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Morphological and molecular identification of screwworm fly in Jeddah city: Genus *Chrysomya* (Calliphoridae)

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Abstract

Genus cChrysomya plays a very important role in the forensic entomology. Chrysomya belongs to family Calliphoridae and subfamily Chrysomynae. Calliphoridae distributed and found in all geographical areas of the world. In the present study, two very important Chrysomya species *C. albiceps* and *C. marginalis* were collected from different areas of Jeddah and identifies using morphological and molecular identifications. In our study for the morphological, dissecting microscope was used, and our results showed similarities with other studies. Mitochondrial cytochrome-c Oxidase-I (COX-1) gene was used for the molecular identification in this study to examine sequences polymorphism in between the collected bluefly samples. The resulting comparative molecular data set did not show any substitutions and thus revealed no information regarding biogeographic relationships. Building upon findings of our research, more morpholigican and molecular studies needed on different stages of chrysomya from different regions of Saudi Arabia.

Keywords: chrysomya, morphological, molecular, *C. albiceps*, *C. marginalis*, COX-1

Introduction

Genus chrysomya plays a very important role in the forensic entomology. Chrysomya belongs to family Calliphoridae and subfamily Chrysomynae. Calliphoridae distributed and found in all geographical areas of the world. These flies are of forensic and medical importance. They are attracted to spoiled meat and other decaying materials. Maggots of these flies are scavengers and feed on decaying animal carcasses. A Female lay up to 200-300 eggs on the flesh dead or wounded animals. Most of the members of Calliphoridae are metallic blue, green, bronze or brown yellow in color (AliKhan *et al*, 2016) [1]. Chrysomya has many species and are commonly referred as carrion flies because these flies are among the first to arrive at dead body (Byrd, 2009) [2]. Chrysomya species also cause myiasis in animals and humans. (Zumpt, 1965) [3]. These flies with other insects are usually used as an evidence during forensic investigations. Therefore, correct, and accurate identification is required. Morphologically the flies can be identified with the help of different available standard taxonomical keys.

Recently molecular techniques (DNA sequencing) are used for correct and reliable identification of different species of forensically important blow flies (Zaidi *et al*, 2011 and Harvey *et al*, 2003a, b) [4, 5, 6].

In the present study two very important Chrysomya species *C. albiceps* and *C. marginalis* were collected from different areas of Jeddah. These two flies already reported from this area of study by many previous workers (AliKhan *et al*, 2018, Al-Shareef *et al*, 2016 and Shaza Al-Qureshi, 2016) [1, 8, 9]. Their identification is purely based on external morphology of adult. In the present work DNA sequencing technique is used to identify *C. albiceps* and *C. marginalis* adults and compare our results with Calliphoridae species samples selected from the GenBank using BLAST software program for online phylogenetic analysis.

Material and Methods

Morphological identification of adults

Six sites were chosen as north, west, east, and south of Jeddah city using GPS (Global Positioning System) Garmin International, Inc., 1200 E, Olathe, KS, 66062, USA. A through sampling was carried out by installing the Final Flight Fly Trap in various habitats of the city. Three days old Beef liver was also used as a bait trap to attract the flies. The collected lived adult flies were brought to the laboratory and were kept in the deep freezer at -4C for 30 minutes after which the Calliphoridae flies were sorted out under the dissecting microscope

Molecular identification

DNA extraction

The collected samples were used in this investigation for total DNA extraction. The genomic DNA extraction and purification were performed. Each adult was homogenized in 50 µl of insect lysis buffer. All the homogenate samples were incubated in a water bath for 45 minutes at 58 ºC with quick addition of 5 µl of Proteinase-K for each homogenate during incubation. Then tubes were transferred and incubated in ice for 30 minutes before centrifugation. The clear supernatant in each tube was transferred into a fresh 1.5 ml Eppendorf tube. The precipitation and purification of the DNA from this supernatant was performed. The solubilized DNA samples was preserved at -24 ºC until the molecular analysis.

PCR teqnique and Nucleotide sequencing

Mitochondrial cytochrome-c Oxidase-I (COX-1) gene was used in this study to examine sequences polymorphism in

between the collected bluefly samples. The total DNA extract samples were used as templates to amplify ~ 670 bp fragments, including primers, specific to COX-1 gene of mt-DNA. Primers used to amplify the COX-I gene were composed of the DNA primers pairs, LCOI490 (forward primer): (5´-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (reverse primer): (5´-TAAACTTCAGGGTGACCAAAAAATCA-3')**.** PCR amplification products were purified using a gel purification kit (Hilden, Germany) and sent for commercial sequencing (Macrogen, Beotkkot-ro, Geumcheon-gu, Seoul, Korea) using the PCR primers in both directions**.**

Phylogenetic and sequences analysis

The obtained sequences of the COX-1 gene were used in this study to demonstrate the evolutionary relationships between the blue fly individuals obtained from the western areas of Saudi Arabia. The COX-1 gene sequences of the collected samples then compared with Calliphoridae species samples selected from the GenBank using BLAST software program for online phylogenetic analysis, embedded in PubMed from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic trees were constructed by using MEGA-X software (Kumar *et al*., 2018), to determine phylogenetic relationships and genetic variability between the collected samples and the selected GenBank Chrysomya species samples. Two phylogenetic trees were constructed, the first tree is based on the UPGMA algorithm within the Tamura-Nei genetic distance model, while the second tree is based on neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model.

