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Research article

Implementing Mitochondrial Cytochrome Oxidase Subunit I (COI) Gene in Identifying *Rhopalosiphum* Koch. spp. In Iraqi Environments

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ABSTRACT

Aphid species are usually identified by microscopic examination of morphological features. This process requires significant expertise and is difficult in immature aphid stages that lack many diagnostic morphological features in addition to the characterization of polymorphism in this group. The current study used molecular technique by detection of mitochondrial cytochrome oxidase subunit I (COI) gene using Lep primer pairs (Forward and Reverse) to identify local *Rhopalosiphum* Koch. spp. these aphid samples were previously characterized by morphological features, and then compared between these two identification methods. Only 5% of samples were not detected by this primer. The DNA amplification product of the mitochondrial COI gene was subjected to direct sequencing. The sequences of the mitochondrial COI gene were obtained from 11 samples and each sample consisted of approximately 710 nucleotides. The results were compared to the Genbank database of the NCBI (National Center for Biotechnology Information), and The sequencing results showed that the 10 samples of *Rhopalosiphum* had percentage similarities between 98-100% with *Rhopalosiphum Padi* and *Rhopalosiphum miadas*, and one sample couldn't find similarity to them in GenBank. The present study approves the importance of designing a specific primer that can differentiate between local *Rhopalosiphum* spp. that could improve the misidentification problems that are intrinsic to morphological features.

Keywords: Mitochondrial cytochrome oxidase subunit I, PCR, Phylogenetic analysis, *Rhopalosiphum* Koch. spp.

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1. INTRODUCTION

Aphids, as an important group of insects that belong to the order Homoptera, are very successful creatures with the most species diversity in temperate regions and worldwide distribution [1]. They may cause loss of plants directly or indirectly, plus the direct loss is made by heavy feeding from sap and includes weakness of plant and finally reduction in yield, they cause indirect loss by honeydew secreting on leaves and branches which absorb dust and also mold will start to grow and finally photosynthesis and yield will be reduced. Furthermore, it helps viruses and fungi to infect the plant [2]. Aphids are usually classified in the order Hemiptera, series Sternorryncha along with the psyllids, whiteflies, scale insects, and mealybugs, another approach puts aphids in the order Homoptera and suborder Sternorryncha. Further phylogenetic studies with molecular techniques are in progress there are only eight subfamilies in the family Aphididae [3]. There are many morphological anatomical studies conducted on aphids that provided a background for systematic

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studies of Aphids. Aphids have a high ability to adaption and change their morphology as a response to environmental factors. Many ecological physiological factors affect the morphology of aphids [4].

Using molecular techniques is a Promising technology for identifying the species of Aphids. Mitochondrial DNA (mt-DNA) was used as a molecular marker for molecular identification of aphid species (mt-DNA) is a small circular molecule ranging in size from 15 to 18 kilobase pairs (bp) [5]. Insect mt-DNA consists of thirty-seven genes including two ribosomal RNA (rRNA) genes, twenty-two transfer RNA (tRNA) genes, and thirteen protein-coding genes [5]. The region of mt-DNA containing cytochrome oxidase I and II (COI-II) has been used in systematic and population genetic studies of insects, including aphids [6]. Molecular identification techniques are not only used to identify Aphid species but they are also used to corroborate morphological identifications that provide good evidence for constructing phylogenetic relationships [7].

In this study we applied common primer pairs (LepF and LepR) to identify local *Rhopalosiphum* aphids specimens, these specimens were previously identified by morphological features.

2. MATERIALS AND METHODS

2.1. Sample collection

In this study, aphid samples were collected from different geographic locations in Iraqi provinces (*Baghdad and Diyala)]* over a period of six months (11.2014 to 4.2015), from various grain crops and grasses. All collected samples were exclusively apterous viviparous females. The samples were kept in plastic containers or bags and then transferred to the laboratory. The data collected at the time of the sample collection such as host plant, locality, and species information. Table 1 shows the sample data. The samples were stored in 90-95% ethyl alcohol at 4 ^{o}C.

Table 1. The sample information about the *Rhopalosiphum* spp.

