INITIAL CLUSTERING OF SOME EGYPTIAN NEMATICIDES *Serratia* ISOLATES BASED ON THEIR *16SrRNAs* GENE SEQUENCES

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Nowadays, most the focus in the agriculture industry is to replace the traditional approach of using chemical compounds for nematode control, which can pollute the plants, soil and the surrounding environment. Also, the usage of fumigation, as an effective method to get rid of the plant-pathogenic nematodes that can reside for a hundred year in the soil in the form of an egg sac, has been banned. So the main focus has been on the formation of eco-friendly products isolated from the soil that can destroy the pest, while the microflora balance of the soil is kept intact.

One of these products is the soil bacteria that reside in the soil but can still be found and isolated from plants, water, insect, nematodes, animals and human, including microorganism of the Family Enterobacteriaceae (Pham *et. al*., 2007). One of the members of this family, which is usually plant-associated bacteria, belonging to Genus: *Serratia* are gram-negative, oxidase-negative and catalase-positive (Hearn *et. al*., 1970; Gerber, 1975).

Some strains of this Genus have unique characteristics that could meet the criteria of an ideal biological control agent for plant-pathogenic nematodes such as *Serratia marcescens* and *Serratia nematodiphila*. Both *Serratia* species have red-pigmented colonies which actually produce a secondary metabolite microbial pigment known as 'prodigiosin' which has nematicide effects on juvenile stages of *Radopholus similis* and *Meloidogyne javanica* (Rahul *et. al*., 2014). Furthermore, they can be exten-

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sively isolated and tested for enhancing plant health with the inhibition of the plant parasitic pathogens. (Kim *et. al*., 2011).

Since it has been found that *Serratia marcescens* and *Serratia nematodiphila* are closely taxonomically related and share close similarity (Ajithkumar *et. al*., 2003; Cho *et. al*., 2020), the identification and the classification of their isolates must be based on molecular techniques such as their unique conservative region of *16SrRNA* gene and through phylogenetic analysis (Zhang *et. al*., 2009).

In this study, we retrieved eight *Serratia* isolates that had been proven to cause nematicide effects on plant-pathogenic nematodes. They were previously collected from tomato-planted soil in Egypt and identified biochemically and phenotypically at the genus level. Our focus in this study is to identify these isolates based on partial sequence of their *16SrRNA* gene, which has been known to a conservative region within each type of bacteria and can help identify it due to its slow evolution in this region. This is just the beginning of initial clustering of some of the Egyptian isolates that show promising results toward better control of plant-pathogenic nematodes.

MATERIALS AND METHODS

This study was conducted at Ain Shams Center of Genetic Engineering and Biotechnology laboratories (ACGEB).

Bacterial isolates

Twenty three samples were collected from the soil of tomato plants grown in Elkhatatba, Egypt (Essam *et. al*., 2008), identified biochemically using API system (Hejazi and Falkiner, 1997), and tested for antibiotic sensitivity using the diffusion method as described by Cheesbrough (2000). Eight samples were selected, plated on Tryptic Soy Broth agar (DSMZ medium 535) and incubated overnight at 33°C. It was gram-stained and catalase and oxidase tests were performed to help identifying the different isolates morphologically.

DNA extraction from bacteria

All bacterial DNA were isolated using PureLink® Genomic DNA kit. Bacterial pellets were collected in 180 µl of digestion buffer and 20 µl proteinase k was added, then vortexed for 5 sec. and incubated at 55°C for 30 min. - 4 hrs until cell digestion. Twenty µl RNase were added for 2 min. at room temperature. Two hundred µl of lysis binding buffer was added, mixed with 200 µl absolute ethanol, then vortexed for 5 sec. To spin column, 640 µl of mix was added and centrifuged for 1 min. at 10000 xg (Repeat until mix finish). The column was transferred to a new tube then 500 µl of wash buffer 1 was added, and centrifuged for 1 min. at 10000 xg. The column was transferred to a new tube then 500 µl of wash

buffer 2 was added then centrifuged for 3 min. at 10000 xg. The column was transferred to a new tube then 100 µl of elution buffer was added then centrifuged for 1 min. at maximum speed.

