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Description of Leafhopper, *Hebata* (*Alboneurasca*) *decipiens* and Its Transmitting Phytoplasma Infesting Sugar Beet Plants

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Abstract: Sugar beet (*Beta vulgaris* L.) is the second sugar crop for sugar production after sugar cane worldwide. This plant is subjected to the infestation of several insect pests that cause considerable damage, especially leafhoppers, that can transmit phytopathogens (e.g., phytoplasma). In this work, we identified and described the leafhopper, *Hebata* (*Alboneurasca*) *decipiens*, in addition to the detection and molecular identification of Candidatus Phytoplasma that was transmitted by this leafhopper on sugar beet plants from naturally infected sugar beet plants to an uninfected one. The 16S rDNA *gene* from phytoplasma was amplified by a nested polymerase chain reaction (PCR) assay and directly sequenced using specific primer pairs DNA fragments. The resulting sequences were analyzed and compared with another phytoplasma sequence available at GenBank sequences performing BLAST using DNAMAN.

1 Introduction

Worldwide, sugar beet (*Beta vulgaris* L.) is the second largest sugar crop for sugar production after sugar cane. This situation is especially true also in Egypt. The total area cultivated during the sugar beet season in 2019 was 608,627 acres, with a production volume of 20,123 tons/feddan. Especially in reclaimed land, it is an important crop that helps in many industries, such as the sugar industry. Sugar beet plants are subjected to the infestation of several insect pests that cause considerable damage, especially piercing-sucking insects.

The Cicadellidae family is a universally distributed group of sap-feeding insects that includes 20,000 described species (Dietrich 2013). They pierce and suck the sap of plants from the xylem, phloem, or mesophyll cells (Knight 1983) and desiccate leaf tissue.

Leafhoppers insert their toxic saliva into the tissue of plants, leading to leaves turning yellow and having dry margins, causing the tissues to wilt, and resulting in plant death (Ebesu 2004). The species *Empoasca decipiens* was transferred to another genus to become *Hebata* (*Alboneurasca*) *decipiens*, according to Xu et al (2021) during their reclassification of the Empoasca generic group. These researchers indicated that *Empoasca* does not occur in Egypt. Therefore, their classification

should be included in the genus *Hebata* and *Hebata* (*Alboneurasca*) *decipiens* and *H.(Signatasca) distinguenda. Empoasca* differs from *Hebata* in having the distal part of the male pygofer appendage excavated and the base of the subgenital plate with a lateral lamella. *Hebata* lacks both traits.

Phytoplasma belongs to the class Mollicutes, Order Acholeplasmatales, and Family Acholeplasmataceae of class prokaryotes (Jones 2002). Leafhoppers or planthoppers naturally transmit them (Maixner 2005).

Most known phytoplasma vectors are from the Cicadellidae family, but numerous are still unknown. Through this family, *Empoasca* sp. (Typhlocybinae) was positive for the existence of several phytoplasmas, and transmission of them was detected in *Empoasca decipiens* (Paoli), where it insured as an experimental vector to Candidatus *Phytoplasma asteris* (Galetto et al 2011). *E. decipiens* has been confirmed in several studies as a potential vector of phytoplasma in different crops.

This work aimed to survey and reidentify leafhopper species infesting sugar beet plants according to world reclassification of leafhoppers (Xu et al 2021) with a special re-description of the most critical leafhopper, *H.* (*Alboneurasca*) *decipiens*. Additionally, the detection and molecular identification of phytoplasma were observed for the first time on a sugar beet plant in Egypt and transmitted by this leafhopper.

2 Materials and Methods

2.1 Leafhopper collection

Leafhopper species were collected from sugar beet plants in different governorates of Egypt, Kafer El Sheikh, Fayoum, Qalyubyia, and Giza.

The samplings occurred from 2017 to 2020, using the two sampling techniques below.

2.1.1 Sweeping net

Leafhoppers were collected by a sweeping net of 37 cm^2 in diameter and 60 cm^2 deep. The samples were collected between 8 and 10 am at distinct locations by applying 50–100 double strokes. Depending on the cultivated area and the size of the plants, two rapid strokes in opposite directions were performed over the plants while walking through the field.