No.	Place of collection	Date	Host plant(s)	Common name
A 10	Baghdad/Abu Ghraib	25\11\2014	*Sorghum halepense*	Johnson grass
D 11	Diyala/ Khan Banisaad	02\12\2014	*Cynodondactylon.*	Bermuda grass
A 16	Baghdad/Abu Ghraib	15\12\2014	*Cyperus* sp.	sedges
D 2	Diyala/ Khan Banisaad	2\12\2014	*Cynodondactylon.*	Bermuda grass
J 5	Baghdad / al-jadiriya	20\11\2014	*Zea mays*	corn
J 6	Baghdad / al-jadiriya	23\11\2014	*Loliumrigidum*	tufted grasses
J 9	Baghdad / al-jadiriya	23\11\2014	*Sorghum halepense*	Johnson grass
J 17	Baghdad / al-jadiriya	13\01\2015	*Cynodondactylon.*	Bermuda grass
A 19	Baghdad/Abu Ghraib	15\01\2015	*Triticum*sp.	Wheat
A 15	Baghdad/Abu Ghraib	15\01\2015	*Zea mays*	corn
D 25	Diyala/ Khan Banisaad	22\01\2015	*Hordeum*sp.	Barley

2.2. Samples preparation

The standard method of Blackman and Eastope, 2000 was followed for preparing balsam mounts. Briefly, samples were heated in 95% ethanol for 10 minutes. Aphids were placed in 10% KOH solution and then heated for 10-20 minutes (or in 40% KOH for 2 to 5 minutes until the body contents were liquefied, and then transferred to 5 ml of distilled water. The brownish fluid streams from the body through the needle hole should appear and the anus and genital opening and left in distilled water for an hour. A drop of balsam was placed on the center of the slide, then the aphid was placed in the drop, the appendages were spread, and the cover slip was gently placed on the specimen then left for 1-2 weeks at 50°C for drying. The Slides were stored vertically in slotted cabinet drawers and boxes. Thick card labels (with collection data) gummed to the slide with adhesive materials. The microscope was calibrated and the samples were examined under the light compound microscope (AmScope B490 Compound Binocular Microscope) [8].

2.3. Measurements

Antenna (ANT): Length of the antenna from the base of segment I to the tip of the *Processus terminalis* (ANT PT). Base antennal VI: Length from basal articulation to the distal end of primary rhinarium. *Processus terminalis* (ANT PT or PT): Length of ultimate antennal segment between the apical ends of the primary rhinarium to the tip of the segment. Body length (BL): Distance from the middle of the front to the base of the cauda. Cauda: Length of cauda from the middle of the base to the apex. Siphunculi: length of siphunculi from its base to apex. Ultimate rostral segment (URS): Length of the Ultimate rostral segment between the basal articulations of segment 4 to the tip of the rostrum. HT II: Length of Second segment of hind tarsus from basal articulation to the tip of the segment.

2.4. Treated with species in this study

Identification was done based on apterae. The Current scientific names were taken from the Survey of the World's Aphids [9]. Common names and keys for identifying the collecting samples are taken from Blackman and Eastop, (2006) [10].

2.5. Polymerase Chain Reaction

DNA was extracted from 82 samples by following the instructions of manfactural company (Geneaid™ DNA Isolation Kit). Three to four individuals (Aphids) were transformed into a 1.5 ml microfuge tube. The provided micro-pestle was used to grind the tissue. 200µL of GT buffer was added and the samples were homogenized sample. 20 µL of proteinase K was added and incubated at 60 ^{o}C for 30 min. 200µL of GBT buffer was added and the mixture was incubated at 60 ^{o}C for 20 min. The tubes were inverted every 5 min. 200µL of absolute ethanol was added to the lysate and the GD column was placed in 2ml collection tube. The mixture was transferred to a GD tube and centrifuge (12000 rpm for 2 min). After that 400µL of W1 buffer was added to the GD column and centrifuged at 14000 rpm for 30 seconds. The flow was discarded and then the GD column back in the 2ml collection tube. 600µL of wash buffer was added to the GD column and centrifuged at 14000 rpm for 30 seconds and then centrifuged for 3 min at 14000 rpm to dry the column matrix. The dried GD column was transferred to a clean 1.5 ml microfuge tube. 20µL of pre-heated Elution buffer was added to the column matrix. The tubes were incubated for 5 min to ensure the elution was completely absorbed. The tubes were centrifuged at

14000rpm for 30 seconds to elute the purified DNA. The last step was repeated twice. The primers were prepared to working stock I (20 pmol) from the original stock (100 pmol) according to the following formula:

$$V1 = 10\ ml\ of\ original\ stock + 40\ ml\ from\ double\ distilled\ water$$

The final concentration of primers was 0,4 µm\ml. Two primers used for the detection and amplification of the cytochrome C oxidase I gene, Lep F (5-ATTCAACCAATCTAAAGATATTGG-3) Lep R(5-TAAACTTCTGGATGTCCAAAAAATCA-3) were added immediately to the master mix (Table 2), then temple DNA (5-10) was added to the PCR tubes, after the optimization condition of PCR cycler (Table 3), using five temperature (45, 45.5, 46, 46.3, 46.7) and the optimal temperature was (46.7) [11].

Table 2. Contents of the PCR tube.

Components	Volume /µL
PCR master mix	lyophilized
Tamplet DNA	5-10µL
Primer Lep F	1 µL
Primer Lep R	1 µL
Double distilled water	38-43 µL

Table 3. Thermal cycler program for DNA amplification.

