.)**:** Subgenital plate nearly rectangular, with 6 macrosetae. Style with small tooth on apophysis, preapical lobe with fine setae. Aedeagus simple, very wide, without any processes, and with small denticles in middle of dorsal margin. Anal collar appendage wide with simple long tapered apex.

Measurements: Body length: Male length 3.37-3.42 mm. Female length 3.75-4.15 mm. Forewing length 2.85-3.10 mm., vertex width with eyes1.2- 1.3 mm; vertex length 0.11-0.13 mm; pronotum width, 0.82-0.95 mm; pronotum length, 0.40-0.47 mm; scutellum width, 0.70-0.80 mm; scutellum length, 0.31-0.34mm.

Material Examined:

Dishna, 30.V.2018 (2); Dishna, 30.VI.2018 (5); Dishna, 30.VII.2018 (10); Dishna, 30.VIII.2018 (9); Al Qlamina,14.V.2018 (21); Al Qlamina,15.VI.2018 (55); Al Qlamina,15.VII.2018 (112); Al Qlamina,15.VIII.2018 (203); Al Qlamina,15.VIII.2018 (158); Qus, 30.V.2018 (12); Qus, 15.VI.2018 (18); Qus, 1.VI .2018 (25), Borg el Arab, 15.V.2018 (15); Borg el Arab, 15.VI.2018 (11); Borg el Arab, 15.VI.2018 (34); Saft, 5.IV.2019 (7); Toshka,15.V.2019 (100); Toshka, 30.VI.2019 (220); Toshka, 15.VII.2019 (109); Toshka, 15.VIII.2019 (205); Saft, 6.II.2020 (3); Saft, 12.VI.2022 (2), (The author collection).

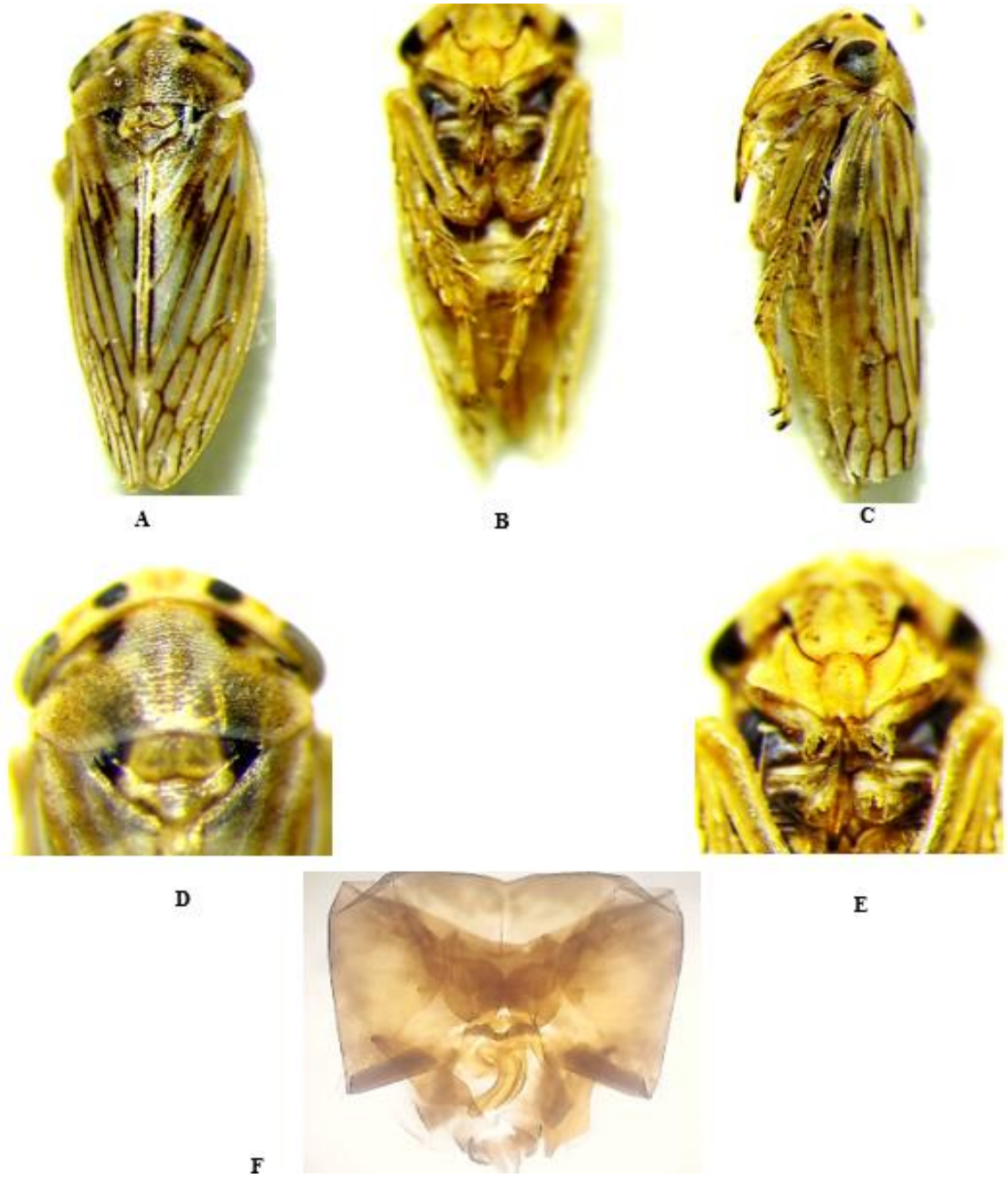


Fig. 2: *Anaceratagallia laevis* (Ribaut). A –C. Habitus, A. dorsal view, B. female ventral view, C. lateral view, D. Pronotum & scutellum, E. Face, F. Male genitalia (pygofer, subgenital plate, valve, styles and connective, aedeagus).

