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RESEARCH ARTICLE

Genetic diversity and symbiotic compatibility among *Rhizobium trifolii*-clover isolated from Sohag governorate.

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Abstract

The present work was undertaken to study the genetic diversity among 25 native *Rhizobium trifolii*-clover isolates obtained from Sohag governorate during 2020 from different locations. morphological markers of the isolated Rhizobium included gram stain; salinity tolerance and pH tolerance were evaluated. In the same time Biochemical markers included Catalase, Citrate utilization, Bromothymol blue, Lysine decarboxylase, and Lipase were tested. Tow of Molecular markers for this isolated Rhizobium included, 16S rRNA, and SRAP marker were also studied. Isolates demonstrated low of similarity coefficient, may reflect a higher diversity of *Rhizobium trifolii* isolates within the fields. A cluster analysis realized using Jaccard's coefficient for the data of SRAP markers, revealed similarity coefficient values ranged from 0.25 to 0.91. The UPGMA cluster analysis based on the SRAP markers according to the data of this work are in full harmony with the previous studies in which PCR genomic fingerprinting is an adequate technique for differentiating Rhizobium strains.

Keywords: Genetic diversity; pH; *Rhizobium trifolii*; Salinity; SRAP marker.

1. Introduction

The Egyptian clover Trifolium alexandrinum, (2n=16) who know as berseem in Egypt one of main important winter forage legume in Egypt, and the Mediterranean region, this is due to its high product yield and quality (Thalooth *et al.*, 2015). Forming root nodules and fixing atmospheric nitrogen two mainly actions do by Rhizobia bacteria for establish symbiosis relation with legumes (Quatrini *et al.*, 2002). Decrease the amount of nitrogenous fertilizers, enrich amount of soil nitrogen direct benefits to this bacteria, while positively affecting some undesirable properties such as temperature, acidity and salinity which lead to severe yield constraint in obtaining plant growth and development were un-direct benefits (Lawson *et al.*, 1995).

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Rhizobia are taxonomically very diverse, so efficient classification methods are needed for determining their diversity. Usually, the diversity of rhizobia was investigated by different morphological and biochemical methods. However, in the past few years, there have been many advances in molecular markers that have provided powerful tools mainly PCR-based which has helped in identifying the genotypic diversity of rhizobia more accurately (Romdhane *et al.*, 2006). Many molecular markers use as tool for genetic diversity and phylogenetic studies between Rhizobia strains such as 16s rRNA, Node genes and sequence-related amplified polymorphism (SRAP).

2. Materials and Methods

2.1. Bacterial Strains

2.1.1. Samples collection

This study was conducted in Laboratories of Genetics Department of Agriculture, Faculty, Sohag University, Egypt.

Twenty-five strains of Rhizobia were isolated from root nodules of Egyptian clover plants locations from Sohag Governorate. These isolates named as S1 to S25.Locations of our samples are shown in, Table 1.

Table 1. Rhizobium isolates Locations from Sohag Governorate.

No	Locations	Strains Name
1	Awlad Salem	S1
2	Awlad Khalaf	S2
3	Naja'a Bardis	S3
4	Alghanimia	S4
5	Alberba	S5
6	Almajabara	S6
7	Al ausayrat	S7
8	Aldowayrat	S8
9	Kharfa sharqia	S9
10	Kharfa gharbia	S10
11	Alsalaa	S11
12	Jazirat Mahrus	S12
13	Alsaahil	S13
14	Aldiyabat	S14
15	Saqulta	S15
16	Altawail	S16
17	Alghorizat	S17
18	Nag Abu Awad	S18
19	Juhayna algharbia	S19
20	Juhayna alsharqia	S20
21	Alsawalem	S21
22	Banho	S22
23	Alsheikh Ammar	S23
24	Tema	S24
25	Faculty's farm	S25