Results

Morphological identification

The available taxonomic keys used for the identification of flies are Mc Alpine, 1989 [10]; Kamal, 1958 [11]; Kurahashi & Afzal, 2002 [12], Zumpt, 1965 [3] and AliKhan, *et al* 2016) [1]. Morphologically the subfamily Chrysomyinae species may be identified by the following diagnostic feature: Presence of a row of bristles on the dorsal surface of Stem-vein (R), Strong setae on greater ampulla, Lower calypter with dense hair, Gena is white, yellow, or orange, Arista plumose, Meso-thorax spiracle white, brown or creamy, Bright green metallic body, Thoracic transverse sutures are complete, Abdominal segments with narrow dark lines on the rear edge, Anterior thoracic spiracle white, 4th wing vein has sharp bend, Ocellar bristles and post ocellar setae are prominent and well developed, Wing with stem vein (R) bearing a row of bristles on the dorsal surface, Arista plumose. Hair on the Gena are silvery or white, 3 Ocelli present.

Chrysomya albiceps: (WEIDMANN, 1819)

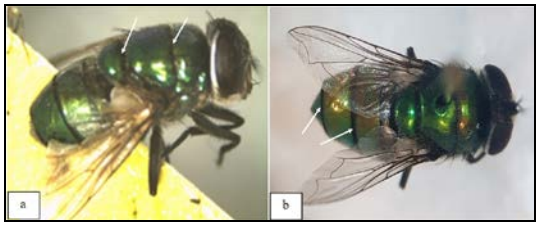

Fig 1 and 2: a-The dark lines on thorax. b-The dark lines on abdomen.

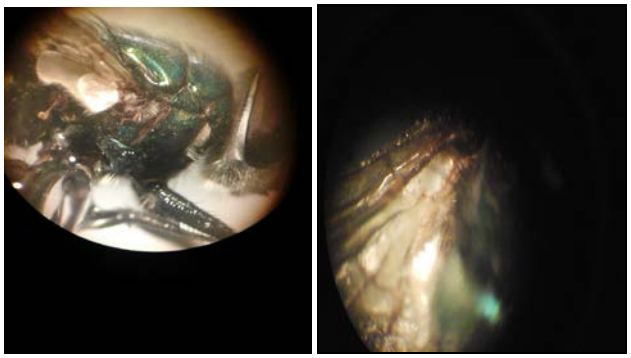

Fig 3 & 4: Thoracic spiracle white, strong setae on greater ampulla and hair on lower calypter, stem vein with row of bristles.

Chrysomya marginalis (WEIDMANN, 1830) **(Also known as *Crysomya regalis*)** (Robineau-Desvoidy, 1830):

Fig 5

Body metallic blue in color, Large with robust and cylindrical body, Thoracic and abdominal segments with dark bands on the rear edge, Head is pale yellow with yellow or orange hair on Gena. Compound eyes are read faceted, Fronto-orbital plate totally orange in female, Vibrissae well developed, Wings are hyaline with dark anterior margins (Main identifying feature among Chrysomya species), Male compound eyes are sharply demarked with large upper facets, Anterior spiracle white or cream color.

Molecular identification

DNA isolation

The DNA was extracted from 16 samples of group 1, and from 16 samples of group 2 using manual technique. DNA was detected by gel electrophoresis using 1% of gel concentration and bands pictures were taken by Ultraviolet as in figure 1 (a and b).

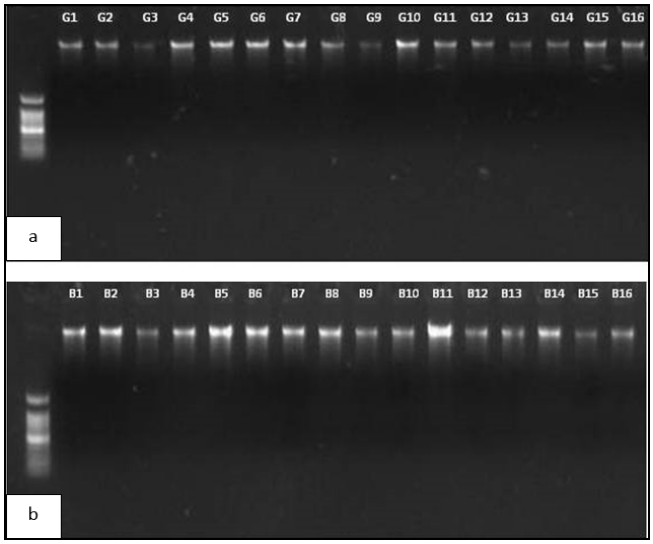

Fig 6: a-DNA bands of *C.albiceps.* b-DNA bands of *C.marginalis.*

PCR technique and Nucleotide sequencing 2.2 PCR technique

Figure 2 (a and b) shows the result of the polymerase chain reaction of the cytochrome c oxidase 1 (COI) gene. The picture shows the doubled bundles of the studied gene at a length of 700 base (bp), which shows the length of the DNA marker bundles, which gives fixed and different lengths of DNA bundles.