Molecular Identification Using 28s rDNA and Mitochondrial COX1 Genes:

A portion of the mitochondrial COX1 gene and the large subunit of the 28s rDNA were amplified by PCR and sequenced in order to molecularly identify the three Megophthalmine species. Subsequently, as shown in Figure 3 (A and B), the PCR products of the 28s rDNA and COX1 genes showed the anticipated size of around 500 and 550 bp, respectively. The Finch TV application was used to modify and visualize the collected sequencing raw reads. The full sequences of the COX1 gene and the 28s rDNA were then obtained by assembling the modified reads using the Bio-Edit software, as shown in Figures (4 and 6). The edited sequencing was aligned using the nucleotide BLAST program on the NCBI database to identify the potential genera of the isolates based on similarity. After that,

the acquired sequences were submitted to the NCBI database. The findings are summarized in Tables (2 and 4).

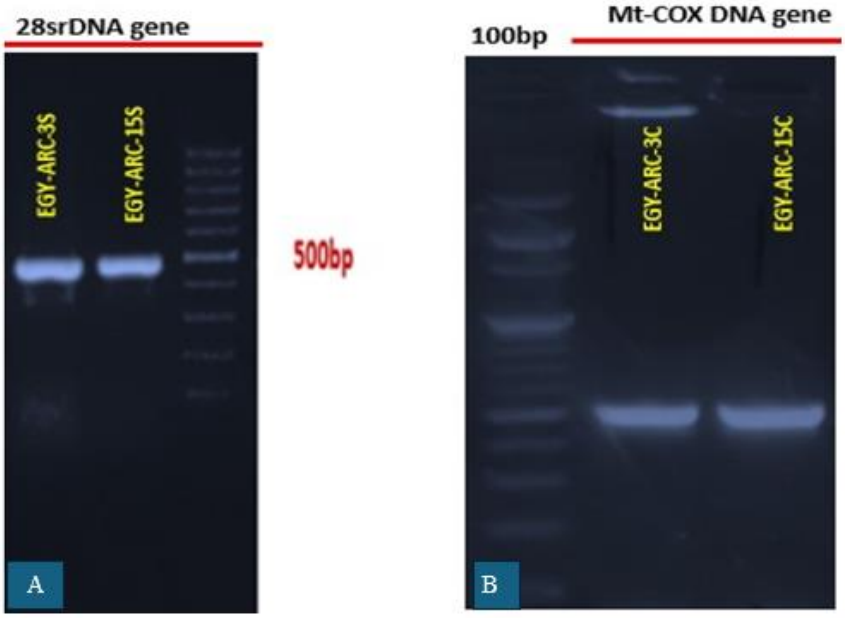


Fig. 3: (A) gel electrophoresis of PCR product profiles of 28S rDNA gene for isolates: Lane (1): DNA marker 100bp, lane (2): EGY-ARC-3S, lane and (3): EGY-ARC-15S isolates. (B) gel electrophoresis of PCR product of mitochondrial COX1 gene for isolates: Lane (1): DNA marker 100bp, lane (2): EGY-ARC-3C, lane (3): EGY-ARC-15C isolates.

In the beginning, we first employed partial sequence analysis of the 28SrDNA gene, and we then used these sequences to clarify the genetic relationships among the Megophthalmine species. Two Megophthalmine species EGY-ARC-3S, and EGY-ARC-15S had alignments with *Japanagallia* sp. (accession number MK064037.1), and *Anaceratagallia* sp. (accession MK063929.1), respectively, with maximum identities of 95.34% and 99.62%. To accurately establish the phylogenetic relationships between Megophthalmine species and identify the existence of phylogenetic markers in DNA sequences, the neighborhood joining (NJ) approach of phylogenetic tree construction was selected (Fig. 5). Additionally, the phylogenetic tree that served as the final guide for the multiple alignments were constructed using the evolutionary distance of 28SrDNA sequences between Megophthalmine species, which was determined for each pair of sequences (Table 3). Furthermore, the 28SrDNA gene sequence analysis revealed that two Megophthalmine species EGY-ARC-3S and EGY-ARC-15S were *Au. sinuata* EGY-ARC3 and *An. laevis* EGY-ARC15. These data were subsequently submitted to the NCBI GeneBank with the corresponding accession numbers, LC670606.1, and LC670615.1 These results were similar to Dietrich (2022) who identified and characterized species of leafhoppers from a genus belonging to Megophthalminae.

Furthermore, the mitochondrial COX1 gene is conserved across species and evolves at a very moderate rate, it is frequently employed in evolutionary research. It is also used for phylogenetic research, species identification, and DNA barcoding. As a result, we employed COX1 gene sequence analysis, and these sequences were then utilized to clarify the genetic relationships among Megophthalmine species. The obtained sequences revealed that two Megophthalmine species (EGY-ARC-3C, and EGY-ARC-15C) significantly aligned with *Austroagallia caboverdensis* (accesssion number MH682020.1), and *Anaceratagallia lithuanica* (accesssion number MZ631325.1), respectively, with maximum identities of 99.99, and 99.98%, respectively. When building phylogenetic trees, the neighborhood

joining (NJ) approach was chosen because it allows for precise phylogenetic tree construction between Megophthalmine species and can identify phylogenetic signals in DNA sequences (Fig. 7). Our goal was to precisely define the evolutionary connections of the Megophthalmine species by analyzing their DNA sequences using the NJ technique. Their evolutionary and genetic relatedness would be shown by the phylogenetic tree that would develop. Furthermore, every pair of sequences in the Megophthalmine species was found to have an evolutionary distance of COX1 (Table 5), which was then utilized to build the phylogenetic tree that served as the final guidance for the multiple alignments.

Furthermore, the sequence analysis of the COX1 gene revealed that the two leafhopper species, designated as EGY-ARC-3C, and EGY-ARC-15C, were found to be *Au. sinuata* EGY-ARC-3C and *An. laevis* EGY-ARC-15C. These were subsequently submitted to the NCBI with the respective accession numbers, LC775124.1 and LC775136.1. Moreover, two leafhopper species' mitochondrial COX1 protein sequences EGY-ARC-3C and EGY-ARC-15C were subjected to amino acid variation and multiple sequence alignment, which were calculated and shown in (Fig. 8).