Temperature	Time period	
95 °C	4min	initial denaturation, Taq activation
95 °C	30 sec.	35 cycles
46.7 °C	30 sec.	
72 °C	60 sec.	
72 °C	5 min.	
4 °C	Forever	

Amplified PCR products were electrophoresis through 1.2 % agarose gel in Tris-Borate-Acetate (TBE) buffer. The target bands with a molecular weight of 710 pb were visualized by staining with ethidium bromide. The procedure depends on gel electrophoresis and was summarized as follows; 0.6 gm of agarose was dissolved in 45 ml of TBE buffer and then heated (till dissolved completely) and then cooled at 45 °C, then 2 µL of ethidium bromide was added. The gel was poured into the tray and put out the comb. The samples were loaded and the power supply selecting 75volt was switched on for 45 min.

2.6. The sequencing for PCR products

The PCR product of the cytochrome C oxidase I (COI) gene was subjected to direct sequencing to confirm the morphological identification, which was carried out using an Applied Biosystem Sequencer apparatus (3730XL USA).

3. RESULT & DISCUSSION

3.1. Classification and Morphological Characters

Aphids are determined mainly based on their morphol-ogical characters and morphometric parameters. Terminology and abbreviations of these characters and parameters according to [12, 8]. These characters are used for the identification of aphids at the genus and species level, at these levels some other characters may also be useful for identifications, such as degree of sclerotization, nature of the cuticular surface, the number, size, and shape of hairs or setae, body shape, particu-lar characters of head, thorax, abdomen, and their respective appendages in addition to the biological, anatomical and cytol-ogical data.

3.2. PCR identification

After optimization of the DNA extracted, we used three individuals from each sample, to improve the DNA concentration and purity was ranged from 9.7 to 131 ng and 1.4 to 2 respectively. These differences may be due to the weight of the insect, the sex male or female, and the aphid stage. Meanwhile, other investigators showed that DNA extraction from mosquito thorax only is better, because some parts of the insect do not give enough good quality DNA, especially the wings and legs; also the abdomen with extremities is full of PCR inhibitors. We used this study on the whole aphid body since apterous aphids lack wings and undifferentiated thorax [13]. The DNA barcoding was originally used for identifying unknown specimens [14]. The rapid DNA extraction method of DNA barcoding may be used as a complimentary tool for the characterization and discrimination of species however some issues need to be carefully considered, The concept of DNA barcoding also has molecular limitations, the examination is based on one gene or a segment of gene, which means that the molecular information available is limited [13].

Some of the insect species are easy to identify and categorize, while others such as aphid species are difficult because of their small size and morphological similarity. Moreover, it is further difficult to identify morphological variation due to environmental factors by available traditional methods [15]. To overcome these problems, advanced molecular techniques, randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and polymerase chain reaction (PCR) have been useful tools in assessing insect genetic diversity [16].

3.3. DNA Amplification

To improve the identification of aphids, this study used the mitochondrial COI gene. The result showed that 96% (24\25) of samples were amplified with common primer pairsLep forward, Lep reverses, to produce a specific band of approximately 710 bp of the mitochondrial genome (Fig. 1). While these pairs of primer failed 4% (1\25) in molecular detection. Other studies that used the mitochondrial cytochrome c oxidase I gene which has been adapted as the standard DNA barcode, that used for testing the effectiveness in the discrimination of over 300 species of aphids in Europe [17].

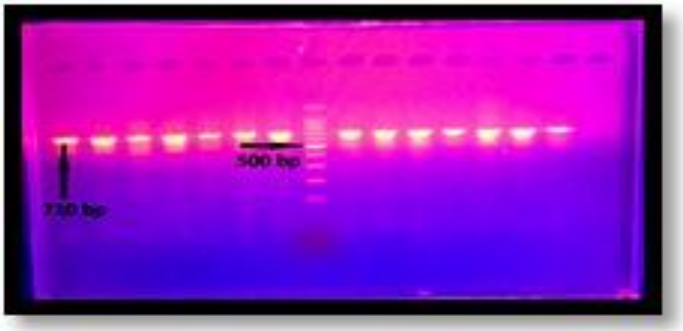


Fig.1. Agarose gel electrophoresis (1.2%) of amplified COI gene (710bp) of *Rhopalosiphum* sp.

Furthermore, other investigators designed new primers from local samples of their fauna, for instance, Wang *et al.* (2011) designed forward and reverse primers and reported that the sequence variation in a partial segment of the mitochondrial COI gene was highly effective for identifying species within Aphidinae, also thirty-six species of aphids were identified in neighbor-joining tree [18].