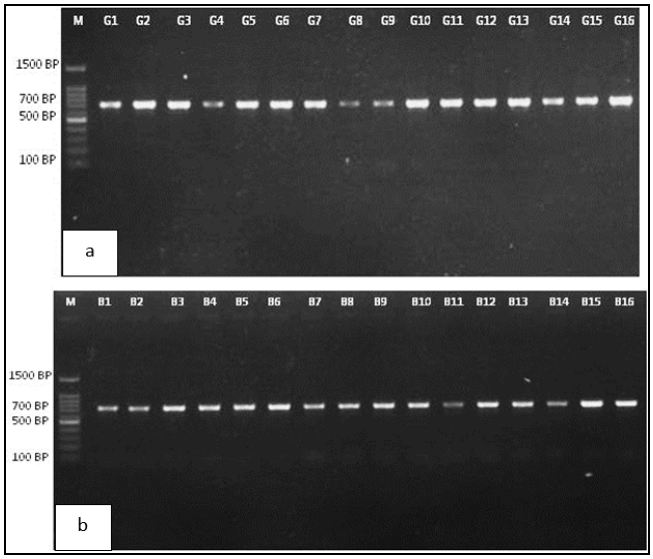

Fig 7: a- PCR products of the COI region of *C.albiceps.* b- PCR products of the COI region of *C.marginalis.*

A comparison was made of the MT-CO1 genetic sequences obtained from Mercogen Corporation - Korea (Macrogen, SEOUL, Korea), and available search tool (BLAST) of the website (www.blast.ncbi.nlm.nih.gov/Blast.cgi), the results showed a high similarity with the MT-CO1 gene sequences found in the NCBI database of Gene-Bank, where the match percentage in the first group was 100-99.52% with *C.albiceps* and the second group was 100-99.52% with *C.marginalis* as shown in Table (1 & 2).

Phylogenetic tree of the study samples

Alignment analysis of the MT-CO1 genetic sequence was done by Maximum Likelihood (ML) method and establishment of a phylogeny showing genetic evolution by determining the affinity distance between the studied samples and the available sequences from Gene Bank (NCBI) using (MEGA X) program**.**

Figure (3 - 4) showes the studied samples of genus *C.albiceps* among themselves and between the comparative samples on the Gene-Bank, where the samples were arranged according to their affinity and self-spacing, dividing the samples into four groups, the main group, showed that the samples G1, G2, G3, G4, G5, G6, G9, G10, G11, G13, G14 formed with the comparison samples a great similarity between them, and the second group branched out from them, G7, G12, while the third group formed it away from the main group, namely samples G15, G16, and the fourth group branched from it with sample G8. This difference resulted between the studied samples and between the comparison samples due to the presence of mutations in the gene sequence as shown in Figure (5-6).

Figure (7-8) refers to the studied samples of the genus *C.marginalis* between them and the comparison samples on the Gene-Bank. The samples were arranged according to their self-convergence and divergence. Dividing the samples into four groups, the main group showed that samples B1, B2, B4, B5, B6, B7, B9, B13, B14, B15, B16 formed with the comparison samples a great similarity and similarity between them, from which the second group branched out B8, B11, B12, as well as the third group branched out with sample B3, and also branched out from the fourth group with sample B10, resulting in This difference between the studied samples and between the comparison samples due to the presence of mutations in the gene sequence as shown in Figure (9-10).

Table 1: The comparison of *C.albiceps* with the data in the Gene Bank (NCBI)**.**

9	Genebank Code	Gene Published date	Organism and Gene Definition	Gene Length (bp)	Pre % Idnt	Country	References
G1, G2, G3, G4, G5, G6, G9, G10, G11, G13, G14	KX161542.1	28-APR-2016	*Chrysomya albiceps isolate P10A12 cytochrome oxidase subunit (COI) gene, partial cds; mitochondrial.*	677 bp	100	Spain	Fuentes-Lopez, A., Ruiz C., Galian, J. and Romera E. (2000) [13]: Molecular identification of forensically important fly species in Spain using COI barcodes. Science & Justice, 60(3): 293-302.
G7, G12	KX161558.1	28-APR-2016	*Chrysomya albiceps isolate P10A12 cytochrome oxidase subunit (COI) gene, partial cds; mitochondrial.*	677 bp	99.84%	Spain	Fuentes-Lopez, A., Ruiz C., Galian, J. and Romera E. (2000) [13]: Molecular identification of forensically important fly species in Spain using COI barcodes. Science & Justice Volume, 60(3): 293-302.

G8	KX161558.1	28-AUG-2014	Chrysomya albiceps haplotype XI cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	1489 bp	99.52%	Egypt	Salem, A.M., Adham, F.K. and Picard, C.J. (2015) [14]. Survey of the Genetic Diversity of Forensically Important Chrysomya (Diptera: Calliphoridae) from Egypt. n Journal of Medical Entomology. 52(3):320-328.
G15	KX161555.1	28-APR-2016	*Chrysomya albiceps isolate P10A12 cytochrome oxidase subunit (COI) gene, partial cds; mitochondrial.*	677 bp	99.84%	Spain	Fuentes-Lopez, A., Ruiz C., Galian, J. and Romera E. (2000) [13]: Molecular identification of forensically important fly species in Spain using COI barcodes. Science & Justice Volume 60(3): 293-302.
G16	KX161543.1	28-APR-2016	*Chrysomya albiceps isolate P10A12 cytochrome oxidase subunit (COI) gene, partial cds; mitochondrial.*	677 bp	99.84%	Spain	Fuentes-Lopez, A., Ruiz C., Galian, J. and Romera E. (2000) [13]: Molecular identification of forensically important fly species in Spain using COI barcodes. Science & Justice, 60(3): 293-302.