EGY-ARC-3S

```
  1 GTGTACGGAA AGACCTCGAC ACCGAAAGGG GAGATTCACG CTCACGCGCA TGAGTCGGCT
 61 CCCACCGGTC CAGATGTCAC CGTCCGCCCG CTCGGCGCAA GTCGTAGCGG GGTCGTGTCG
121 GTGGTCACTC GGAGCCGGTG GGGGTTATGC CGGCCGCGCT GGGCCGCACT TCTCCCTCAG
181 TAGGACGTAG CGACCTGTTG GGCGACGGTC GACGGCCTCG GTGGTAGCCC GGGACGGTGC
241 TTTCGGGCTC TCGTCCCGGA CCCCGGGAGT CCTGGCCGAC CGCCAGACGG TCTGAAACGC
301 AGGTGTTGGA CCGCCCCCTC GTAGGGCGTC CGGGCCGGTC GCAAGCTCGG GCGTTGCTCG
361 GGATGACGGA CCCCTGGTCC GGCTCCGGGG GAGTCCAAAT GTGGGGGGGG GCGTCATCCT
421 CCCCTTTGGG GATCCAAGGC TTTTCGTTTC CCGGGGTGCT TTCCCCCCCT TGTAAACCCT
481 ATCAACAAAC
```

EGY-ARC-15S

```
  1 ACATTTCTTT TAAAAAAATT TTAAAGGTAT TTTCTGCTCA ATGATTAATT TTAAATAGCT
 61 GCAGTATTTT GACTGTACAA AGGTAGCATA ATAATTAGTT TATTAATTGT AAACTGGAAT
121 GAATGAAAAT ATGAGGGATT AACTTTATTA TTAAAATTAT TAAAATTTTA TTTTTAAGTT
181 AAAAAGCTTA ATTGTATTTT AGGGACGATA AGACCCTATA GAGATTGATT TTCAATTTAT
241 ATTTTTTTAT TGTTAAATTT TGTTGATAAT TATTTATTGA AATTTTTGTT GGGGTGATAG
301 ATAAATTACA AACTTTATTA TTATATATCA ATTATTTTTG ATTAAATTGA TCTGTTATAA
361 AAATGATTAT AAGATTAAGC TACCTTAGGG ATAACAGCGT AATTTTAATG GGGAGTTCTT
421 ATTTATATTA AATTTTGCGA CCTCGATGTT GAATTAAGAT TAGATTTTGG GGTAGGTTTT
481 AATTTTTCTA AGTCTGTTCG ACTTTTAAAT TCTTACATGA TCTG
```

Fig. 4: The 28S rDNA subunit gene sequence of *Austroagallia sinuata* EGY-ARC-3S and *Anaceratagallia laevis* EGY-ARC-15S

Table 2: Similarity percentage of 28S rRNA gene for Megophthalminae generated by BLAST tools.

No.	Isolate code	Significant Alignments	E value	Per. Ident	Retrieved Accession	Strains	Submitted accession no.
	EGY-ARC-3S	*Austroagallia sinuata EGY-ARC-3S*	0.0	100.00%	LC670606.1	*Austroagallia sinuata EGY-ARC-3S*	LC670606
		Japanagallia sp. 1 QX-2018 DC91	6e-170	95.34%	MK064037.1		
		Platyproctus maculipes	1e-166	94.82%	AF304620.1		
		Japanagallia sp. 2 QX-2018 DC92	2e-164	94.55%	MK064038.1		
		Dryodurgades lamellaris AG2	3e-163	94.30%	MK064018.1		
3	**EGY-ARC-15S**	*Anaceratagallia laevis EGY-ARC-15S*	0.0	100.00%	LC670615.1	*Anaceratagallia laevis EGY-ARC-15S*	LC670615
		Anaceratagallia sp. DC94 large	0.0	99.62%	MK063929.1		
		Anaceratagallia venosa DC79	1e-167	87.59%	MK063930.1		
		Anaceratagallia venosa	1e-167	87.59%	MF162927.1		
		Japanagallia neohamata	7e-150	85.61%	MF162935.1		