Collected specimens were placed in small plastic containers covered with muslin and labeled with their host plant, date, and locality of collection. Then, the specimens were transferred to the laboratory and kept in the freezer for 5 min to slow down the movement of the insects and facilitate the process of species separation.

2.1.2 Aspirator

Leafhoppers were aspirated off using an aspirator and transferred to the rearing cage. The previously collected insects were cleaned from the plant residues, sticking dust, and other unwanted insects using a camel's hairbrush with a hand lens (Viraktamath 2005).

The sample was prepared for mounted microscopic slides and identified leafhoppers were based on examining their morphological characters using available description and identification keys (Blocker 1967, Abul-Nasr and Samy 1967, Nielson 1968, Ibrahim 2016, Xu et al 2021). An Olympus light microscope model, CHS with a magnification of 40x, was used to examine the morphological characteristics.

2.2 Phytoplasma source

Sugar beet plants that were expected to be naturally infected by phytoplasma had different symptoms, such as stunting, leaf curling, increased spacing between branches, and little leaf symptoms, from other governorates, primarily from El Fayoum and Kafer El Sheikh. These symptoms were observed in the open field of the sugar beet cultivated area **(Fig 1)** during two successive seasons, 2018/2019–2019/2020. Plants were evaluated with nested PCR on the existence of phytoplasma, and plants that gave positive results were used for isolation. A high population of leafhoppers associated with the symptoms of the infested field was collected from a sugar beet plantation and kept in an insect-proof greenhouse for transmission. Infected sugar beet plants were collected from the central growing sugar beet governorates, such as Giza, Qalyubyia, Sharkia, Fayoum, and Kafr El Sheikh, during the seasons from 2017 until 2020.

Fig 1. Naturally infected sugar beet plants with phytoplasma in the open field show different symptoms of leaf stunting, loss of apical dominance of the shoots stunting, leaf curling, increased spacing between branches, and little leaf symptoms

2.3 Rearing leafhoppers (maintaining a pathogen-free colony)

A leafhopper culture was initially started from live samples collected by sweeping the net from the sugar beet fields. The culture was also maintained with the collection of nymphs, as follows.

2.3.1 Isolating cage

A wooden cage was used to isolate the required leafhopper species from other species and different insects collected in the sweeping net. The back of this cage was covered with a white cloth sheath. There was a small lamb outside one of its corners. The front was covered with a transparent cloth sheath.

2.3.2 Rearing cage

Whole-infested sugar beet plants with leafhopper nymphs were transferred from the field to the laboratory of the Piercing-Sucking Insect Research Department at the Plant Protection Research Institute at the Agricultural Research Center. Afterward, they were replanted in pots and then transferred to rearing cages made of steel covered with fine mesh wire. The whole cage measured 60 × 50 × 44 cm (**Fig 2).**

Pots of 15 cm in diameter were cultivated with barley plants. After 11–14 days, the seedlings were covered with glass or plastic cages with an upper opening covered with muslin and fixed with rubber bands. The cages were pushed into the soil of the pot around the plant. The isolated insects were transferred to the barley seedling using the isolating cage and then kept to feed on and serve as an oviposition site for several days.

2.3.3 Transmission of Phytoplasma by leafhoppers

Leafhopper *H. decipiens* colonies were collected from sugar beet fields. Adult leafhopper individuals were reared on barley plants in insect-proof cages. After that, they were allowed to oviposit on barley seedlings. The hatching nymphs were transferred onto healthy barley plants in chimney glass cages to maintain a leafhopper colony free from viruses or phytoplasma, as shown in **Fig 3.** Nested PCR confirmed the results. After starving for 24 h, phytoplasma-free insects were allowed to feed on infected sugar beet plants for one week (Ahmed et al 2014, Gad et al 2019) as an acquisition period. Twenty insects/plants were placed on healthy sugar beet plants for an inoculation access period of 30 days. The plants were then sprayed with pesticides to kill adult insects. Finally, the plants were monitored until symptoms appeared.

2.4 Detection of phytoplasma using nested PCR

2.4.1 DNA extraction

DNA was extracted from sugar beet and leafhopper samples using the method proposed by Dellaporta et al (1983).