Table 2: The comparison of *C.marginalis* with the data in the Gen Bank (NCBI)*.*

No. Samples	Gene bank Code	Gene Published date	Organism and Gene Definition	Gene Length (bp)	Pre % Idnt	Country	References
B1, B2, B4, B5, B6, B7, B9, B13, B14, B15, B16	KM434353.1	28-AUG-2014	*Chrysomya marginalis haplotype V cytochrome coxidase subunit I (COI) gene, partial cds;mitochondrial*	1509 bp	100	Egypt	Salem, A.M., Adham, F.K. and Picard, C.J. (2015) [14]. Survey of the Genetic Diversity of Forensically Important Chrysomya (Diptera: Calliphoridae) from Egypt. N Journal of Medical Entomology. 2(3):320-328.
B3	KM434353.1	28-AUG-2014	*Chrysomya marginalis haplotype V cytochrome coxidase subunit I (COI) gene, partial cds;mitochondrial*	1509 bp	99.68%	Egypt	Salem, A.M., Adham, F.K. and Picard, C.J. (2015) [14]. Survey of the Genetic Diversity of Forensically Important Chrysomya (Diptera: Calliphoridae) from Egypt. N Journal of Medical Entomology. 52(3): 320-328.
B8, B10, B11, B12	KM434353.1	28-AUG-2014	*Chrysomya marginalis haplotype V cytochrome coxidase subunit I (COI) gene, partial cds;mitochondrial*	1509 bp	99.52%	Egypt	Salem, A.M., Adham, F.K. and Picard, C.J. (2015) [14]. Survey of the Genetic Diversity of Forensically Important Chrysomya (Diptera: Calliphoridae) from Egypt. N Journal of Medical Entomology. 52(3): 320-328.

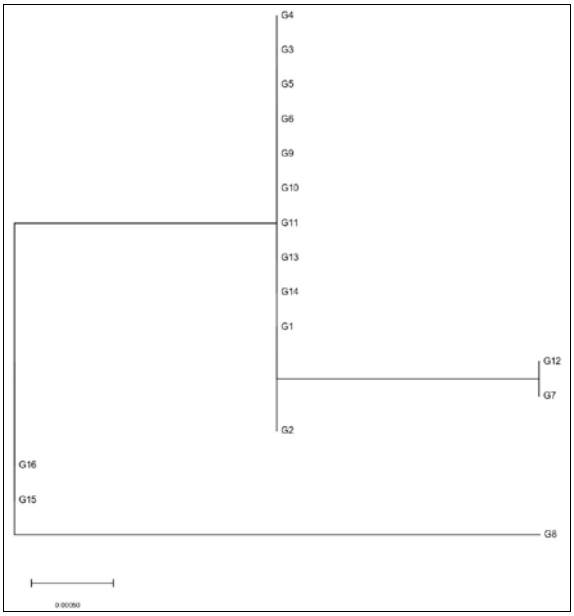

Fig 8: The phylogenetic tree of *C.albiceps* is illustrated by the evolutionary analysis by Maximum Likelihood method, using the MEGA X program

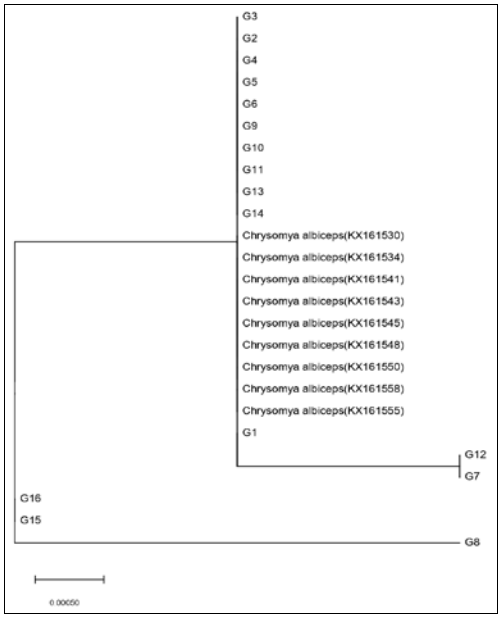

Fig 9: The phylogenetic tree of *C.albiceps* is illustrated by the evolutionary analysis by Maximum Likelihood method, using the MEGA X program.