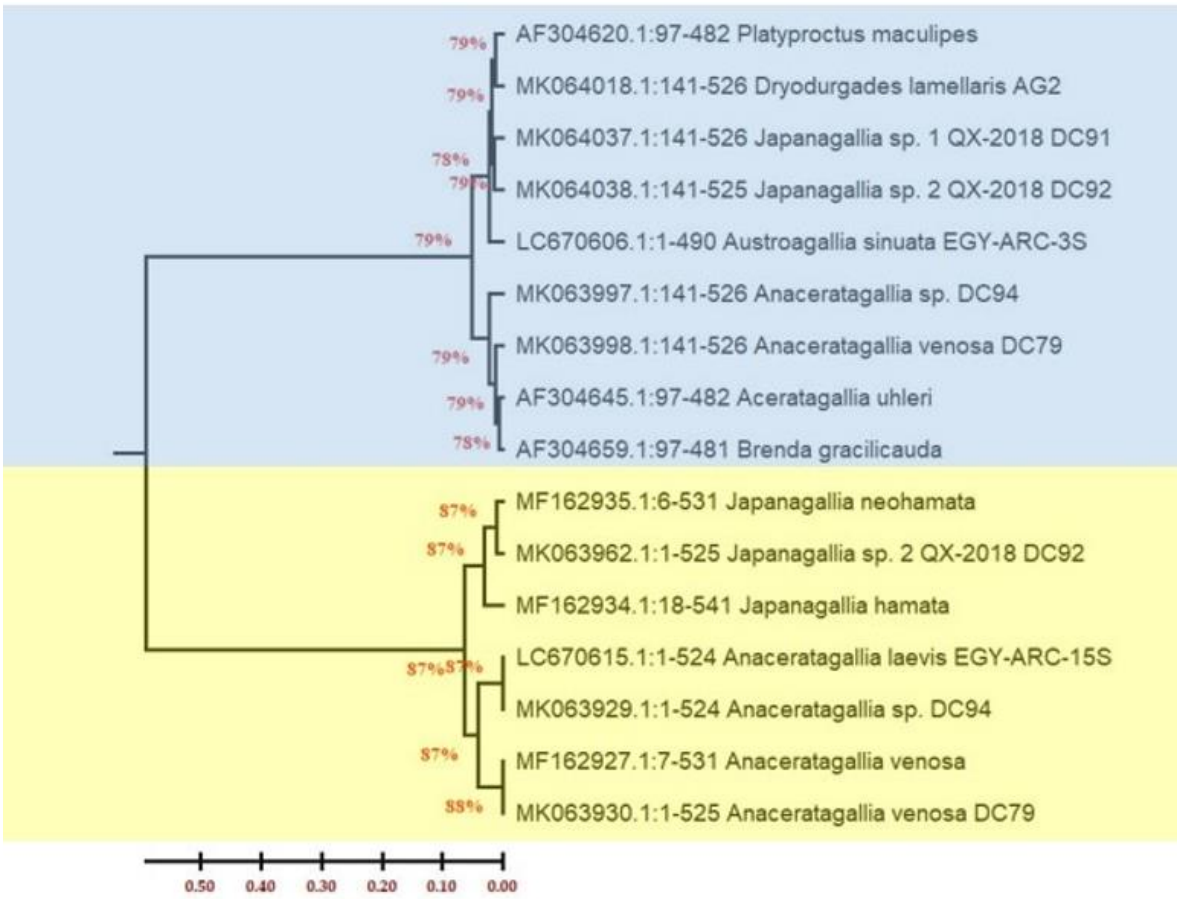


Fig. 5: NJ tree constructed using 28S gene sequence of Megophthalminae species from Egypt, with related species

Table 3: Estimates of Evolutionary Divergence of 28S sequences between Megophthalminae.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
LC670615.1:1-524_Anaceratagallia_laevis_EGY-ARC-15S															
MK063929.1:1-524_Anaceratagallia_sp._DC94	0.0000														
MK063930.1:1-525_Anaceratagallia_venosa_DC79	0.0823	0.0823													
MF162927.1:7-531_Anaceratagallia_venosa	0.0823	0.0823	0.0000												
MF162935.1:6-531_Japanagallia_neohamata	0.1088	0.1088	0.1263	0.1263											
MK063962.1:1-525_Japanagallia_sp._2_QX-2018_DC92	0.1149	0.1149	0.1417	0.1417	0.0192										
MF162934.1:18-541_Japanagallia_hamata	0.1301	0.1301	0.1476	0.1476	0.0603	0.0657									
LC670606.1:1-490_Austroagallia_sinuata_EGY-ARC-3S	1.1760	1.1760	1.2203	1.2203	1.1373	1.1780	1.2034								
MK064037.1:141-526_Japanagallia_sp._1_QX-2018_DC91	1.2213	1.2213	1.2648	1.2648	1.1521	1.2038	1.1944	0.0408							
AF304620.1:97-482_Platyproctus_maculipes	1.1651	1.1651	1.2191	1.2191	1.1379	1.1884	1.1811	0.0463	0.0376						
MK064038.1:141-525_Japanagallia_sp._2_QX-2018_DC92	1.1827	1.1827	1.2267	1.2267	1.1335	1.1846	1.1990	0.0491	0.0295	0.0347					
MK064018.1:141-526_Dryodurgades_lamellaris_AG2	1.2077	1.2077	1.2513	1.2513	1.1577	1.2094	1.2240	0.0520	0.0486	0.0266	0.0346				
MK063998.1:141-526_Anaceratagallia_venosa_DC79	1.1573	1.1573	1.2126	1.2126	1.1149	1.1573	1.1541	0.0913	0.1034	0.0992	0.0970	0.1090			
AF304645.1:97-482_Aceratagallia_uhleri	1.1216	1.1216	1.1875	1.1875	1.1010	1.1431	1.1189	0.1031	0.1154	0.1048	0.0998	0.1209	0.0292		
AF304659.1:97-481_Brenda_gracilicauda	1.1296	1.1296	1.1848	1.1848	1.0878	1.1296	1.1268	0.0973	0.1033	0.0991	0.0880	0.1088	0.0213	0.0132	
MK063997.1:141-526_Anaceratagallia_sp._DC94	1.1994	1.1994	1.2609	1.2609	1.1556	1.1994	1.1725	0.1030	0.1153	0.1049	0.1057	0.1147	0.0347	0.0538	0.0457

EGY-ARC-3C

```
1 ATAATTATTC GAATTGAATT AGGTCAACCT GGATCATTAA TTAATAATGA CCAGGTATAT
61 AATGTAGTAG TTACATCACA TGCTTTTATT ATGATCTTCT TCATAGTTAT ACCAATTATA
121 ATTGGGGGGT TCGGCAATTG ACTTTTACCT TTAATAATTG CTGCACCTGA CATAGCTTTT
181 CCACGATTAA ATAACATAAG ATTCTGATTA CTTCCCCCAT CTGTAACACT ATTACTGTCT
241 AGATCAATAG TAGAAACAGG GGCAGGGACA GGATGAACAG TTTACCCACC TTTATCCTCT
301 AATATTGCTC ATTCAGGAGC TAGAGTTGAT TTAGCTATTT TTTCTCTTCA CCTTGCAGGA
361 ATTTCTTCAA TTTTAGGAGC AATTAACTTC ATCACTACAG TTATTAATAT GCGTTCCCCA
421 GGAATAAAGA TAGACCAAAC ACCATTATTT GTTTGGTCAG TACTTATTAC AGCTATTCTA
481 CTTCTCCTGT CTCTACCAGT TTTAGCAGGA GCTATCACAA TACTATTAAC AGACCGAAAT
541 TAA
```