Molecular identification: 16SrRNA gene sequence and phylogenetic analysis

PCR amplification of partial 16SrRNA gene was carried out with 50 ul reaction volume containing 2X-my Taq (25 ul), 50 ng bacterial DNA (1 ul), H_2O (22 ul), and universal primers: 10 mM Forward (F515) 1 ul; 10 mM Reverse (R800) 1 ul (Oligo DNA synthesis Service, Macrogen Europe Meibergdreef 31, 1105 AZ., Amsterdam, Netherlands). PCR program was 95°C / 2 min. - (94°C / 1 min., 55°C / 1 min., and 72°C / 90 sec.) x 35 cycle - 72°C / 10 min., - 4°C / ∞. Primers sequences were as follows: 518F-5'-CCAGCAGCCGCGGTAATAC-3' and 806R- 5'-TACCAGGGTATCTAATCC-3' (Ghyselinck *et. al*., 2013). All bacterial PCR products were separated on 1.5% agrose gel electrophoresis then purified using (GeneJET™ PCR Purification) kit following the manufacture protocol. Only eight individual isolates (ACGEB Ser1, ACGEB Ser 4, ACGEB Ser 6, ACGEB Ser 8, ACGEB Ser 10, ACGEB Ser 12, ACGEB Ser 13, and ACGEB Ser 22) (Table 1) were sequenced based on their partial 16SrRNA gene sequences. Direct sequencing of PCR purified product of 16SrRNA gene was carried out by a private service of (Macrogen, Netherlands) company using the automated sequencer ABI 3100 with Big Dye Terminator Kit v. 3.1. Using http://www.ncbi.nlm.nih.gov/, Microbial nucleotide Blastn (Altschul *et. al*., 1997) were conducted and Max. score and E-values were reported. Pairwise and multiple alignments were done using ClustalW algorithm (Thompson *et. al*., 1994) to detect similarities between the different sequences and *Bacillus thuringiensis JN 315886.1* from NCBI GenBank database was used as a reference sequence. Phylogenetic tree analyses were conducted by MEGA7 program (Kumar *et. al*., 2015) using neighbor-joining method (Saitou and Nei, 1987; Tamura *et. al.*, 2004) with 1000 bootstrap (Felsenstein, 1985).

RESULTS AND DISCUSSION

Twenty three isolates collected from the soil were identified using API 20E kit (Biomérieux, Rome, Italy) according to manufacture instructions. Eight isolates that were chosen in this study based on their nematicidal effect on plant-pathogenic nematodes. Three of these isolates were used as a nematicides (ACGEB Ser1, ACGEB Ser 4 and ACGEB Ser 6), while five (ACGEB Ser 8, ACGEB Ser 10, ACGEB Ser 12, ACGEB Ser 13, and ACGEB Ser 22) were tested as a suspension on *C. elegans* (larvae and adults) bioassay which were killed within the first 24 hrs of the exposure to the suspension (in press). Eight isolates were tested on biochemical and molecular levels. All isolates were streaked on TSB agar medium as a selec-

tive medium for *Serratia* which gave bright red-pigmented circular colonies. Biochemical tests confirmed gram-negative, catalase positive and oxidase negative isolates.

The DNA isolated from the eight isolates was amplified using universal primers. PCR products were eluted and *16SrRNAs* gene was sequenced. The sequenced fragments were trimmed, pairwise and multiple aligned and the pairwise distance was performed and the overall mean distance was 0.58. The sequence analysis of the isolates in the BLAST microbial genomes revealed that all isolates were within the Genus *Serratia* with a sequence identity average of 99.4-99.8% and high homology with *Serratia marcescens* and *Serratia nematodiphila* which were used as biocontrol agents (Fig. 1). Some of these species *16SrRNAs* gene were downloaded from the NCBI GenBank database. Using ClustalW, the eight isolates and the downloaded related sequences were aligned pairwise and multiple alignments. Table (1) shows the similarities of *16SrRNA* gene sequences between the isolates and the closely related strains showed that: seven out of eight isolates (ACGEB Ser1, Ser4, Ser10, Ser12, Ser13, Ser22 and Ser6) were most closely related to *Serratia marcescens* with (99.4%-99.8) while ACGEB Ser8 shared even higher similarities with *S. nematodiphila* (99.6-99.7%).

Phylogenetic analysis of all isolates and strains *16SrRNA* gene sequences was performed using MEGA version 7 program based on the neighbor-joining method with a bootstrap values of 1,000 replications and *Bacillus thuringiensis* SV2 JN315886.1 was used as a tree root (Patil *et. al*., 2012). The phylogenetic tree separated the isolates within three clusters. The first cluster included ACGEB Ser1; Ser4 and Ser10 isolates along with one strain of *S. marcescens* subspecies, the second cluster comprised ACGEB Ser12, Ser13, and Ser22 with another type of *S. marcescens* subspecies and the third cluster contained ACGEB Ser6 with two strains of *S. marcescens* subspecies while Ser8 clustered with two types of *S. nematodiphila* (Fig. 1).

Genus *Serratia* especially *S. marcescens* and *S. nematodiphila* species can be found and isolated from different organisms such as plants (Cho *et. al*., 2020), and nematode (*Zhang et. al.*, 2008). Whereas, some of these species shares the same characteristic of biocontrol agent against plant-pathogens such as the genome of one of the Serratia strain (M24T3) that have been discovered to carry many genes involved in nematotoxic activity (Proença *et. al*., 2012).