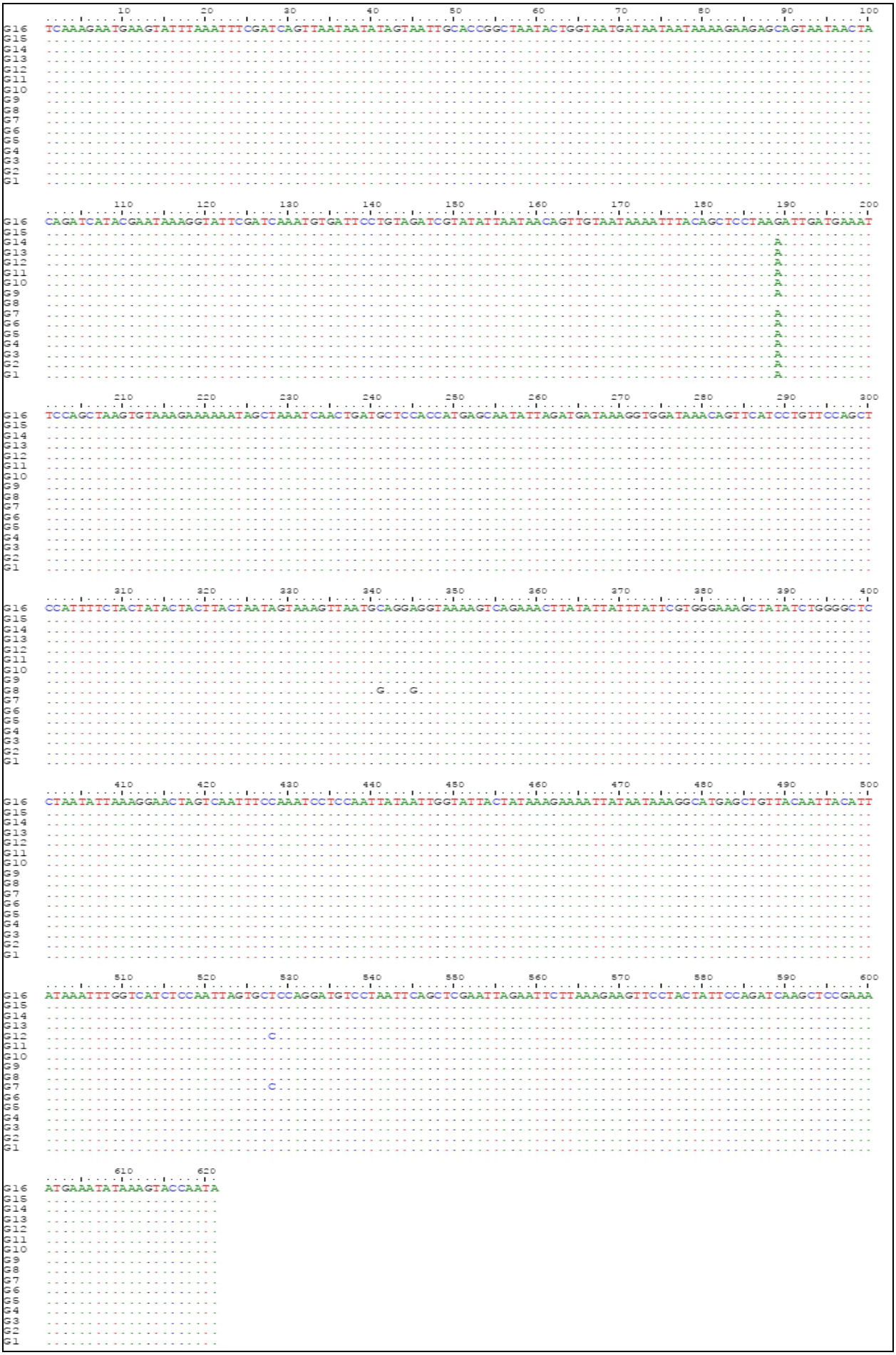

Fig 10: The comparison between the sequence of nitrogenous bases of *C.albiceps* MT-CO1 gene showing similarities and differences between samples.