Since the amplification of the COI gene to separate Iraq's local *Rhopalosiphum* species using common primer pairs as LepF and LepR failed to give a good difference in size bands between *Rh. padi*, *R. maidis,* and other *Rh.* species, also In this respect, not only the COI analysis did not recover the differences between *Rh.*spp. but also doesn't improve the differences between several aphid genera [19]. So this study confirmed the importance of designing specific primers that show a size difference between *Rh. padi* and the other *Rh.* species.

3.4. Identification of *Rhopalosiphum* species by Sequencing:

The DNA amplification product of the COI gene was subjected to direct sequencing to confirm the morphological identification. The sequences were obtained from 11 samples for a forward primer and each sample consisted of 710 nucleotides. The obtained sequences of samples A15, A16, A19, D11, D25, J2, J10, J5, J6, J9, and J17 were analyzed and compared with the reference species available in the Genbank Database National Center for Biotechnology Information (NCBI). After Blast basic local alignment search tool, the sequencing results of COI gene confirmed that the 10 samples of *Rhopalosiphum* **s**p. had percentage similarities between 99-100% with *Rh. maidis* **(**5 samples) (Fig. 2) and *Rh. padi* (5 samples) (Fig. 3) species, while one sample was unknown *Rhopalosiphum* **s**pp.in GenBank (Fig. 4).