EGY-ARC-15C

```
1 ATATACTTTA TCTTTGGTAT ATGATCAGCA ATAGTAGGAA TAATACTTAG AATAATTATC
61 CGAATTGAAC TGGCTCAACC AGGCTCAATT ATTGGGAACG ATCAAGTATA TAATGTAATA
121 GTAACTTCAC ATGCATTTGT AATAATTTTC TTTATAGTAA TACCTATTAT AATTGGTGGT
181 TTCGGGAACT GACTACTACC TCTAATAATT GCAGCACCAG ACATAGCATT TCCTCGATTA
241 AACAACATAA GATTTTGACT TCTTCCTCCT TCAATCTCAT TATTATTAAC AAGATCATTA
301 ATTGAAATAG GTGCTGGGAC CGGATGAACA GTATACCCTC CCCTATCATC TAATATTGCA
361 CACGCTGGTC CAAGAGTAGA CATGGCAATT TTCTCACTTC ACTTAGCAGG AATTTCTTCA
421 ATCTTAGGAG CTATTAACTT TATCACTACC GTAATTAATA TACGATCTCC CGGTATGAAA
481 ATAGATCAAA CACCTCTTTT TGTATGATCA GTGCTAATTA CAGCAATTTT ATTACTTCTT
541 TCACTACCAG TTCTAGCGGG TGCTATTACT ATATTATTAA CAGATCGAAA TATTAACACA
601 TCATTCTTTG ACCCATCGGG AGGGGGTGAT CCAATCTTAT ATCAACACTT ATTTTAA
```

Fig. 6: The mitochondrial COX1 gene sequence of *Austroagallia sinuata* EGY-ARC-3C and *Anaceratagallia laevis* EGY-ARC-15C

Table 4: Similarity percentage of COX1 gene for Megophthalminae generated by BLAST tools.

No	Isolate code	Significant Alignments	E value	Per. Ident	Retrieved Accession	Strains	Submitted accession no.
2	**EGY-ARC-3C**	*Austroagallia sinuata EGY-ARC-3C*	0.0	100.00%	LC775124.1	*Austroagallia sinuata EGY-ARC-3C*	LC775124.1
		Austroagallia caboverdensis 18/6342	0.0	100.00%	MH682020.1		
		Austroagallia caboverdensis 18/6434	0.0	100.00%	MH682019.1		
		Austroagallia caboverdensis 18/6251	0.0	100.00%	MH682018.1		
		Austroagallia caboverdensis 18/6246	0.0	100.00%	MH682017.1		
3	**EGY-ARC-15C**	*Anaceratagallia laevis EGY-ARC-15C*	0.0	100.00%	LC775136.1	*Anaceratagallia laevis EGY-ARC-15C*	LC775136.1
		Anaceratagallia lithuanica TR983	0.0	99.98%	MZ631325.1		
		Anaceratagallia lithuanica TR982	0.0	99.79%	MZ631056.1		
		Anaceratagallia sp. isolate 1/2017_CBI	0.0	99.69%	OK205264.1		
		Anaceratagallia fragariae ANACER_02	0.0	98.93%	OQ469522.1		

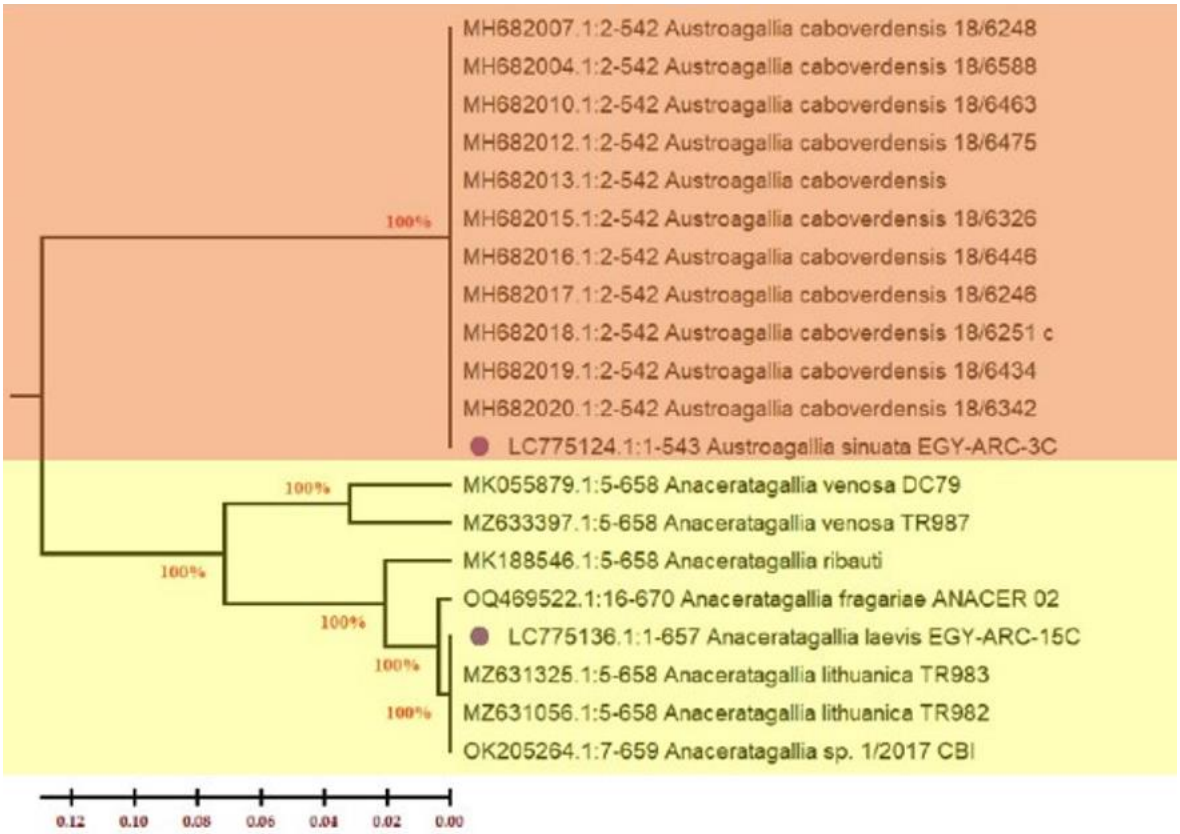


Fig. 7: NJ tree constructed using COX1 gene sequence of *Megophthalminae species* from Egypt, with related species.