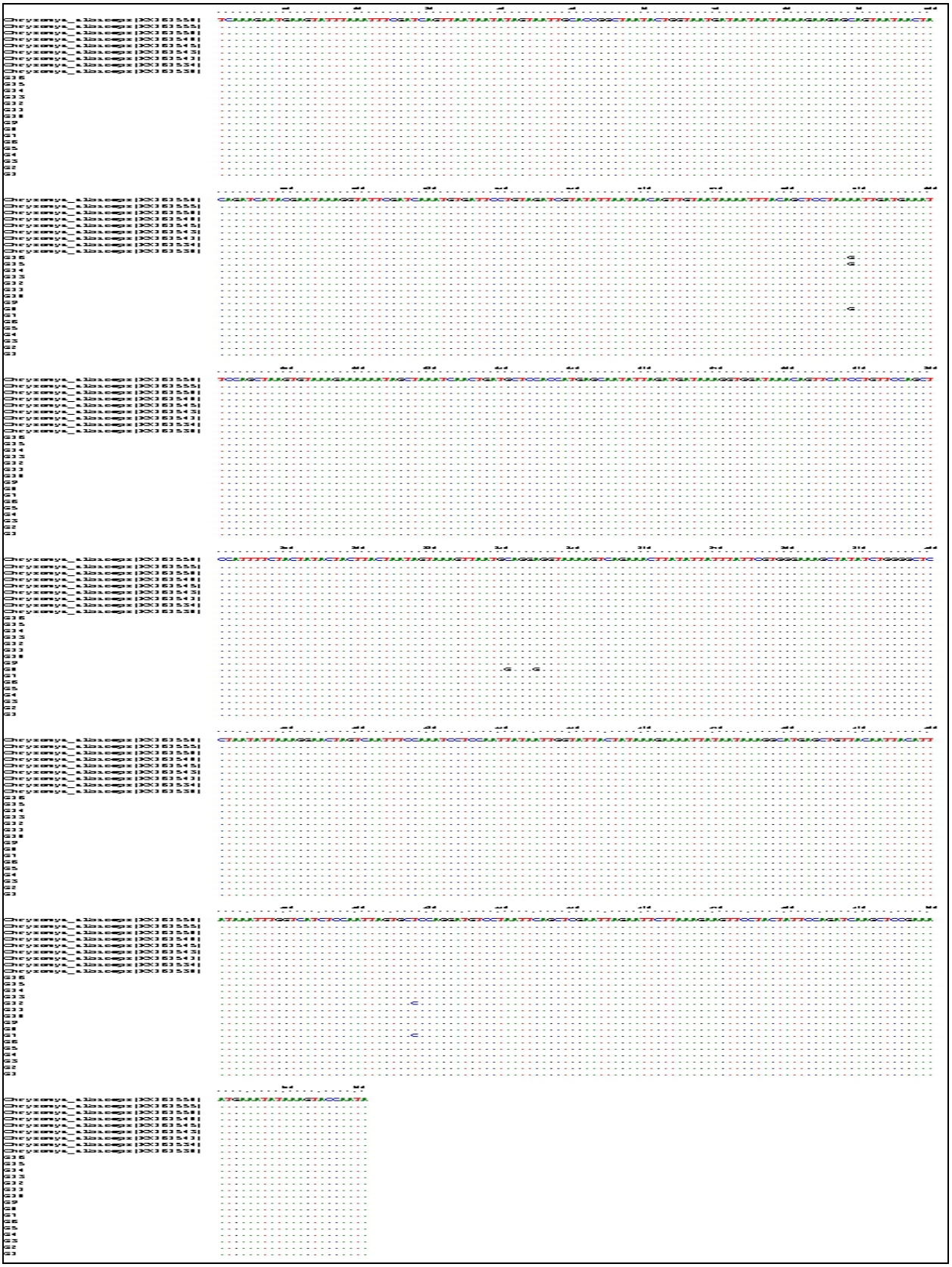

Fig 11: The comparison of the MT-CO1 gene base sequences between *C.albiceps* and the comparison samples from the Genebank (NCBI) showing the similarity and the difference between the samples.

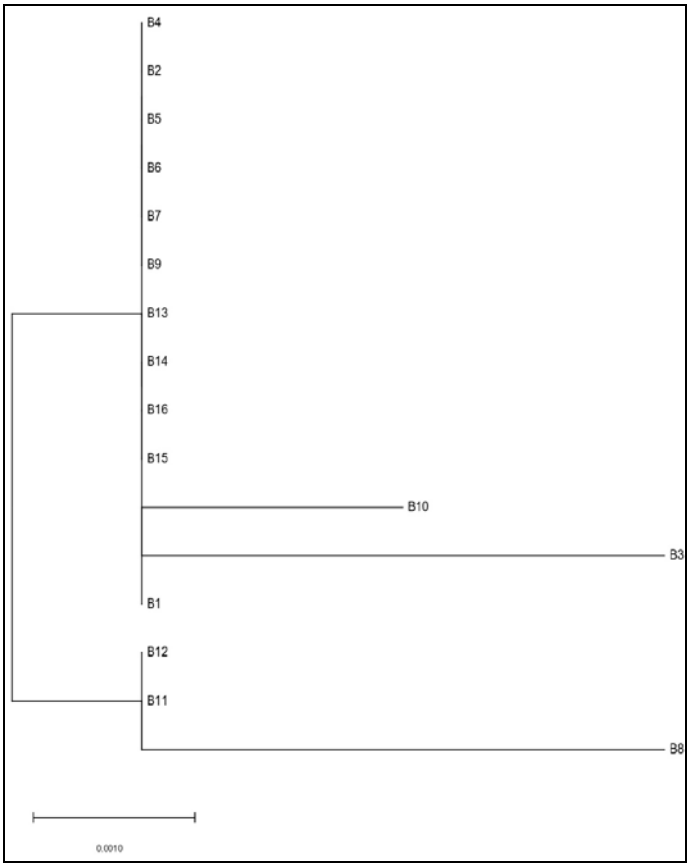

Fig 12: The phylogenetic tree of *C.marginalis* is illustrated by the evolutionary analysis by Maximum Likelihood method, using the MEGA X program.

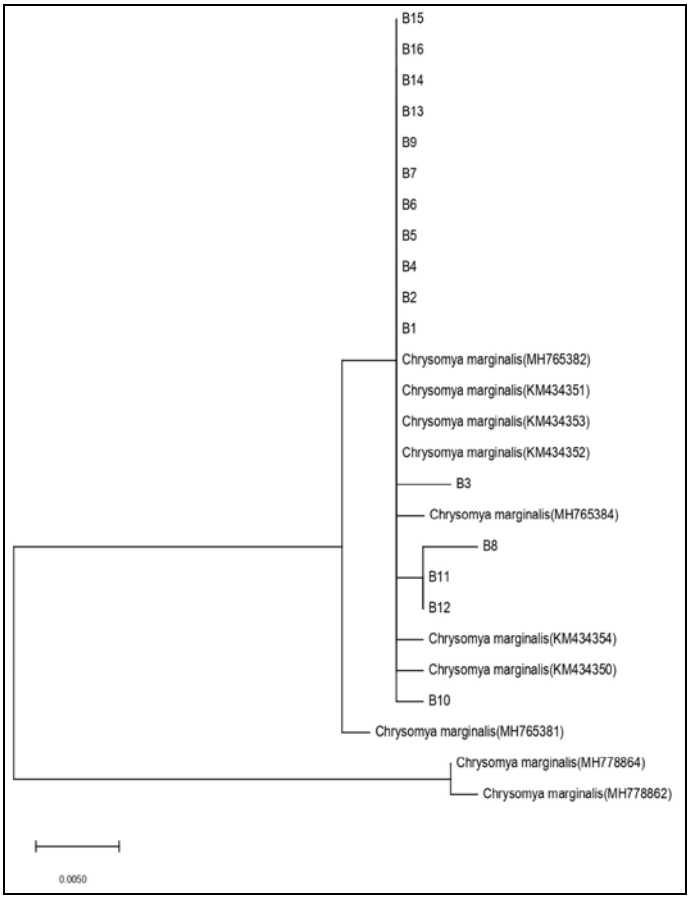

Fig 13: The phylogenetic tree of *C.marginalis* is illustrated by the evolutionary analysis by Maximum Likelihood method, using the MEGA X program.