2.1.2. Bacterial isolation

Rhizobia were isolated from clover plants root nodules were washed under tap water to clean it from mud and soil remains. Under complete sterilized conditions Individual nodules were cut out from the secondary roots, taking into consideration very small nodules which will be attached either side of the nodule with little root tissue. For maximum thirty minutes according to the nodule size Nodules were washed thoroughly in RO water and the non-ionic surfactant 'Tween 80' (100 μL.L $^{-1}$) to remove all traces of soil. The nodules were then transferred to a sterile petri dish and surface sterilized by immersion in 10 mL of a 5% solution of commercial sodium hypochlorite (final concentration: 0.25 g.L $^{-1}$ NaOCl) and Tween 80 (10 μL.L $^{-1}$), sterile mQ water were rinsed more than one time in Individual nodules which crushed and streaked on Yeast Extract Manitol Agar (YEMA) plates and incubated at 26°C for between three and ten days. Individual colonies appearing over this period were re-streaked onto YEMA plates, and sub-cultured onto YEMA slopes in test tubes for short-term storage at 8°C.

2.2. Morphological Characterizations

The test of gram stain type was made and the ability to grow at different pH degree start from pH 4.0 to pH 12.0 was test too by adjust of YEMA pH using NaOH and HCL. Salinity test was made by growth of *Rhizobiu leguminosarum bv.trifolii* in different concentration (1% - 5%) of

NaCl add to Mannitol Yeast Extract Agar medium and observing the bacterial growth and tolerance to exceeding salt concentration comparing to the control the colonies were observed after 24 to 72 hours of incubation (Al-Taie, 2008).

2.3. Biochemical Characterizations

The isolates were tested for presence of enzyme catalase, Lipase presence around bacterial colonies, and Lysine decarboxylase test fowling the methodology of (Wadhwa *et al.*, 2017). Citrate utilization as a carbon source test and Bromothymol blue test were done used. (Datta *et al*., 2015)

2.4. Molecular Characterizations

2.4.1. DNA extraction

Total DNA were isolated from Fresh bacteria of twenty-five isolates using cetyl trimethyl ammonium bromide (CTAB) protocol Porebski *et al.* (1997), at Biotechnology Laboratory of the Genetics Department, Faculty of Agriculture, Sohag University. Primer pairs 16S-1F/16S-1509R were amplified for 16S rRNA gene to (Table 2) yielding 1300-bp and 1500-bp products respectively.

2.4.2. Data analysis and dendrogram construction

To analyze the SRAP banding patterns which generated by marker Gene Profiler (version 4.03) computer programme was used. The presence (1) or absence (0) of each band was recorded for each sample for all studied primers and Genetic distance was estimated according to Jaccard's (1908). Formula of Ghislain *et al.* (1999) as PIC= 1- [(p)2 + (q)2], was used to megarment the informativeness of the SRAP technique in differentiating among genotypes, the polymorphic information content (PIC), where p is the frequency of allele band present and q is frequency of allele band absent across the studied bacteria samples. The genetic similarities among the tested Rhizobium bacteria samples were computed and UPGMA-dendrogram was performed according to Jaccard's coefficient of similarity using NTSYS-pc version 2.20 (Applied Biostatics Inc.) (Rohlf, 2000).

Table 2. SRAP primers pairs, their sequences and temperatures (Tm ºC) used in this study.

Primers	Sequences (5' – 3')	Tm ºC
ME-1(F)	TGAGTCCAAACCGGATA	49
ME-2 (F)	TGAGTCCAAACCGGAGC	54
ME-7(F)	TGAGTCCAAACCGGACG	54
ME- 9(F)	TGAGTCCAAACCGGTGC	56
EM-3(R)	GACTGCGTACGAATTGAC	50
EM-5(R)	GACTGCGTACGAATTAAC	47
EM-6(R)	GACTGCGTACGAATTGCA	53
16S-1F	AGC GGCGAC GGG TGA GTA ATG	65
16S-1509R	AAG GAG GGG ATC CAG CCG CA	64

3. Results and discussion

3.1. Morphological characters

Nitrogen-Fixation plants effect by soil negative changes like high Salinity and low pH which considered as limiting factors to the growth and activity of nitrogen-fixing. So, morphological characterization of the 25 isolates of *Rhizobium trifolii* including gram stain, pH and salinity tolerant was studied. The results showed that all 25 isolates were found gram negative as the cells appeared pink in color after gram staining. In addition, it could be observed that all 25 isolates showing mucoid white colonies on YEMA plates. The visual examination of the cells revealed that all isolates were short rod in shape. So, these isolates were preliminarily identified as *Rhizobium trifolii* based on our data. Similar findings were obtained by (Datta *et al*., 2015;

Kapembwa *et al.*, 2016; Tanim *et al*., 2019; Hossain *et al*., 2019). They found that all isolates strain were shown gram negative and short rod and motile in shape.