Table 5: Estimates of Evolutionary Divergence of COX1 sequences between Megophthalminae.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	MZ631325.1:5-658_Anaceratagallia_lithuanica_TR983																		
2	MZ631056.1:5-658_Anaceratagallia_lithuanica_TR982	0.000																	
3	OK205264.1:7-659_Anaceratagallia_sp._1/2017_CBI	0.000	0.000																
4	OQ469522.1:16-670_Anaceratagallia_fragariae_ANACER_02	0.007	0.007	0.007															
5	MK188546.1:5-658_Anaceratagallia_ribauti	0.040	0.040	0.040	0.048														
6	MK055879.1:5-658_Anaceratagallia_venosa_DC79	0.141	0.141	0.141	0.146	0.139													
7	MZ633397.1:5-658_Anaceratagallia_venosa_TR987	0.143	0.143	0.143	0.148	0.143	0.064												
8	LC775124.1:1-543_Austroagallia_sinuata_EGY-ARC-3C	0.265	0.265	0.265	0.271	0.254	0.248	0.244											
9	MH682020.1:2-542_Austroagallia_caboverdensis_18/6342	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000										
10	MH682019.1:2-542_Austroagallia_caboverdensis_18/6434	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000									
11	MH682018.1:2-542_Austroagallia_caboverdensis_18/6251_c	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000								
12	MH682017.1:2-542_Austroagallia_caboverdensis_18/6246	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000							
13	MH682016.1:2-542_Austroagallia_caboverdensis_18/6446	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000						
14	MH682015.1:2-542_Austroagallia_caboverdensis_18/6326	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000					
15	MH682013.1:2-542_Austroagallia_caboverdensis_18/6552	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
16	MH682012.1:2-542_Austroagallia_caboverdensis_18/6475	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
17	MH682010.1:2-542_Austroagallia_caboverdensis_18/6463	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
18	MH682007.1:2-542_Austroagallia_caboverdensis_18/6248	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
19	MH682004.1:2-542_Austroagallia_caboverdensis_18/6588	0.263	0.263	0.263	0.268	0.251	0.245	0.242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

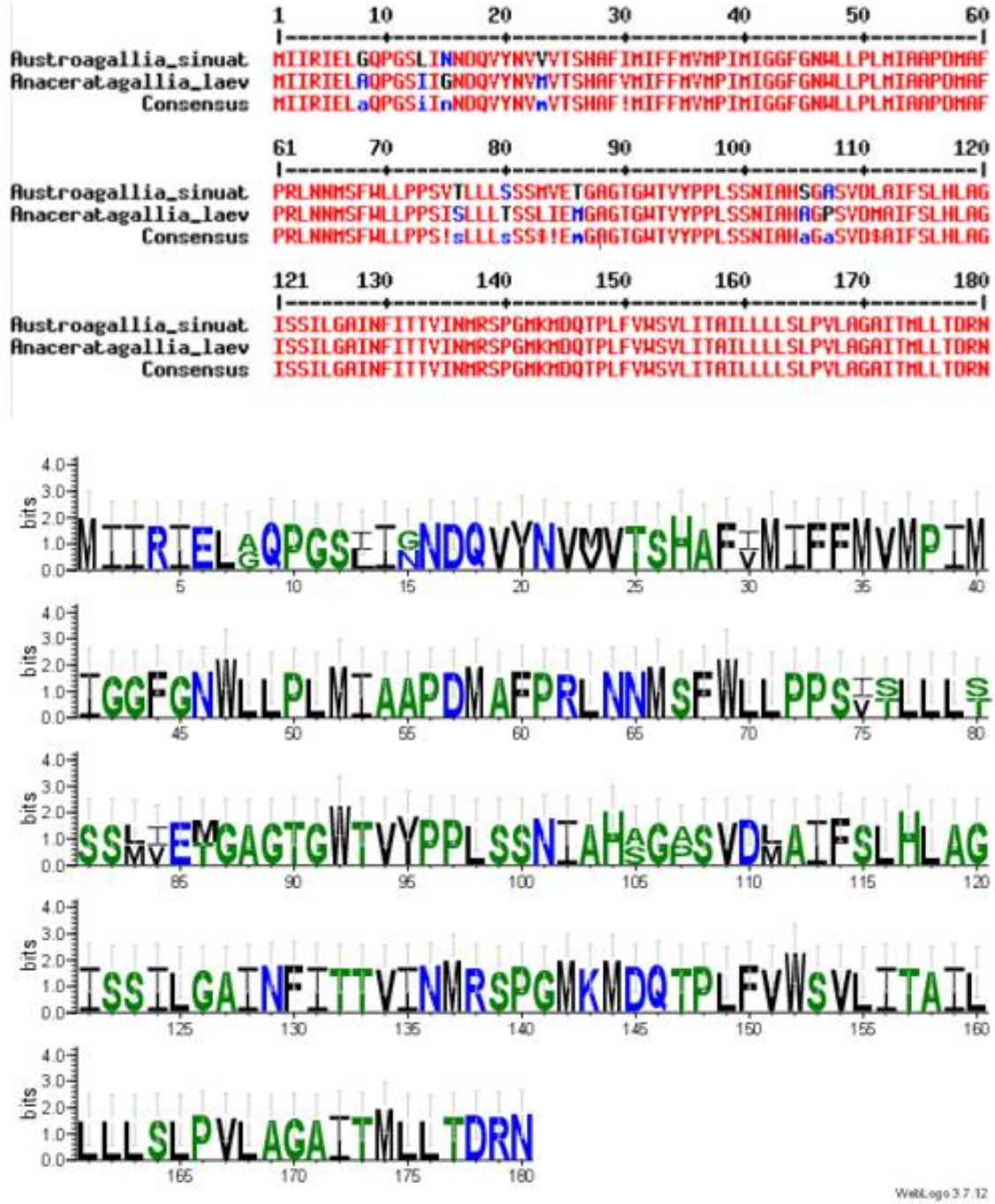


Fig. 8. (A) Amino acids variations of the COX1 gene generated by WebLogo3 server. (B) Multiple amino acids alinments for selected *Megophthalminae* isolates generated by MultAlin server