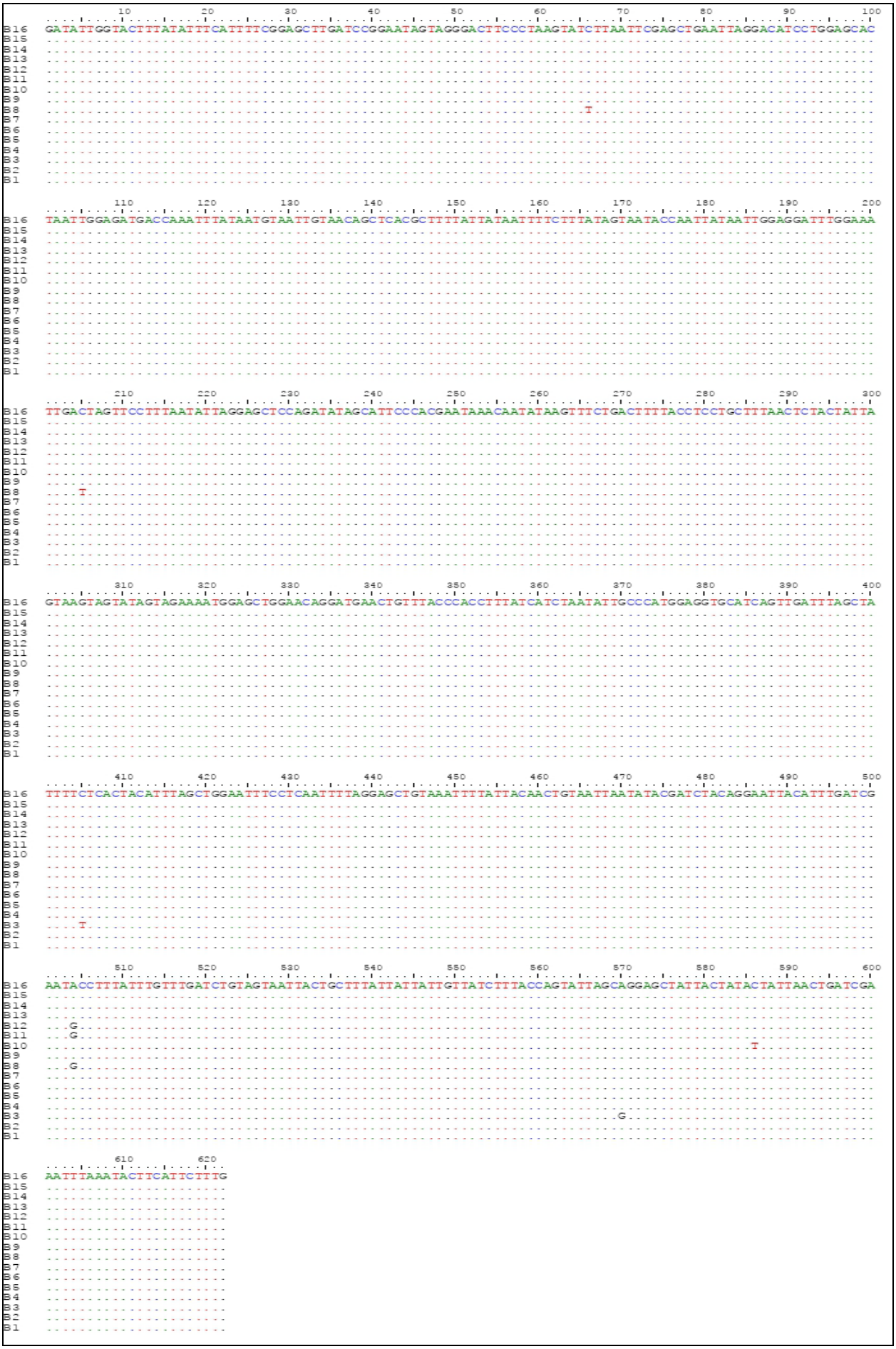

Fig 14: The comparison between the sequence of nitrogenous bases of *C.marginalis* MT-CO1 gene showing similarities and differences

between samples.