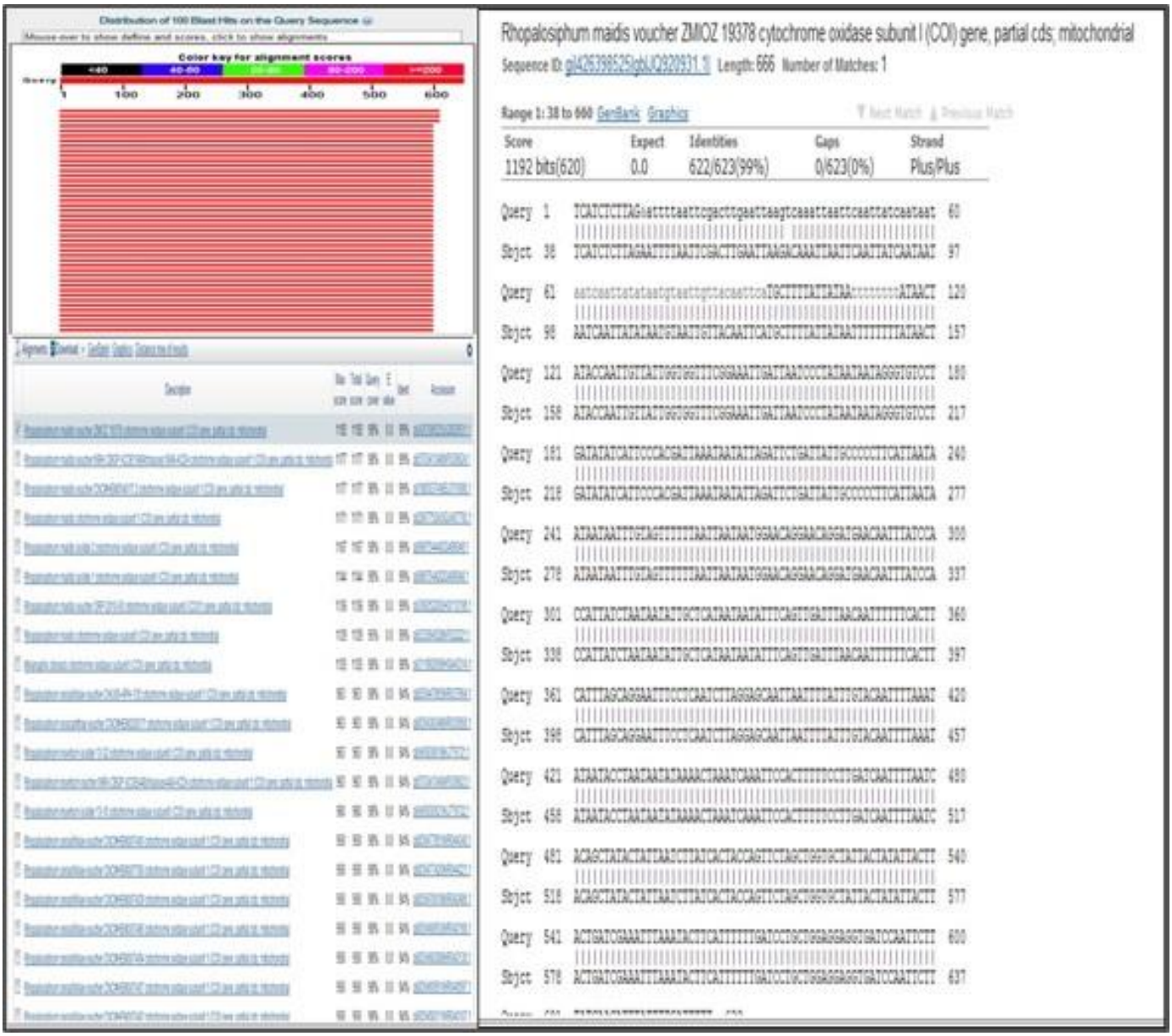
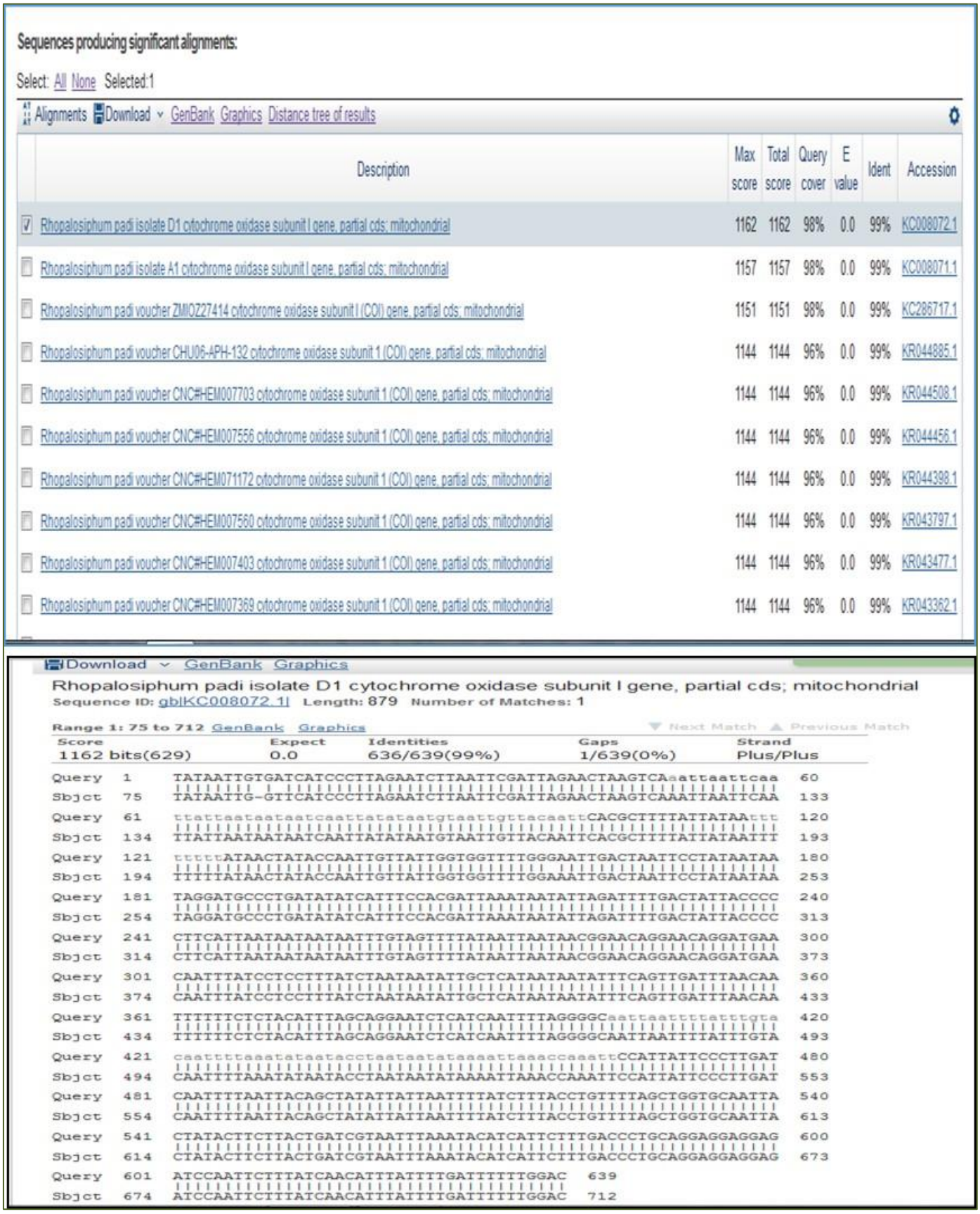


Fig. 2. Blasting and alignment against *Rhopalosiphum maidis* voucher ZMIOZ 19378 COI gene, partial cds; mitochondrial with accession number gil426398525uq920931.1 in Genbank of NCBI and high alignment scores ≥200. Identifies about 610 nucleotides with Gap 0% for J2, J6, J9, J10 and A19 Iraqi samples. (J2\ *Rhopalosiphum maidis).*



Sequences producing significant alignments:

Select: All None Selected:1

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	Rhopalosiphum padi isolate D1 cytochrome oxidase subunit I gene, partial cds; mitochondrial	1162	1162	98%	0.0	99%	KC008072.1
☐	Rhopalosiphum padi isolate A1 cytochrome oxidase subunit I gene, partial cds; mitochondrial	1157	1157	98%	0.0	99%	KC008071.1
☐	Rhopalosiphum padi voucher ZMI0Z27414 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1151	1151	98%	0.0	99%	KC286717.1
☐	Rhopalosiphum padi voucher CHU06-APH-132 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR044885.1
☐	Rhopalosiphum padi voucher CNC#HEM007703 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR044508.1
☐	Rhopalosiphum padi voucher CNC#HEM007556 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR044456.1
☐	Rhopalosiphum padi voucher CNC#HEM071172 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR044398.1
☐	Rhopalosiphum padi voucher CNC#HEM007560 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR043797.1
☐	Rhopalosiphum padi voucher CNC#HEM007403 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR043477.1
☐	Rhopalosiphum padi voucher CNC#HEM007369 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	1144	1144	96%	0.0	99%	KR043362.1

Download GenBank Graphics

Rhopalosiphum padi isolate D1 cytochrome oxidase subunit I gene, partial cds; mitochondrial