2.4.2 Nested polymerase chain reaction (PCR)

A nested PCR was used to amplify the 16S rDNA gene of phytoplasma. The first PCR round was conducted using universal primers (P1, P7), **Table 1**. as described by Deng and Hiruki (1991), Smart et al (1996) and EMPPO (2018). The second PCR round was nested using pair of primers R16F2n/R2 **(**Gundersen and Lee 1996). The PCR reaction was done by mixing the following: 10 µl amaR One PCR master mix (GenedireX.INC), 3 µl of 10 pmol of each primer, 3 µl of DNA sample, and 1 µl distilled water. The PCR

Fig 2. Rearing cages

Fig 3. Chimney glass cages to maintain a leafhopper colony free from virus or phytoplasma

Table 1. Sequences of the oligonucleotide primers used in nested PCR for phytoplasma detection

Primer	**Sequence**
P1 (forward)	5′ -AAG AGT TTG ATC CTG GCT CAG GAT T-3′
P7 (reverse)	5′ -CGT CCT TCA TCG GCT CTT-3′
R16F2n (forward)	5′ -GAA ACG ACT GCT AAG ACT GG-3′
R16R2 (reverse)	5′ -TGA CGG GCG GTG TGT ACA AAC CCC G-3′

amplification profile was initiated at 94°C for 3 min, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, and extension for 1 min at 72°C. The last extension remained for 10 min at 72°C. The PCR product in the second PCR round was diluted in a concentration of 1:10 in distilled water, and 2 µl was used in nested PCR as a template with primer pair R16F2n/R2 (Gundersen and Lee 1996). The PCR products were visualized by 1% agarose gel stained with EZ view stain (Biomatik-Canada).

2.4.3 PCR sequencing and analysis

Samples with positive results were cut and clarified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan). Fragments of DNA were delivered to South Korea at Macrogen Inc. for sequencing. The resulting sequences were analyzed and compared with another phytoplasma sequence available on GenBank to perform a BLAST comparison with DNAMAN.

3 Results and Discussion

The most important and abundant leafhopper was *H.* (*Alboneurasca*) *decipiens* Paoli from the Typhlocybinae subfamily and Empoascini tribe.

3.1 Diagnostic characteristics

The specimens are green or light green. Tibia and tarsus are bluish-green. The last segments of the abdomen are bright and light green. Vertex is semi-rounded, making an obtuse angle. The fore wing is membranous, thicker than the hind wing, smooth with yellow veins, four apical cells, and without an appendix. The hind wing is membranous and hyaline. The veins end in a margin with one apical cell. The male abdominal apodeme is as long as two abdominal segments, diverging toward the apex, with a crescentic shape at the apex. Pygofer has chitinized processes. The genital plate is elongated, curved, and provided with numerous marginal microsetae and long macrosetae at the upper one-third of the inner side. Two pairs of the stylus are present, the outer one slightly curved as an arc along its length ended with a tapered chitinized end, the inner one longer, more curved, its apex pointed and dentated. Connective anterior lateral arms are bilobed with a cleft at the apex, robust, chitinized and connected with the aedeagus. The aedeagus is short and spatulated, with a developed preatrium (**Fig 4)** similar to the apodemes adopted by Ibrahim (2016).

3.2 Transmission of Phytoplasma by the leafhoppers

The collected infected leafhopper *H. decipiens* individuals with phytoplasma from sugar beet fields could successfully transmit the same symptoms of disease when fed on new sugar beet plants. Phytoplasma transmission has long been considered due to their similar morphology and disease symptoms as the source of infection. Several symptoms appeared on sugar beet plants, such as stunting, leaf curling, little leaf symptoms, increased spacing between branches, and loss of apical dominance of the shoots 30–60 days after feeding on healthy plants, as shown in **Fig 5**.

3.3 Nested PCR for the detection of phytoplasma using nested PCR

Nested PCR successfully distinguished phytoplasma from sugar beet leaves and leafhoppers. The PCR products were visualized using agarose gel electrophoresis at a 50 bp gene ruler DNA ladder as a DNA size marker. A DNA fragment of 1,200 bp was observed in both the positive and infected sugar beet symptomatic samples (**Fig 6**). Such a band was not found in the symptomless (NC) sample.

3.4 PCR cleanup and sequencing analysis

The presence of phytoplasma in different sugar beet samples was revealed and confirmed by genetic sequencing analysis. The partial 16S rDNA gene region of one positive sample was bidirectionally sequenced at Macrogen Korean company by automated DNA sequencing using the same primers used in the PCR reaction.