3.2. Salinity Tolerance

Result showed that all of isolates grown in 1% salinity but 16 isolates grown at 2.0% NaCl. Moreover, 14 isolates strains were grown under 3.0% NaCl. Two isolated showed Promising results by grown at 4.0 % NaCl but all strains were unable to grow at 5.0% NaCl (Fig 1). The ability of growth in alkaline soils under salt stress for Rhizobial strains mentioned by (Surange *et al.*, 1997). Survival isolates from Rhizobia Bacteria was notice at 4% and 4.5% NaCl by Kucuk *et al.* (2006). Shamseldin and Werner (2004) stated that two isolated Rhizobial strains were highly tolerant to a salt concentration up to 4% NaCl (EBRI 21 and EBRI 26) and three strains were moderately tolerant up to 3% NaCl (EBRI 24, 27, and 29). Similar findings were obtained by (Al-Taie, 2008; Gauri *et al*., 2012; Fentahun *et al*., 2013; Datta *et al*., 2015; Wadhwa *et al.*, 2017; Kumar *et al.*, 2018). They found that all isolated Rhizobial strains were grown in medium having 1.0 to 4% concentration of consequently, high levels of salt have inhibitory effect on the growth of Rhizobium. It could be observed that these salt inhibitory concentrations varied among strains. So, the genetic variations among the isolated strains were tolerant to salt stress. Therefore, selection of salt tolerant strains of *Rhizobium trifolli* could become a good screening criterion for symbiotic nitrogen fixation in unfavorable environment**.**

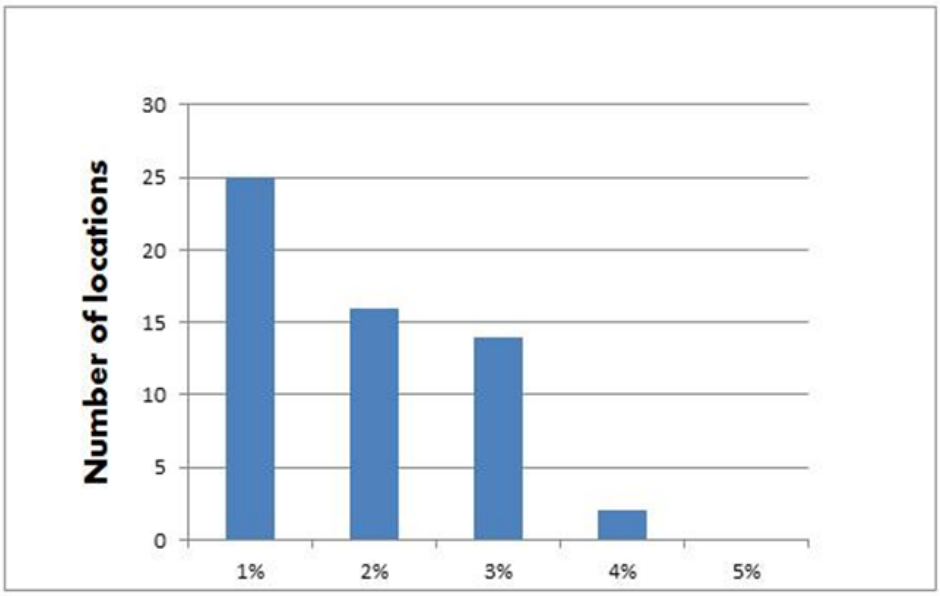

Fig 1. Sodium Chloride solution NaCl %

3.3. pH Tolerance

The effect of pH levels on the bacterial growth presented in Fig 2. Generally, the most isolated strains grew efficiently at different pH levels The results showed that none of all tested isolates grown at pH 4.0, revealing that isolated strains were sensitive to acidity. Whereas, all strains grown efficiently at pH levels from 5.0 to 10.0. However, 16 and 12 out of 25 isolates exhibited good growing at pH 11.0 and 12.0, indicating that these strains were tolerant to pH 11.0 and 12.0 levels, respectively.