Furthermore, *Serratia nematodiphila* has been used as a microbial control agent against insect pests such as New Zealand grass grub (Johnson *et. al*., 2001; Townsend *et. al*., 2003). While some subspecies of the *Serratia*

nematodiphila were found to share symbiotic life cycle with entomopathogenic nematode such as *S. nematodiphila* DSM 21420^T whose genome was the first to be sequenced within the subspecies of *S. nematodiphila* (Kwak *et. al*., 2015) and *S. nematodiphila* DZ0503SBS1T (Zhang *et. al*., 2008). Also, *S. marcescens* has been found and used to be a very effective biocontrol agent against fungal plant pathogens such as C-1 strain (Harris *et al*., 2004) and against insect pathogens such as 14 RPW strains (Scrascia *et. al*., 2016). Furthermore, the lethal effects on plant-pathogenic nematodes such as *Radopholus similis* and *Meloidogyne javanica* were reported (Rahul *et. al*., 2014).

It is quite evident that *S. marcescens* is more abundant in the soil and associated with fresh produce plants compared to *S. nematodiphila* and *S. ureilytica* Cho *et. al*., (2020) who reported that fifteen isolates of *Serratia spp*. that were collected from fresh vegetables. Twelve out of fifteen isolates, based on its *16SrRNA* gene sequences, were identified as *S. marcescens*, with 99.50-99.93% similarity, while the rest of the isolates were identified as *S. nematodiphila* and *S. ureilytica* with 99.86-100.00% similarity. Meanwhile, Zhang *et. al*., (2009) compared his isolate (DZ0503SBS1T) using partial 16SrRNA gene sequence and confirmed that it is closely related to *S. marcescens* with 99.8% similarity and less than this percentage with other *Serratia* (*S. ureilytica*, *S. odorifera*, *S. entomophila*, *S. ficaria*, *S. plymuthica*, *S. grimesii*, *S. proteamaculans*, *S. fonticola* and *S. rubidaea*).

The only argument that can separate *S. marcescens* from *S. nematodiphila* is its genetic makeup which is represented in housekeeping genes such as *16SrRNA* gene. In our study we had three clusters of isolates distributed within the Genus *Serratia* with subspecies *S. marcescens* and *S. nematodiphila*. While ACGEB Ser1, Ser4, Ser10, Ser12, Ser13 and Ser22 shared high similarities with *S. marcescens* of different strains within two clusters, however, there were a couple of isolates that shared S. *marcescens* and *S. nematodiphila* within the same cluster, it has been found that *S. marcescens* is very closely related to *S. nematodiphila* and their gene similarities explain their roles in the soil as biocontrol agents (Ajithkumar *et. al*., 2003; Cho *et. al*., 2020).

SUMMARY

Biocontrol is known to be the safest agriculture practice for pest control in comparison with pesticides. In addition, pests can be very detrimental to crops, destroying them within a few days. One of the pests the farmers have to deal with on a regular basis is nematodes, which can be extremely destructive to seedlings and whole plants. In our study we shed some light on promising bacterial isolates found to belong to Genus *Serratia* which

can be a good candidate for nematode biocontrol. Eight *Serratia* isolates were collected from tomato-planted Egyptian soil. They were chosen for this study based on their nematicidal effect. DNA isolation, PCR amplification and partial sequence analysis of *16SrRNA* gene were performed. They were aligned using ClustalW with similar strains from the NCBI GenBank database, compared with reference strain of *Bacillus thuringiensis JN 315886.1* (as a tree root). BLASTn Microbial genome database, based on their *16SrRNA* partial gene sequence, were used to identify the isolates to be mostly correlated to Genus *Serratia*. Phylogenetic analyses were conducted by MEGA7 using neighbor-joining method with 1000 replicates for bootstrap analysis which indicates 99.4-99.8% similarities with the *Serratia nematodiphila* and *Serratia marcescens* species.

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Table (1): Identification *of* ACGEB *Serratia* isolates based on their 16SrRNAs partial gene sequences and similarities with related sequences from NCBI GenBank database.