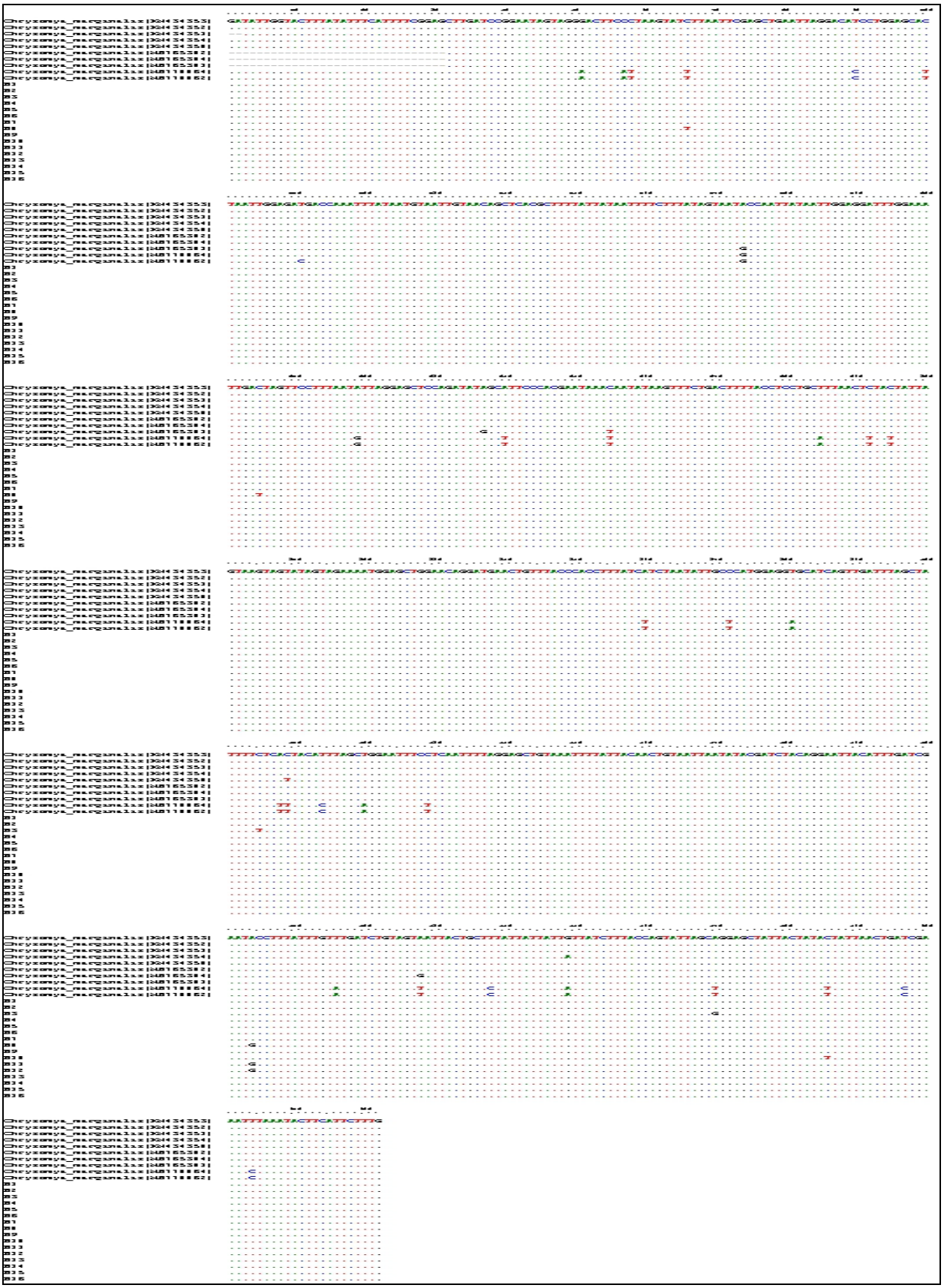

Fig 15: The comparison of the MT-CO1 gene base sequences between *C.marginalis* and the comparison samples from the Genebank (NCBI) showing the similarity and the difference between the samples.

Discussions

Due to the close phylogenetic relationship of species of the genus Chrysomya (Diptera: Calliphoridae), morphological characters are not always sufficiently distinguishable. In our study, *C.albiceps* has a bright green metallic body and the abdominal segments with narrow dark lines on the rear

edge, which agree with many authores (Smith, 1986; Rognes 2002; Kotrba *et al*., 2012) [15, 16, 17]. For *C. marginalis*, we found in our study that the sample has a metallic blue body in color, large with robust and cylindrical body, thoracic and abdominal segments with dark bands on the rear edge, ans the head is pale yellow with yellow or orange hair on gena, which agree also with many authors (Zumpt, 1956) [3]. As for the molecular studies in kingdom of Saudi Arabia, they are rarely on screwworm fly. The mitochondrial genome of the screwworm fly was completely sequenced using Cytochrome C Oxidase subunit I gene (COI) DNA barcode for the identification of screwworm fly species of the genus Chrysomya to confirm the molecular identification which has continuously generated discussion about its taxonomic position over the last century. The resulting comparative molecular data set did not show any substitutions and thus revealed no information regarding biogeographic relationships. In our finding, we used barcode region of mt-DNA gene of DNA extracted from two groups of Chrysomya, *C.albiceps* and *C.marginalis*. The PCR generates a single band wit size 700 bp of all study specimens. Our results also showed a high similarity with the CO1 gene sequences found in the NCBI database of Gene-Bank, where the match percentage in the first group was 100-99.52% with *C.albiceps* and the second group was 100-99.52% with *C.marginalis*. The phylogenetic trees did not show any species-level paraphylies beside our sampling was collected from Jeddah. Our results demonstrate that among the eixteen samples of two species, Anyway, the molecular identification studies of Chrysomya genus are not much studied in Saudi Arabia in general and in Jeddah governorate. But, many researchers have studied the molecular identifications of *C.albiceps* and *C.marginalis* in other countries (Aly and Wen, 2013; Marquez-Acero *et al*., 2017; Al-Mekhlafi *et al*., 2020) [18, 19, 20].

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