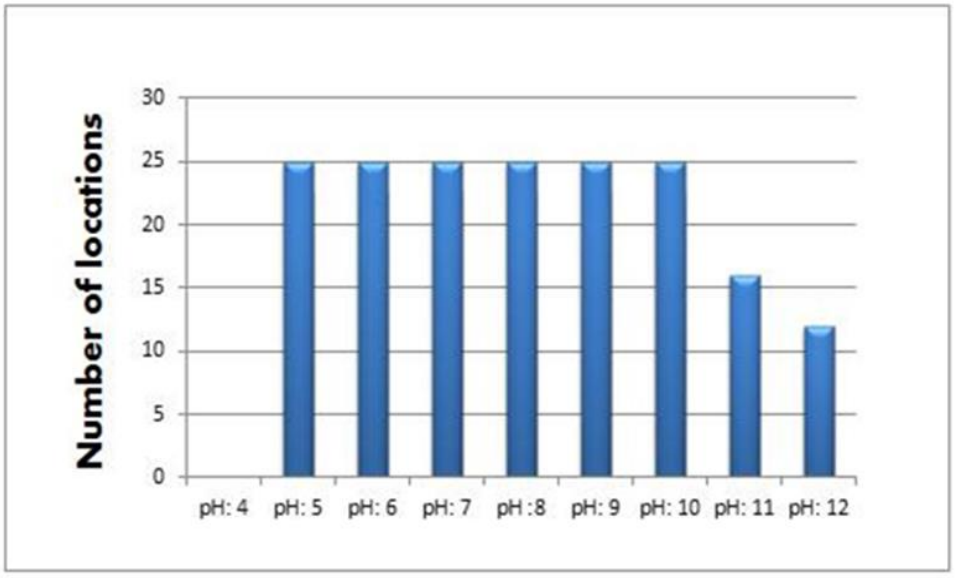

Fig 2. Effect of pH levels on growth of Rhizobium isolates.

Consequently, these tolerant strains are highly recommended for forming good nodulations in Egyptian clover in alkaline soils. Jida and Assefa (2014) evaluated acidity tolerance of *Rhizobium leguminosarum* strains for their symbiotic performance under different acidic conditions. Their results showed that all acid tolerant strains were recovered from highly acidic soil (4.8- 5.2) and the acid sensitive strain was isolated from neutral soil. So, our results revealed that these isolates have a good potential efficient inoculant for Egyptian clover in alkaline soils. Therefore, selection of alkaline tolerant Rhizobia to inoculate clover hosts under alkaline conditions may help the establishment of the symbiosis and also may improve the alkaline tolerance of Egyptian clover.

3.4. Biochemical characters

Our results showed that all isolates were positive for catalase test except three strains (S1, S13 and S14) see Table 5, However, all isolates were positive for Lipase test. Whereas, all isolates were negative for Citrate utilization and Lysine decarboxylase. While, 17 out of 25 isolated strains were positive for Bromothymol blue test. Singh and Sewak (2013) also observed positive results for catalase activities. Wani and Khan (2013) and Gauri *et al.* (2012), also reported that *Mesorhizobium* isolates were positive for catalase production, citrate utilization and were negative for lipase.

Generally, our results of biochemical markers confirm that the isolated bacterial strains are belong to Rhizobium. These results are similar to those previously obtained by Singha *et al.*, 2015; Tyagi *et al*., 2017; Kumar *et al*., 2018).