No.	Isolate	Size (bp)	Similarities %	Accession #	Strain(*16S ribosomal RNA* gene, partial sequence)	
1	ACGEB Ser1	782	99.5	MK209062.1	*Serratia marcescens* strain 4.2.5.2.X-17	**Cluster I**
2	ACGEB Ser4	700	99.4	MK209062.1	*Serratia marcescens* strain 4.2.5.2.X-17	
3	ACGEB Ser10	783	99.4	MK209062.1	*Serratia marcescens* strain 4.2.5.2.X-17	
4	ACGEB Ser12	662	99.7	JF429937.1	*Serratia marcescens* strain 21-3	**Cluster II**
5	ACGEB Ser13	558	99.6	JF429937.1	*Serratia marcescens* strain 21-3	
6	ACGEB Ser22	485	99.8	JF429937.1	*Serratia marcescens* strain 21-3	
7	ACGEB Ser6	694	99.5	MN173861.1	*Serratia marcescens* strain MRK2	**Cluster III**
			99.6	HM590891.1	*Serratia marcescens* strain SYBCT02	
8	ACGEB Ser8	704	99.7	KY963567.1	*Serratia nematodiphila* strain SG1	
			99.6	MN548419.1	*Serratia nematodiphila* strain RPD31	

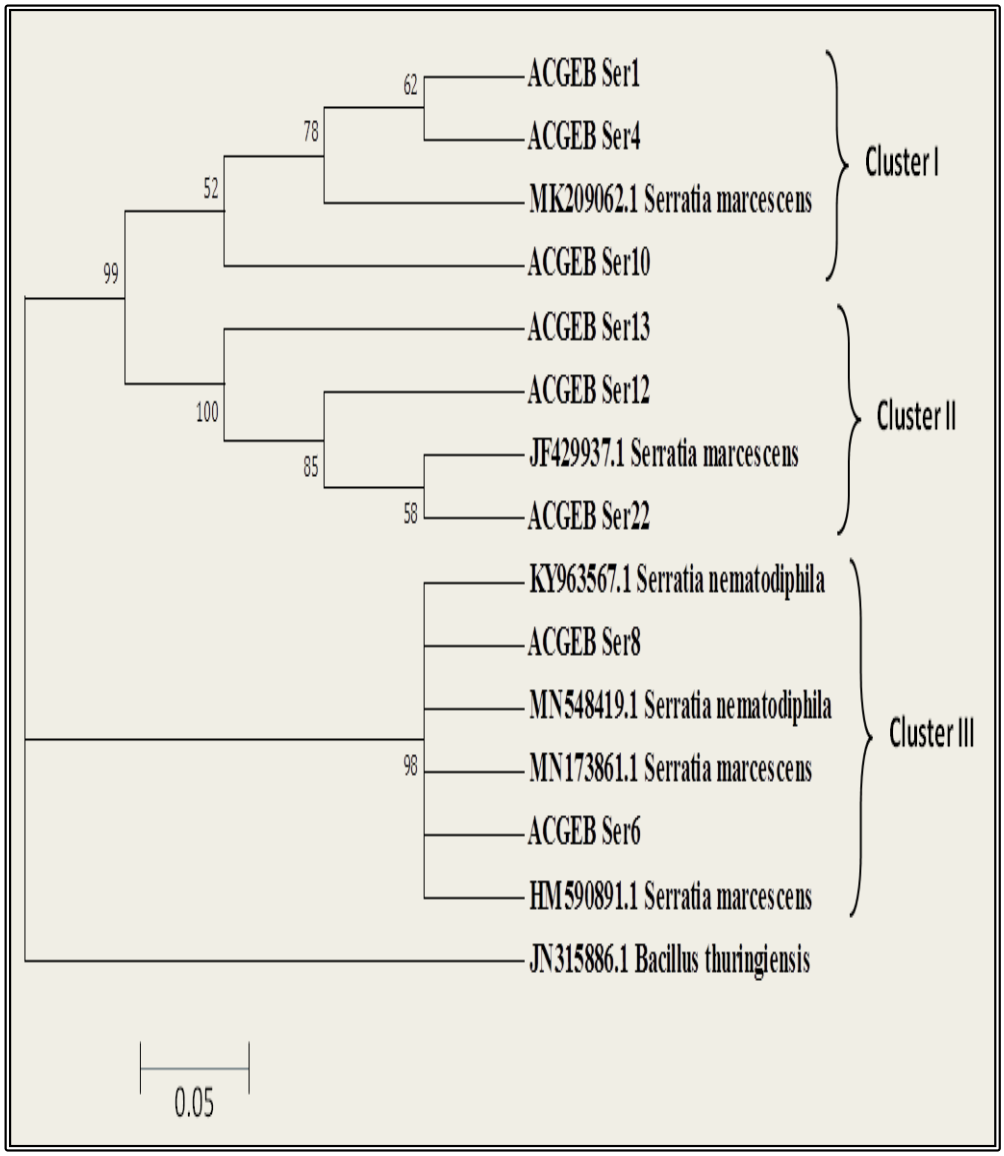

Fig. (1): Phylogenetic tree of ACGEB *Serratia* isolates based on their *16SrRNA* partial gene sequence viewing their similarities with *Serratia* strains from NCBI GenBank database. Scale bar: 0.05.