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Near-full length sequencing of 16S rDNA and RFLP indicates that *Rhizobium etli* is the dominant species nodulating Egyptian winter Berseem clover (*Trifolium alexandrinum* L.)

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ABSTRACT

Egyptian winter Berseem clover (EWBC) is one of the main important forage legume crops in Egypt that is used for animal feeding in winter and it occupies about 2.5 million feddans (Feddan = 4200 m^2) in winter agricultural rotation systems. Forty-eight rhizobial isolates that nodulated this legume host from different geographical regions within Egypt were isolated. RFLP analyses of 16S rDNA (1.5 kb) and whole ribosomal DNA (5 kb), the sequencing of 16S rDNA, and the sequencing of *nodC*, *nifH* and house keeping genes were used to identify these isolates. The RFLP analysis of 16S rDNA (1.5 kb) among 15 representative strains with three enzymes generated two genotypes. The largest genotype was similar to *Rhizobium etli* CFN42T (93.33%) except for strain 902 that failed to re-nodulate EWBC. RFLP analysis of complete ribosomal DNA (5 kb) produced five genotypes. The majority of tested strains shared the genotype with *R. etli* CFN42T (53.33%). Only one strain (1002) shared the genotype with *Rhizobium leguminosarum* sv. *trifolii* 3023. The other four strains were comprised of two unique genotypes. Phylogenetic analysis of 16S rDNA sequences revealed that seven representative strains could be divided into two genetic clusters sharing the ancestral clad with *R. etli* CFN42T. A phylogenetic tree based on *nodC* gene sequence confirmed that all the examined strains shared the genetic lineage with *R. leguminosarum* sv. *trifolii* WSM1325. The phylogenetic trees of house keeping genes are supported strongly the identification of majority of strains as a novel symbiovar of *R. etli* with new lineages.

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Introduction

Bacteria belonging to the family *Rhizobiaceae* fix atmospheric nitrogen through symbiotic association with legumes. Worldwide about 44–66 million tons of nitrogen is biologically fixed annually and providing nearly half of all requirements used in agriculture [3]. Due to their considerable agricultural and environmental significance the legume-symbionts have been extensively used as an alternative to synthetic fertilizers to supply the nitrogen requirements of plants in agro-ecosystems [19].

Forage crops play important roles for animal production. Egyptian winter Berseem clover (*Trifolium alexandrinum* L.) and abbreviated as (EWBC) has considerable significance among those forage crops due to its limited bloat problems as animal fodder, fast winter growth rate, and a relatively long growing season [22].

Numerous reports on the identification and biodiversity of legume symbionts isolated around the world have been reported [4,10,35,36,46]. However despite its cultivation in Egypt for many centuries little information is available about the population of diversity of symbiotic N_2 fixing bacteria nodulating EWBC [34]. Restriction fragment length polymorphism (RFLP) analysis of rDNA genes has been used successfully to identify numerous phylogenetically diverse bacterial genera [25,37,45,50]. Analysis of 16S rDNA sequences is widely used to define the taxonomic status of rhizobia [57,24,43,28].

In rhizobia many of the genes for symbiotic interaction and nitrogen fixation with legumes are localized on large symbiotic (pSym) plasmids. Several investigators [7,9,15,38] have confirmed

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the ability of pSym to transfer among soil bacteria via conjugation. It has been suggested that *Rhizobium leguminosarum* sv. *trifolii* frequently transfers its symbiotic plasmid to other rhizobial and non rhizobial strains in natural environments [1,13,14,41,53]. Since pSym plasmids are often not correlated with a specific chromosomal genotype among rhizobia, the use of 16S rDNA sequences alone to identify new rhizobial isolates is problematic [54]. Consequently, in this study we examined the relationship between sym plasmid localized (*nodC* and *nifH*) and chromosomal localized (16S rDNA, *recA*, *glnII* and *atpd*) genes to strongly solve the controversial of identifying these EWBC microsymbionts obtained from a variety of geographic locations in Egypt.

Materials and methods

Rhizobial isolation

Forty-eight rhizobial isolates were obtained after surface sterilization of randomly collected nodules of clover according to the method described by [48]. Yellow colonies grown on yeast extract mannitol agar amended with bromothymol blue and free of contamination were collected and kept in 50% glycerol at −80 °C for further analysis. Standard rhizobial strains of *Rhizobium etli* CFN42T and *R. leguminosarum* sv. *trifolii* 3022 and 3023 were kindly provided by Mike Sadowsky.

Plant infection technique

Seeds of Egyptian winter Berseem clover cultivar Meskawy and common bean cultivar Perto 143 were surface sterilized by brief immersion in 95% ethanol followed by 3 min in 1% sodium hypochlorite. Seeds were washed several times with sterile water and left to imbibe 4 h except for seeds of clover which were soaked overnight at 5 °C. Seeds were distributed onto the surface of 1% water agar plates then and incubated at 30 °C in the dark for germination. After germination seedlings were cultivated in Leonard jars [48] and incubated in a plant growth chamber under the following conditions 10–12 light, 70 humidity and day/night temperatures of 28 °C and 20 °C, respectively. Three replicates of each strain were examined for nodulating ability and un-inoculated plants served as negative controls.