Rapid and unambiguous identification of marker strains among field isolates has greatly benefited from recent advances in DNA fingerprinting methods based on the polymerase chain reaction (PCR). In this study, two primers 16S-1F and 16S-1509R were used to amplify of 16S rRNA gene among the 25 Rhizobium bacteria samples. The results showed that all of isolates generated size of bands about1500 bp (Fig 3), suggesting that these isolates belonged to the genus rhizobium. It could be noticed that the PCR results of 16s rRNA did not show any polymorphism between studied isolates. So, it is necessary to use other genetic markers which should have a higher molecular evolution rate than the 16S rRNA Li *et al.* (2011) and Youssef *et al.* (2019).

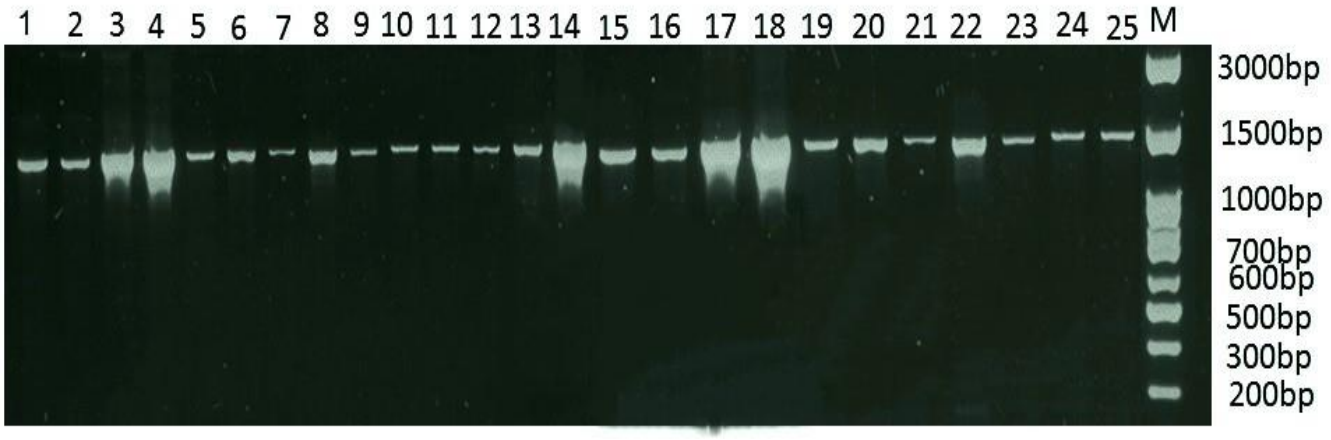

Fig 3. Gel profile of 16s rRNA for 25 samples of Rhizobium bacteria.

3.5. SRAP Marker

Sequence-related amplified polymorphism (SRAP) markers are based on two primers amplification, which preferentially amplifies open reading frames (ORFs) or coding regions using polymerase chain reaction (PCR). It could provide high polymorphism and plentiful information to assess the genetic diversity. In this study, fifteen pairs of SRAP primers were screened among the 25 Rhizobium bacteria samples under study and 4 pairs of them (forward and reverse) were polymorphic. A total of 45 bands were amplified, of which 40 bands (88.88%) were found polymorphic. The total number of bands was from 4 (ME7-EM5) to 16 (ME1-EM6). Adding, the polymorphic band numbers varied from 2 to 16 bands for the same primer combinations, respectively. The percentage of polymorphism (P%) ranged from 25.00 to 100.00% was detected by ME7F-EM5R and ME9F-EM3R primer combination, respectively, with an average of 76.91% see (Table 3). The mean number of total bands and polymorphic bands were 11.25 and 10.0 per primer, respectively. The smallest and largest size of bands were 65 bp and 2300 bp, generated by ME7-EM5 and ME2-EM5 primers (Fig 4), respectively.