Sequence ID: gb|KC008072.1| Length: 879 Number of Matches: 1

Range 1: 75 to 712 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1162 bits(629)	0.0	636/639(99%)	1/639(0%)	Plus/Plus

```
Query  1    TATAATTGTGATCATCCCTTAGAATCTTAATTCGATTAGAACTAAGTCAaattaattcaa  60
Sbjct  75   TATAATTG-GTTCATCCCTTAGAATCTTAATTCGATTAGAACTAAGTCAAATTAATTCAA  133
Query  61   ttattaataataatcaattatataatgtaattgttacaattCACGCTTTTATTATAAttt  120
Sbjct  134  TTATTAATAATAATCAATTATATAATGTAATTGTTACAATTCACGCTTTTATTATAATTT  193
Query  121  tttttATAACTATACCAATTGTTATTGGTGGTTTTGGGAATTGACTAATTCCTATAATAA  180
Sbjct  194  TTTTTATAACTATACCAATTGTTATTGGTGGTTTTGGGAAATTGACTAATTCCTATAATAA  253
Query  181  TAGGATGCCCTGATATATCATTTCCACGATTAAATAATATTAGATTTTGACTATTACCCC  240
Sbjct  254  TAGGATGCCCTGATATATCATTTCCACGATTAAATAATATTAGATTTTGACTATTACCCC  313
Query  241  CTTCATTAATAATAATAATTTGTAGTTTTTATAATTAATAACGGAACAGGAACAGGATGAA  300
Sbjct  314  CTTCATTAATAATAATAATTTGTAGTTTTTATAATTAATAACGGAACAGGAACAGGATGAA  373
Query  301  CAATTTATCCTCCTTTATCTAATAATATTGCTCATAATAATATTTCAGTTGATTTAACAA  360
Sbjct  374  CAATTTATCCTCCTTTATCTAATAATATTGCTCATAATAATATTTCAGTTGATTTAACAA  433
Query  361  TTTTTTCTCTACATTTAGCAGGAATCTCATCAATTTTAGGGGCaattaatttttatttgta  420
Sbjct  434  TTTTTTCTCTACATTTAGCAGGAATCTCATCAATTTTAGGGGCAATTAATTTTATTTGTA  493
Query  421  caattttaaatataatacctaataatataaaattaaaccaaattCCATTATTCCCTTGAT  480
Sbjct  494  CAATTTTAAATATAATACCTAATAATATAAAATTAAACCAAATTCCATTATTCCCTTGAT  553
Query  481  CAATTTTAATTACAGCTATATTATTAATTTTATCTTTACCTGTTTTAGCTGGTGCAATTA  540
Sbjct  554  CAATTTTAATTACAGCTATATTATTAATTTTATCTTTACCTGTTTTAGCTGGTGCAATTA  613
Query  541  CTATACTTCTTACTGATCGTAATTTAAATACATCATTCTTTGACCCTGCAGGAGGAGGAG  600
Sbjct  614  CTATACTTCTTACTGATCGTAATTTAAATACATCATTCTTTGACCCTGCAGGAGGAGGAG  673
Query  601  ATCCAATTCTTTATCAACATTTATTTTGATTTTTTGGAC  639
Sbjct  674  ATCCAATTCTTTATCAACATTTATTTTGATTTTTTGGAC  712
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Fig. 3. Blasting and alignment against *Rhopalosiphum padi* isolate D1 COI gene, partial cds; mitochondrial with accession number KC008072.1 in Genbank of NCBI. Identifies about 610 nucleotides with Gap 0% for J5, J17, A15, A16 and D11 Iraqi samples. A16\ *Rhopalosiphum padi.*

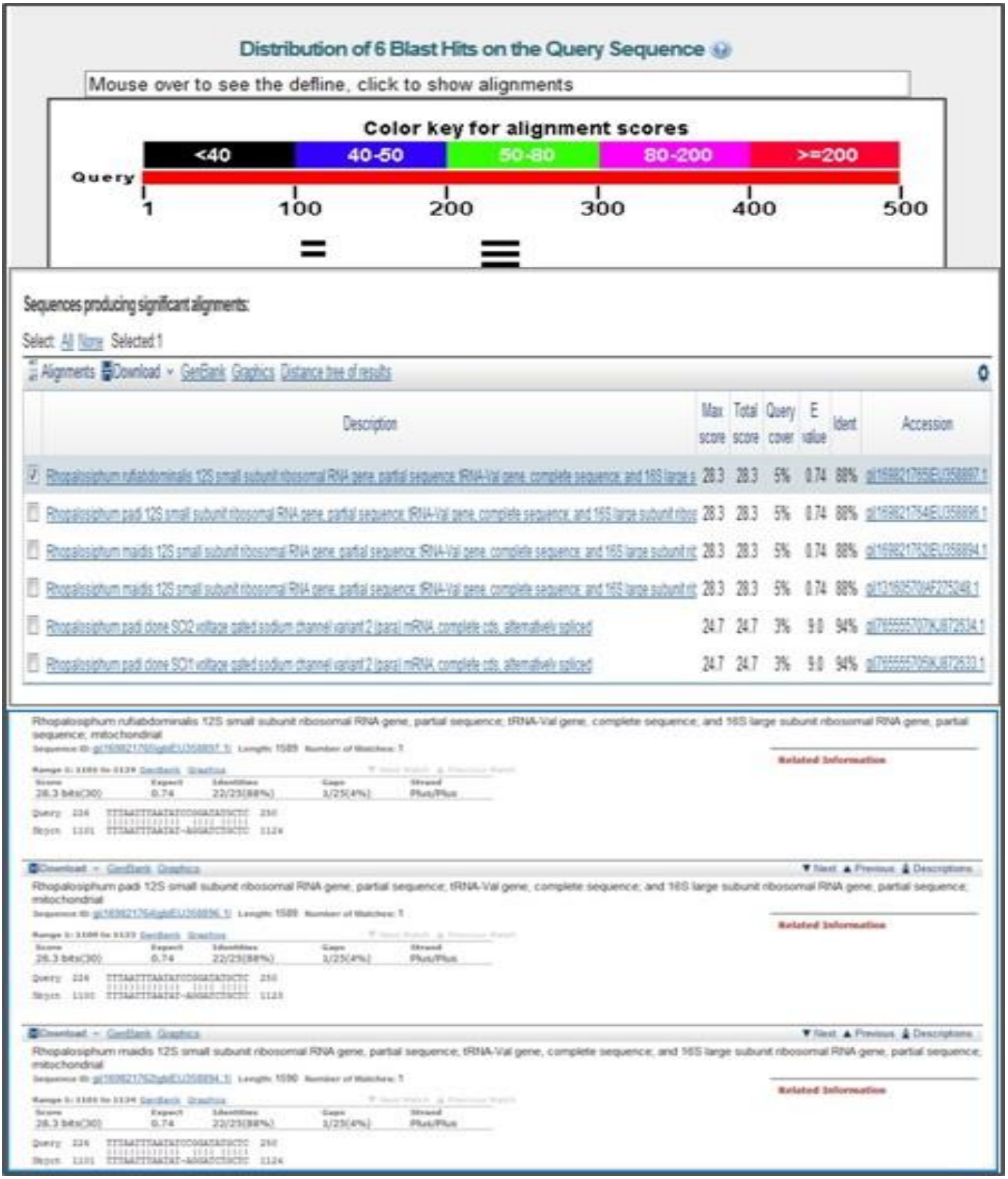

Fig 4. Blasting and alignment against *unknown Rhopalosiphum sp.*With low alignment scores (<40) in Genbank of NCBI. Identifies about 22 nucleotides with Gap 4% for the D25 Iraqi sample.

Similarly, the sequencing of *Rhopalosiphum* spp. and other aphid species has been carried out by other researchers comparing two gene sequences. Significant differences were also observed between DNA sequences from New Zealand *Rh.insertum* and a European *Rh. insertum* sequence deposited in GenBank [19].