Prior research on Megophthalminae taxonomy has predominantly depended on morphology-based categorization, as stated by (Duan & Zhang 2014). Nevertheless, insufficient taxa have been included in previous phylogenetic studies to examine the connections between species within the genus or assess the borders between species. This emphasizes the necessity of more studies employing molecular methods to look into the taxonomic categorization and evolutionary links of Megophthalminae species. According to the findings of our investigation, species of Megophthalminae may be distinguished from one another using molecular identification based on the 28S DNA and COX genes. In particular, GenBank validated the sequences we sequenced for two species *Au. sinuata*

EGY-ARC-3 and *An. laevis* EGY-ARC-15. Table (6), contains a list of the accession numbers for these sequences. A dependable and precise way for differentiating between Megophthalminae species is provided by this genetic barcoding methodology, which has applications in conservation biology, pest control, and biodiversity evaluation.

Table 6: NCBI accession numbers of the studied Megophthalminae, the accession numbers provided from NCBI for the submitted sequences.

No	Code	Strain	Subfamily	Accession number submitted to GenBank	
				28SrDNA	COX1
1	EGY-ARC-3	*Austroagallia sinuata* (Mulsant and Rey)	Megophthalminae	LC670606.1	LC775124.1
2	EGY-ARC-15	*Anaceratagallia laevis* (Ribaut)	Megophthalminae	LC670615	LC775136.1

Conclusion:

The inability to differentiate between leafhopper species using only male genitalia and the instability of morphological traits that altered in response to host plant impacts and geographical connections are two drawbacks of traditional morphological approaches. Molecular barcoding using the 28S DNA and COX sequence provides a great alternative marker for characterizing and identifying two different species of Megophthalminae obtained from Egypt, at any stage of their life cycle, and for resolving other morphological identification problems.

Declarations:

Ethical Approval: Not applicable.

Authors Contributions: I hereby verify that the author mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

Competing Interests: The authors declare that they have no competing interests.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Source of Funding: The current research was not funded.

Acknowledgements: The authors appreciate to the staff members of the Insect Taxonomy Department, Plant Protection Research Institute, Dokki, Giza, for providing critical assistance throughout this study.

REFERENCES

Campbell, B. C., Steffen-Cmpbell, J.D., Werren, J.H. (1993) Phylogeny of the Nasonia species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology,* 2 (4): 225-237.

Dietrich, C. H. (2022). A New genus of flightless leafhoppers (Hemiptera: Cicadellidae: Megophthalminae) from the Pramo of Northern South America. *Zootaxa,* 5141(3), 270-276.

Dietrich, C.H. & Rakitov, R.A. (2002) Some remarkable new deltocephaline leafhoppers (Hemiptera: Cicadellidae: Deltocephalinae) from the Amazonian rainforest canopy. *Journal of the New York Entomological Society*, 110, 1–48.

Dietrich, C.H. (2005) Keys to the families of Cicadomorpha and subfamilies and tribes of Cicadellidae (Hemiptera: Auchenorrhyncha). *Florida Entomologist,* 88(4), 502-517. https://doi.org/10.1653/0015-4040

Dietrich, C.H., Fitzgerald, S.J., Holmes, J.L., Black, W.C., IV & Nault, L.R (1998) Reassessment of *Dalbulus* leafhopper (Homoptera:Cicadellidae) phylogeny based on mitochondrial DNA sequences. *Annals of the Entomological Society of America*, 91, 590–597.

Dietrich, C.H., Whitcomb, R.F. & Black, W.C., IV. (1997) Phylogeny of the grassland leafhopper genus Flexamia (Homoptera: Cicadellidae) based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 8, 139–149.

Duan, Y and Zhang Y. 2014 Review of the grassland leafhopper genus *Nephotettix* Matsumura (Hemiptera: Cicadellidae: Deltocephalinae: Chiasmini) from the Chinese mainland. *Zootaxa*, Vol. 3755 No. 3.

Evans, J.W. (1935) The Bythoscopidae of Australia (Homopera: Jassoidea). Papers and Proceedings of Royal Society of Tasmania, 1935, 61–63.

Fang Q.Q., Black 4th W.C., Blocker H.D. & Whitcomb R.F. 1993. A phylogeny of New World Deltocephalus-like leafhopper genera base on mitochondrial 16S ribosomal DNA sequences. *Molecular Phylogenetics and Evolution,* 2 (2): 119–131. https://doi.org/10.1006/mpev.

Fieber, F. X. (1868) Europäische neue oder wenig bekannte Bythoscopida. *Verhandlungen der Zoolosch-botanischen Gesellschaft in Wien*, 18. 449–464.

Fieber, F. X. (1872) Berichtigungen zu Dr. Kirschbaum's Cicadinen der Gegend von wiesbaden, Frankfurt a. M. und anderer Gegenden. Wiesbaden (Niedner) 1968 und Aufschlüsse über einige Cicadinen in der vorm. Germar'schen Sammlung. *Verhhandlungen der Zoolosch-botanischen Gesellschaft in Wien,*22, 27–34.

Flor, G. (1861) Die Rhynchoten Livlands in systematischer Folge beschrieben. Zweiter Teil: Rhynchota gulaerostria Zett. (Homoptera Auct). Cicadina und Psyllodea. *Archiv für die Naturkunde Liv.-Est-u.Kulands*, 4 (2), 1–567.