In this regard, Abi-Ghanem *et al*. (2013) found that DNA of 95 Rhizobial isolates was successfully amplified by ten SRAP primer pairs, and a total of 102 markers were resolved. Also, the number of markers produced by each primer set ranged from 8 to 14 with an average of 10 markers per primer pair. Likely, Shalaby *et al*. (2014) used six SRAP combination primers to identify the genetic variability and genetic relationship among the three Rhizobia genotypes. High level of polymorphism was observed among these three genotypes. They found, four out of six SRAP combinations produced amplified bands, however, ME1-EM2 primers gave highest percentage of polymorphism 57.1% followed by ME3-EM2 gave 50% polymorphism. Also, a total of 21 amplified bands, out of them nine polymorphic bands. The highest P% (57.1) was generated by combination primers ME1-EM2, scored seven bands followed by primer ME3-EM2, gave eight bands with P% of 50.0%. These findings imply that SRAP markers are useful and efficient to estimate genetic diversity level in Rhizobial bacteria.

3.6. Cluster analysis

A cluster analysis realized using Jaccard's coefficient for the data of SRAP markers, revealed similarity coefficient values ranged from 0.25 to 0.91.

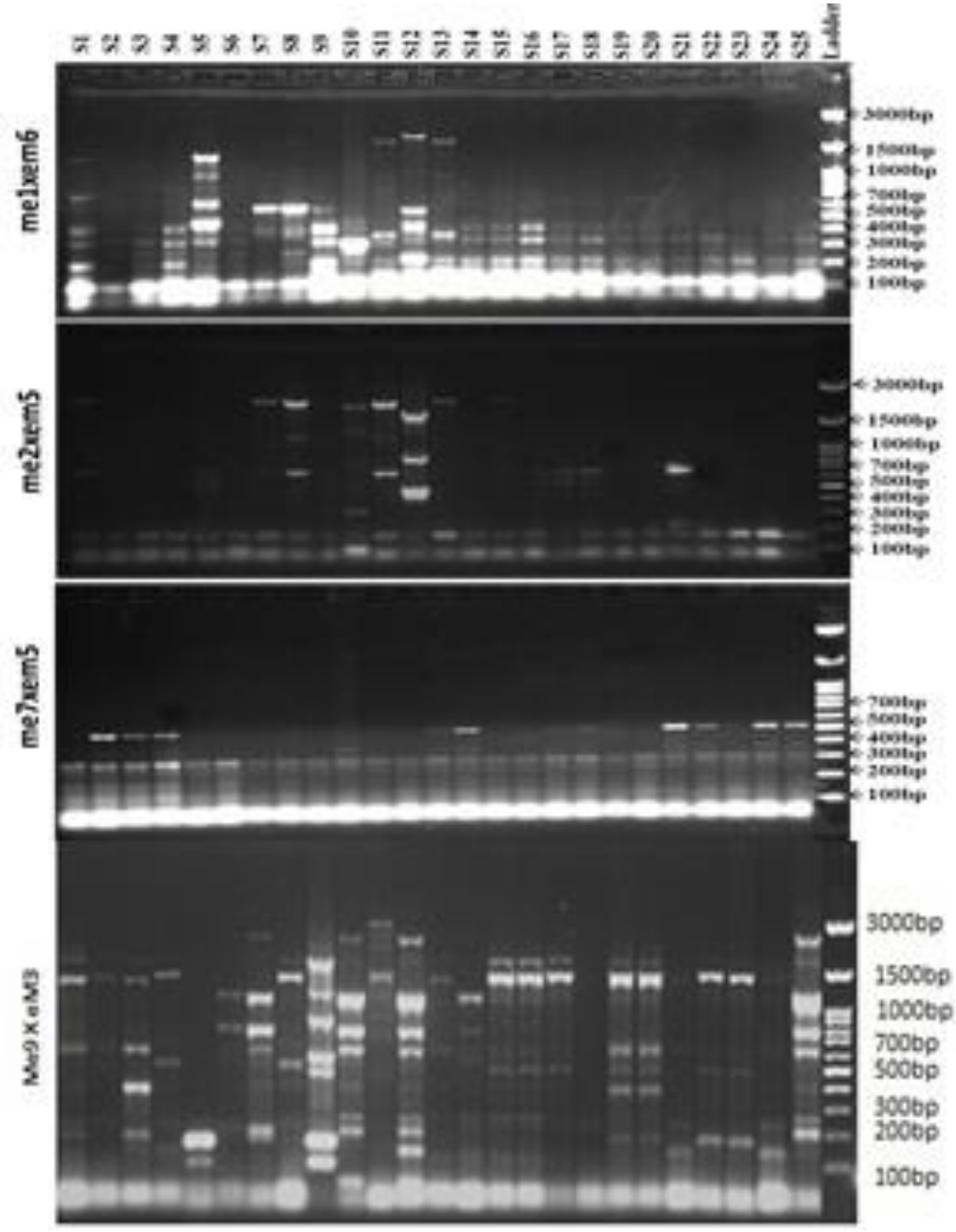

Fig 4. SRAPs profiles obtained for 25 Rhizobium samples from different locations amplified with four primer combinations and ladder = 100bp.

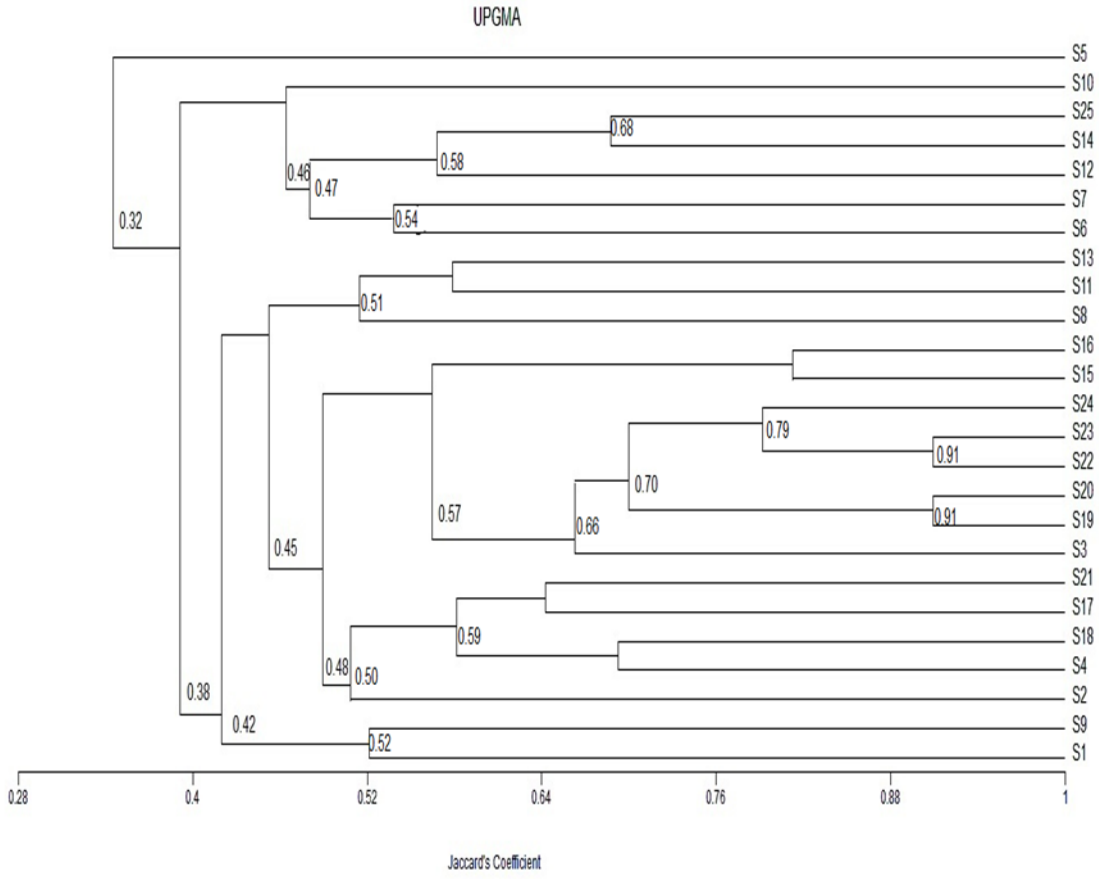

Fig 5. UPGMA-Dendogram of genetic similarities using SRAP data based on Jaccard's coefficient among 25 studied Rhizobium bacteria isolates.