Phylogenetic and molecular evolutionary analyses by neighbor-joining and 1,000 bootstrap replicates were performed on the chosen GenBank data to visually assess the genetic relationships of sugar beet plant matter from 16S rDNA sequences. A partial sequence of the 16S rDNA region from the sugar beet sample containing 1200 bases was deposited in GenBank using BLAST analysis. The resulting sequence was compared with the sequence from the phytoplasma found in GenBank.

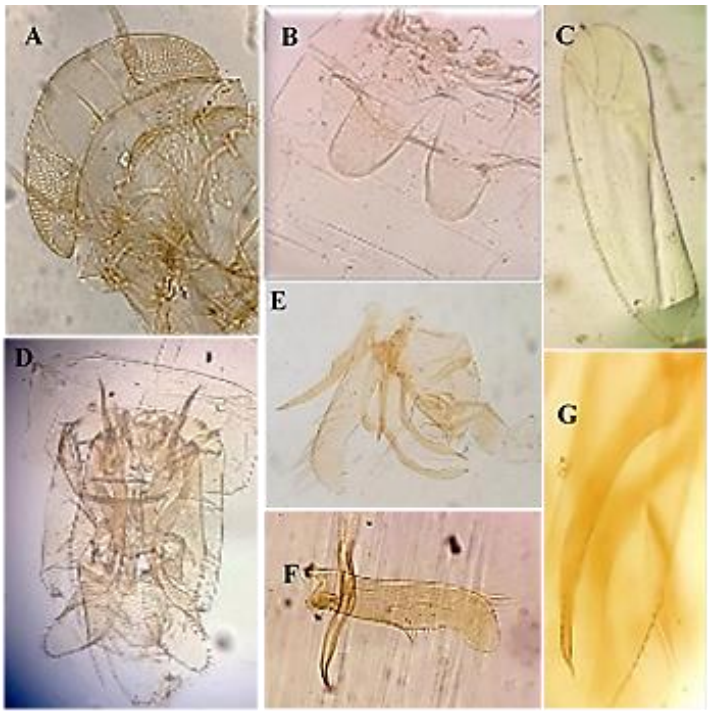

Fig 4. Morphological diagnostic characters of *Hebata* (*Alboneurasca*) *decipiens* Paoli: Vertex, pronotum**. B.** Abdominal apodeme. **C.** Adult forewing. **D.** Male genitalia, ventral view. **E.** Male genitalia, lateral view. **F.** Genital plate. **G.** Stylus

Fig 5. Sugar beet plants infected with phytoplasma by *Hebata (Alboneurasca) decipiens* at the greenhouse after 30–60 days showing stunting, leaf curling, increased spacing between branches and little leaf symptoms

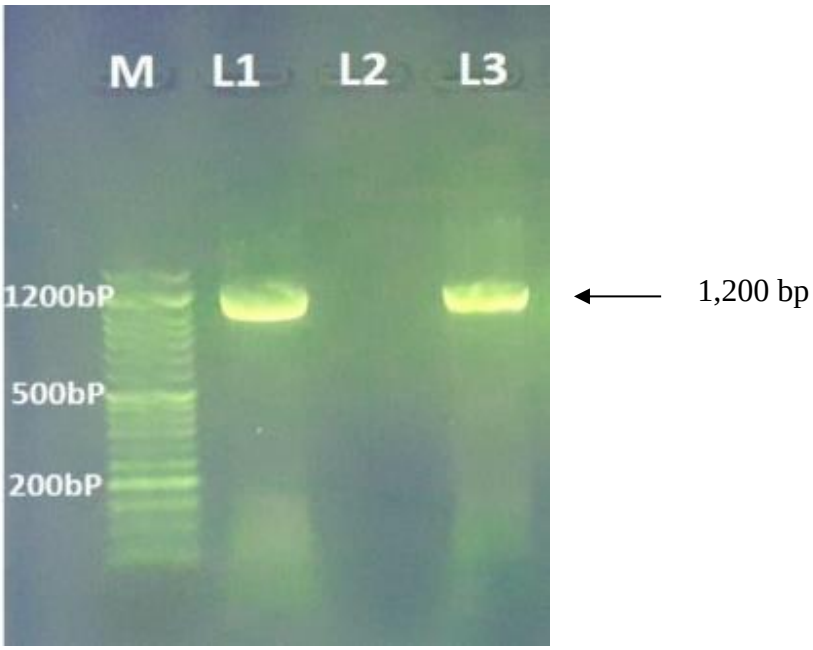

Fig 6. Phytoplasma detection using nested PCR with R16F2n/R2 Primers from infected plants. M: 1200 bp GeneRuler DNA ladder; L1: Positive control sample (PC); L2: healthy plant control (NC), L3: positive sample affected with phytoplasma