Foottit *et al*. (2009) studied the application of DNA barcoding in which approximately 3600 specimens representing 568 species and 169 genera of the major subfamilies of aphids, they tested the effectiveness in the discrimination of over 300 species of aphids from more than 130 genera. Most (96%) species were well differentiated, and sequence variation within species was low, averaging just 0.2%, despite the complex life cycles and parthenogenesis reproduction of aphids [20]. The current study showed the Blasting and alignment in the Gene bank that the max score ranged from 1136 to 1194, query cover ranging from 97% to 99% and the E value was 0.0 for five samples as shown in J2 sample **(**Fig. 2), also for samples (J6, J9, J10, A19) have the same results with few alternations.

The max score ranged from 1109 to 1177, query cover ranged from 94% to 99%, E value was 0.0 for five samples as shown in the A16 sample **(Fig. 3**), also for samples (J6, J9, J10, A19) have the same results with few alternations.

Sample D25 had 610bp when sequenced but during blasting in the gene bank there were a few nucleotides (22bp) belonging to the same genus, and the max score was very low as shown in Fig 4, this referred to misidentification for this species in Genbank.

3.5. Molecular phylogenetic analysis

The relationships between *Rhopalosiphum* spp. samples used in this study were depicted in a dendrogram using the neighbor-joining method from the MEGA6 package program. The obtained sequences were analyzed using ClustalW for multiple alignments (11 sequences). The phylogenetic tree was rooted in D-25 which was the out group. Phylogenetic analysis in Fig 5, gave away 11 Iraqi samples and showed that closely related to each other with *Rh.maidis* and *Rh. padi.* Five samples were clustered in separate clades with *Rh.maidis* with a bootstrap value of 100% and another 5 samples located with *Rh.padi* with a bootstrap value of 90% that high similarity to each other, while the D25 sample appeared as out group from them.

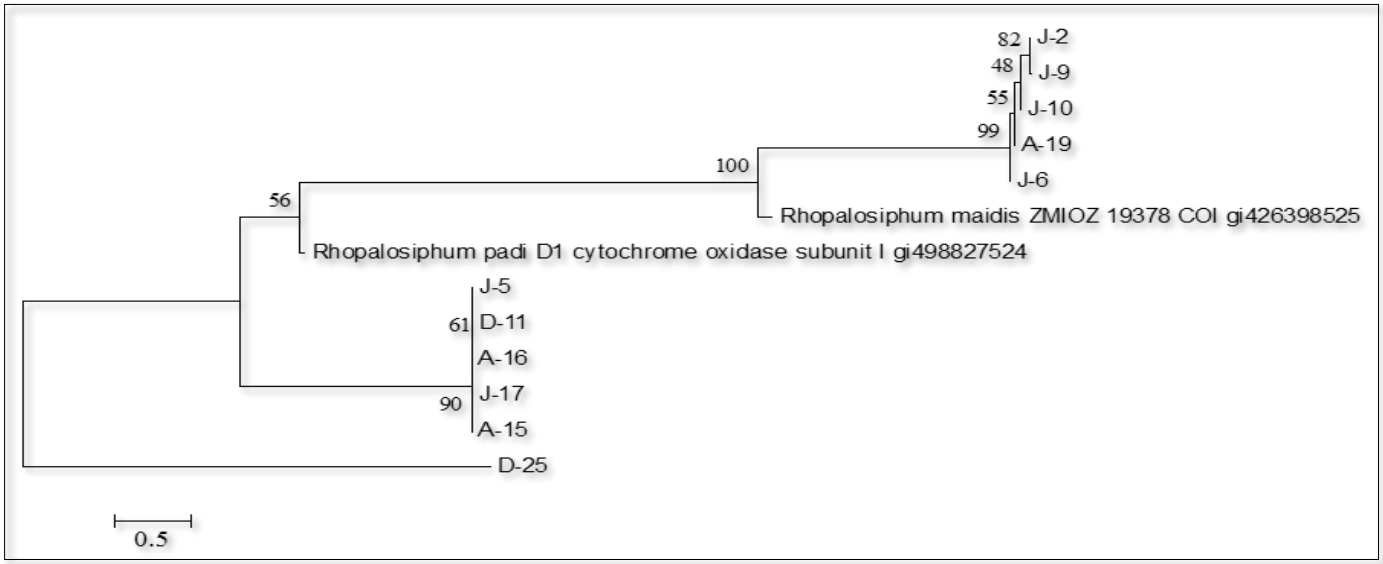

Fig. 5. Molecular phylogenetic analysis by Neighbor-joining method. Bootstrap values were 90-100, while the branches with lower values are shown with checkerboard lines. The analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 660 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

4. Conclusion

The current study demonstrated that it is possible to rely on molecular biology techniques in classifying aphids, as this technique can be an aid to phenotypic classification and can also be supportive of it as well, as sequencing of the Mitochondrial COI gene showed the close relationship between the Rhopalosiphum species collected from Iraqi environments with standardized mitochondrial COI gene in GenBank. The results showed that most of the isolates were genetically similar, except for only one isolate, D25 post applying a dendrogram using the neighbor-joining method from the MEGA6 package program.

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Conflict of interest

The authors declare that they have no conflict of interests.

Ethical Approval

This review was approved by the Ethical Committee of the University of Baghdad, Baghdad, Iraq (No 1094, 2020).

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