Gnaneswaran R, Hemachandra K, Wijegunasekara H, Ahangama D. 2010. A key for identification of Deltocephaline genera (Hemiptera: Cicadellidae: Deltocephalinae) associated with vegetable ecosystem in Sri Lanka. *Tropical Agriculture Research*, 21:421–426.

Habib A, El Kady E, Herakly F. 1975a. Taxonomy of Jassids infesting truck crops in Egypt, (Hemiptera: Jassids). I Taxonomy of Subfamily Typhlocybinae. *Bulletin of the Entomological Society of Egypt,* 59:331–343.

Habib A, El Kady E, Herakly F. 1975b. Taxonomy of Jassids infesting truck crops in Egypt, (Hemiptera: Jassids). II. Taxonomy of subfamily Euscelinae, tribe Euscelini. *Bulletin of the Entomological Society of Egypt,* 59:363–380.

Habib A, El Kady E, Herakly F. 1976. Taxonomy of jassids infesting truck crops in Egypt (Hemiptera: Jassidae). Taxonomy of subfamily Euscelinae, tribe Macrostelini. *Bulletin of the Entomological Society of Egypt,* 60::197–211

Hancock, J.M., Tautz, D., Dover, G.A. 1988. Evolution of the secondary structure and compensatory mutation of the ribosomal RNAs of Drosophila melanogaster. *Molecular Biology and Evolution,* 5 (4): 393-414.

Kameswara Rao, P., Ramakrishnan, U. & Ghai, S. (1979) Description of *Austroagallia* afganistanensis sp. nov. (Agallidae: Homoptera). *Current Science*, 48, 655–656.

Kirschbaum, C.L. (1868) Die Cicadinen der gegend von Wiesbaden und Frankfurt A. M. nebst einer anzahi neuer oder Schwer zu unterscheidendender Arten aus anderen Gegenden Europ's Tabellarisch Beschriben. *Jahrbuch des Nassauischen vereins für Naturkunde,* 21–22, 1–202.

Knight WJ. 1983. The leafhopper genus *Batracomorphus* (Cicadellidae, Iassinae) in the eastern, Oriental and Australian regions. *Bulletin of the British Museum Entomology*, 47:27–210.

Linares, A.R., Hancock, J.M., Dover, G.A. 1991. Secondary structure constraints on the evolution of Drosophila 28S ribosomal RNA expansion segments. *Molecular Biology and Evolution,* 219 (4): 381-390.

Löw, P. (1885) Beitrage zur Kenntinis der Cicadinen. *Verhandlungen der Zoolosch-botanischen Gesellschaft in Wien,*35, 343–358.

Mohammad, Z.K. (1996): Homoptera (in El-Moursy, A.A. ed.), Biological Diversity of Egypt (Insecta). The United Nations Environmental programme-published by the Egyptian Environmental Affairs Agency.

Mulsant, M.E. & Rey, C. (1855) Description de quelques Hémiptères-Homoptères nouveau ou peu conus. *Annales de la Société linnéene de Lyon,* 2, 197–249.

Nast, J. (1972) Palaearctic Auchenorrhyncha (Homoptera) an annotated checklist. Polish Sceintific Publisher, Warszawa. 550 p.

Nielson, M. W. (1979) Taxonomic relationships of leafhopper vectors of plant pathogens. In: Maramarosch, K. and Harris, K. F. (eds) Leafhopper vectors and plant disease agents. pp. 3–27. Academic Press, New York.

Oman PW, Knight WJ, Nielson MW. 1990. Leafhoppers (Cicadellidae): a bibliography, generic checklist and index to the world literature 1956–1985. Leafhoppers (Cicadellidae): a bibliography, generic check-list and index to the world literature 1956–1985.

Oman, P.W. (1949) The Nearctic leafhoppers: a generic classification and checklist. *Memoirs of the Entomological Society of Washington,* 3, 1–253.

Ribaut, H. (1935) Espèces nouvelles du genre *Agallia*. *Bulletin de la Société d'Histoire Naturelle de Toulouse,* 67, 29–36.

Tamura, K., Stecher, G., Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution*, 38:3022-302.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research*, 22:4673-4680.

Tishechkin D. YU. (2017). On the taxonomy of *Anaceratagallia* Zachvatkin, 1946 (Homoptera: Auchenorrhyncha: Cicadellidae: Agalliinae) of Western Kyrgyzstan. *Zootaxa,* 4273 (1) © 2017 Magnolia Press. https://doi.org/10.11646/ zootaxa. 4273.1.4

Viraktamath CA. (2005) Key to the subfamilies and tribes of leafhoppers (Hemiptera; Cicadellidae) of the Indian Subcontinent. *Bionotes,* 7(1):20–4.

Viraktamath, C.A. (2011) Revision of the Oriental and Australian Agalliini (Hemiptera: Cicadellidae: Megophthalminae). *Zootaxa,* 2844, 1–118.

Viraktamath, C.A., Dai, W. & Zhang, Y.-L. (2012) Taxonomic revision of the leafhopper tribe Agalliini (Hemiptera: Cicadellidae: Megophthalminae) from China, with description of new taxa). *Zootaxa,* 3430, 1–49.

Vismara, F. (1878) Note emitterologiche. Nota III. Sopra I generi Deltocephalus Burm. ed Agallia Curt. *Bollettino della Societá entomologica italiana,* 10, 34–42.

Weintraub, P.G. & Beanland, L. (2006) Insect vectors of phytoplasmas. *Annual Review of Entomology,* 51, 91–111.

Zahniser JN, Dietrich CH. 2010. Phylogeny of the leafhopper subfamily Deltocephalinae (Hemiptera: Cicadellidae) based on molecular and morphological data with a revised family-group classification. Systematic *Entomology,* 35:489–511.

Zakhvatkin, A.A. (1946) Studies on the Homoptera of Turkey. I–VII. *Transactions of the Royal Entomological Society of London,* 97 (6), 149–176.https://doi.org/10. 1111/j.1365-2311.1946.tb00278.