The presence of phytoplasma in different sugar beet samples was detected and confirmed by the results. A 98% identity with isolates FR822709, FR822713, MH011394, MK377249 and MPLDRRI, 97% identity with isolateKX670809, 91% identity with isolates AJ550984, AY197655, EU498728, EF666051, JQ044393, KC412029, and Y14175, and 90% identity with isolates AY688362, DQ286953, KF826615, EF546439, and MT940834, are shown in the results in **Fig 7** and **Table 2**. The Egyptian isolate was submitted to GenBank under accession No. OP032749.

The similarity percentage shown in **Fig 7** and the phylogenetic tree for the 16S rDNA nucleotide sequences of the phytoplasma were ascertained in sugar beet. In the present work, this is the first statement of the existence, symptomatology, and partial genetic characterization of a 16SrXIV phytoplasma-infected sugar beet in Egypt.

Plant viruses and phytoplasmas are one of the most severe pathogen groups affecting economic loss. The present work successfully detected the incidence of phytoplasma in sugar beet plants and its insect vector, leafhoppers [*H.* (*Alboneurasca*) *decipiens*]. Phytoplasma symptoms recorded on sugar beet plants were stunting and leaf area reduction (little leaf symptoms). Thilagavathi et al (2011), and Shazly et al (2016) obtained the same results. The detection shown in the leafhopper samples indicated that all tested samples were positive for phytoplasma. However, sugar beet samples resulted in negative consequences in the Giza governorate, suggesting that not all phytoplasma groups or sub-groups can infect sugar beet plants. Also, detecting phytoplasma in their insect vectors gave an early alert for the appearance of phytoplasma diseases in the same and next growing seasons for sugar beet plants and other crops. These results agree with those found by Trivellone and Dietrich (2021). An identity sequence analysis between 90% and 98% has been shown with those isolates available at GenBank.

4 Conclusion

Candidatus phytoplasma sp. was detected and isolated for the first time from naturally infected sugar beet plants and then transferred to healthy sugar beet plants by the leafhopper *H.* (*Alboneurasca*) *decipiens o*f nine tested leafhopper species belonging to two subfamilies and five genera found on sugar beet plants, only *Hebata* (*Alboneurasca*) *decipiens* proved its ability to transmit phytoplasma. It is crucial to conduct regular taxonomic revisions for leafhoppers, especially those capable of transmitting pathogens to plants. With such an analysis, it will be possible to detect plant pathogens inside insects to predict the type and strain of the pathogen. Such information will allow us to prevent the infection of economic crops and take the necessary measures to limit the spreading of phytoplasma.