Phenotypic characterization of strains

Several phenotypic characteristics were applied to test the ability of strains to resist antibiotics such as kanamycin (40 ppm), neomycin (15 ppm), growth on LB medium and resistant to environmental stresses such as salinity, alkalinity and high temperature [44].

DNA isolation

Total genomic DNA was isolated from mid log phase rhizobial cultures using Promega DNA isolation Kits according to the manufacture's protocol. The purity of DNA was checked on 0.8% agarose gels, run in TAE buffer, and samples were diluted to ~50 ng μl^{-1} and kept at 4 °C for further PCR analysis.

BOX-AIR PCR finger printing

Rep-PCR DNA fingerprints of isolates were obtained by using the Box-AIR primer (5-CTACGGCAAGGCGACGCTGACG-3) as previously described [12]. Gel images were captured with a FOTO/Analyst Archive electronic documentation system (Fotodyne Inc, Hartland, WI).

Normalization of BOX-AIR patterns

Gel images were normalized using Bionumerics software (Version 1.5; Applied Maths, Kortrijk, Belgium) in the presence of 1 kb plus DNA ladder for comparison. Similarity coefficients were generated by the band-based method of Jaccard. A dendrogram was constructed by using Jaccard similarity coefficients and a binary band-matching character table was generated by using the BOX-derived PCR DNA fingerprint data. Data was analyzed by using multivariate analysis of variance (MANOVA), a form of discriminate analysis accounting for covariance structure.

Amplification of whole rDNA, 16S rDNA, nodC, nifH, recA, glnII and atpD genes

About a 5 kb ribosomal DNA fragment was amplified using primers 16S rDNA forward primer 63f (5′-CAGGCCTAACACATGCAAGTC-3′) corresponding to *Escherichia coli* 16S rDNA bases 63–84 [29] and 23S rDNA reverse primer P4 (5-CCCGCTTAGATGCTTTCAGC-3′) corresponding to *E. coli* 23S rDNA bases 2744–2763 bp [50]. Reactions were done in 50 μl as previously described [45]. An approximately 1.5 kb ribosomal DNA fragment was amplified using primers fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) as described by [55]. The 940 bp DNA fragment of *nodC* gene was amplified using primers nodCF4 (5′-AYGTHGTYGAYGACGGATC-3′) and nodCI (5′-CGYGACAGCCANTC KCTATTG-3′) while the 720 bp of *nifH* gene was generated using primers *nifHF* (5′-TACGGNAARGGSGGNATCGGCAA-3) and *nifHI* (5′-AGCATGTCYTCSAGY TCNTCCA-3′) as described by [26]. To amplify the specific region of the three house keeping genes of *recA*, *glnII* and *atpD* the following primers recA6f (5′-CGKCTSGTAGAGGAYAAATCGGTGGA-3′) and recA640r (5′-ACATSACRCCGATCTTCATGC-3′); glnII 12F (5′-YAAGCTCGAGTACATYTGGCT-3′) and glnII 689R (TGCATGCCSGAGCCGTTCCA-3′) and atpDf (5′-GCTSGGCCGCATCMTSAACGTC-3′) and atpD782R (GCCGACACTTCMGAACCNGCCTG-3′) were used on respectively as mentioned by [30]. PCR reactions (50 μl) were performed in 1× PCR buffer, 1.5 mM MgCl, 5% dimethyl sulfoxide, 200 μM of each dNTP, 15 pmol of each primer, 1 U of Taq and 50 ng of purified DNA. The temperature program was as follows: initial denaturation at 95 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, except for the *glnII* where the annealing temperature was 58 °C. A final extension was conducted at 72 °C for 7 min. About 10 μl of each PCR products were electrophoresed on 0.8% agarose gels containing ethidium bromide in TAE buffer and bands were visualized under UV light.

RFLP analysis of 5 kb ribosomal DNA and 1.5 kb 16S rDNA

PCR product of the 5 kb ribosomal DNA and the 1.5 kb 16S rDNA were digested to completion using restriction endonucleases *Hinf*I, *Cfo*I and *Msp*I (USA Amersham International) using conditions as recommended by manufacture. DNA fragments were resolved on 1.5 agarose gels, in 0.5× TAE buffers, to examine differentiation among rhizobial genotypes [45].

16S rDNA, nodC, nifH, recA, glnII and atpD sequencing

Fragments generated from *16S rDNA* (1.5 kb), *nodC* (940 bp), *nifH* (720 bp), *recA*, *glnII* and *atpD* were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacture's instructions and each product was sequenced with the same primers that were used before for amplification. Sequencing reactions was done at Sigma Company (5 Eschenstrasse, 82024 Taufkirchen, Germany). Sequences reads were

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