The UPGMA cluster analysis based on the SRAP markers separated the studied bacterial samples into sixth significantly different clusters (Fig 5). The first cluster was with genotypes "S1 and S9". The second contains five strains S4, S18, S17, S21 and S2 which branched at 0.50. The third cluster contains eight strains S3, S19, S20, S22, S23, S24, S15 and S16 branched at 0.57, the second and third cluster with gathered at 0.48 similarity coefficient (Fig 5). The fourth cluster contains the strains S8, S11 and S13 at 0.51 similarity coefficient, this cluster linked with cluster second and third at 0.45 similarity coefficient. The genotype S16 placed alone in the fourth cluster. The fifth cluster was with six strains (S6, S7, S12, S14, S25 and S10), branched at 0.48 similarity coefficient. The Rhizobium strain S5 placed on sixth, the strain shows very low similarity coefficient (0.32) with the other studied isolates (Fig 5). Isolates demonstrated low of similarity coefficient, may reflect a higher diversity of Rhizobium trifolii isolates within the fields.

Among all molecular markers, SRAP was firstly applied in Rhizobia strains for successive resources conservation, maintainable utilization and further research. SRAP aims at the amplification of open reading frames and its markers more reliable than RAPD, it has been widely applied in genetic diversity analysis, molecular identification, genetic linkage map construction, gene tagging (Ren *et al.*, 2010; Cai *et al.*, 2011). SRAP markers were used to examine genetic diversity among isolates of *R. leguminosarum.* The phylogenetic tree constructed by SRAP analysis demonstrated

higher variability among rhizobial isolates than that from 16S rRNA sequences (Abi-Ghanem *et al.*, 2013).

The data of this work are in full harmony with the previous studies in which PCR genomic fingerprinting is an adequate technique for differentiating Rhizobium strains (Zribi *et al*., 2004; Ogutcu *et al*., 2009). Contrary, Blazinkov *et al.* (2007) reported that PCR was less reliable for differentiating among different strains of *Rhizobium leguminosarum* isolated from different locations.

Table 3. Primers used for SRAP marker, total number of fragments detected by each pair of primers, %P, PIC, MI, RP and fragments sizes.

Primer combinations	Amplified bands		%P	PIC	MI	RP	Fragments size (bp)	
	Bands number	Polymorphic bands					Larger	Smaller
ME1F-EM6R	16	15	93.75	0.17	2.55	3.52	2020	870
ME2F-EM5R	9	8	88.89	0.12	0.01	2.24	2300	70
ME7F-EM5R	4	1	25.00	0.12	0.12	0.88	520	65
ME9F-EM3R	16	16	100.00	0.37	5.92	9.28	1975	110
Total	45	40						
Means	11.25	10	76.91	0.20	2.15	3.10		

%P: Percentage of polymorphism, **MI**: Marker index, **PIC:** Polymorphic information content, **RP**: Resolving power.

4. Conclusion

In conclusion, the present study was undertaken to study the genetic diversity among 25 native *Rhizobium trifolii*-clover strains isolated from Sohag Governorate. The isolated bacteria were identified on the basis of morphological, biochemical and molecular characters. Our results showed that all isolate strains were preliminarily identified as *Rhizobium trifolii* according to the visual examination and biochemical markers. Moreover, the existence of genetic variation among the isolated strains in this study for tolerance to salt and pH stresses, suggesting that these isolates have a good potential efficient for Biological Nitrogen Fixation (BNF) for Egyptian Clover in unfavorable environment inoculant. Concerning molecular markers assessment, the results of 16S rRNA gene based on PCR showed that all of isolates are belonged to the genus Rhizobium. In addition, the results of SRAP analysis for isolate strains demonstrated low of similarity coefficient, may reflect a higher diversity of *Rhizobium trifollii* isolates within the fields.

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There is no fund in this research.
Institutional Review Board Statement
All Institutional Review Board Statement are confirmed and approved.
Data Availability Statement
Data presented in this study are available on fair request from the respective author.
Ethics Approval and Consent to Participate
This work carried out at genetics department and followed all the department instructions.
Consent for Publication
Not applicable.
Conflicts of Interest
The authors declare no conflict of interest.

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