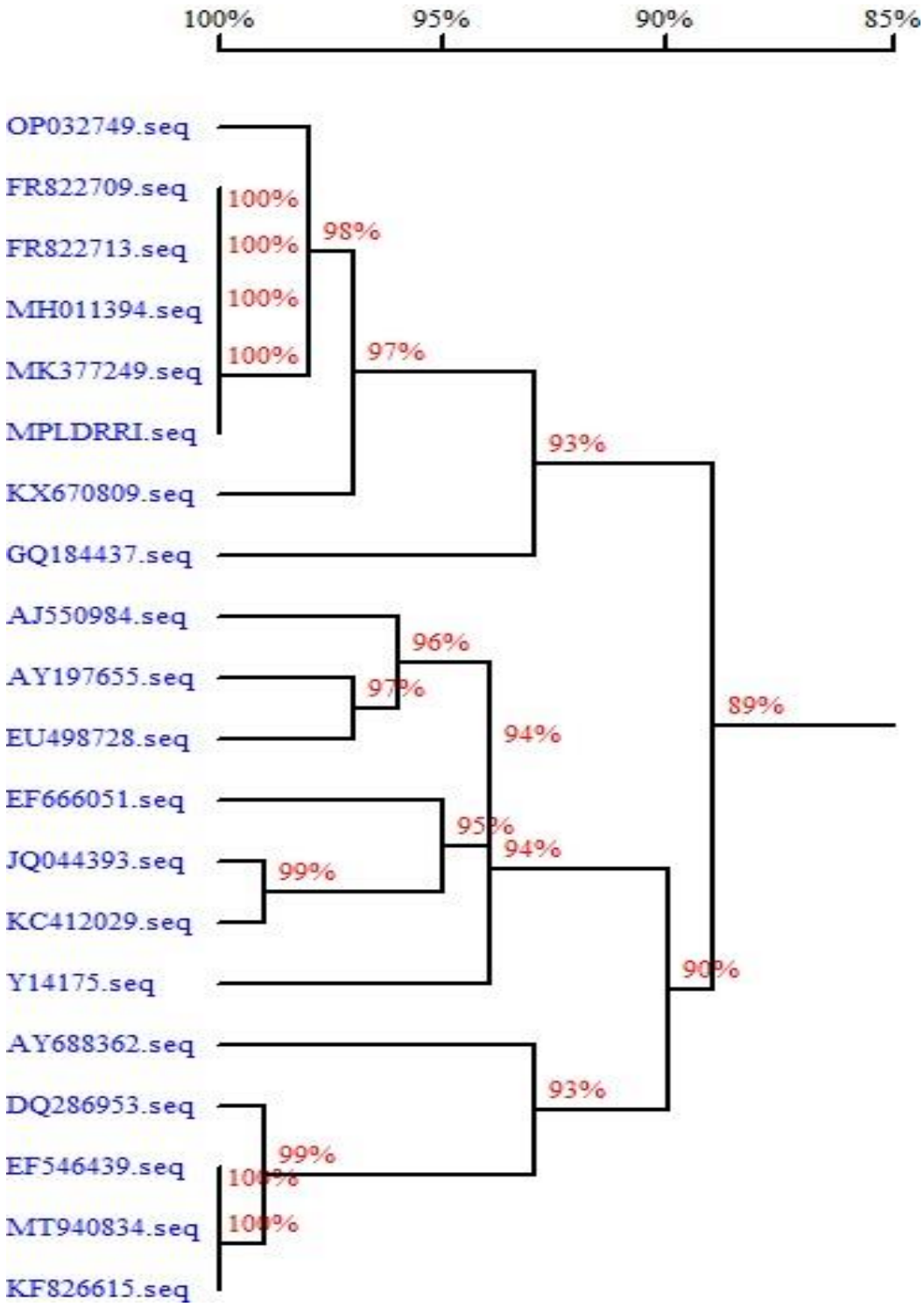

Fig 7. Phylogenetic tree showing relationships among reported isolates of phytoplasma at the gene bank and the sugar beet Egyptian isolate based on the nucleotide sequences

Table 2. Accession number and similarity matrix for isolates of phytoplasma at the gene bank and the sugar beet Egyptian isolate based on nucleotide sequences

Accession Number	Host	Country	Identity
OP032749	Sugar beet	Egypt	100%
MH011394	Sesame	Egypt	98.1%
MK377249	African Daisy (Gazania)	Egypt	98.0%
MPLDRRI	Peanut	Beltsville,	98.0%
FR822709	Squash	Egypt	98.0%
FR822713	Tomato	Egypt	98.0%
KX670809	Grapevine	USA	96.2%
GQ184437	Sugar beet	India	93%
KC412029	Romerillo	Argentina	90.5%
JQ044393	Peach	USA	90.3%
Y14175	Coconut	UK	89.8%
AJ550984	Bermuda grass	Italy	89.4%
EF666051	Legume tree	Oman	89.3%
AY688362	Apricot	Tunisia	88.7%
EU498728	Oil palm	Malaysia	88.7%
EF546439	Periwinkle	Egypt	88.5%
MT940834	Hop bush	Egypt	88.5%
AY197655	Elm	USA	88.4%
DQ286953	Broad bean	Cuba	88.1%
KF826615	date palm	Egypt	88.0%

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