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The symbiovar trifolii of *Rhizobium bangladeshense* and *Rhizobium aegyptiacum* sp. nov. nodulate *Trifolium alexandrinum* in Egypt

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ABSTRACT

In the present work we analyzed the taxonomic status of several *Rhizobium* strains isolated from *Trifolium alexandrinum* L. nodules in Egypt. The 16S rRNA genes of these strains were identical to those of *Rhizobium bangladeshense* BLR175^T and *Rhizobium binae* BLR195^T. However, the analyses of *recA* and *atpD* genes split the strains into two clusters. Cluster II strains are identified as *R. bangladeshense* with >98% similarity values in both genes. The cluster I strains are phylogenetically related to *Rhizobium etli* CFN42^T and *R. bangladeshense* BLR175^T, but with less than 94% similarity values in *recA* and *atpD* genes. DNA–DNA hybridization analysis showed 42% and 48% average relatedness between the strain 1010^T from cluster I with respect to *R. bangladeshense* BLR175^T and *R. etli* CFN42^T, respectively. Phenotypic characteristics of cluster I strains also differed from those of their closest related *Rhizobium* species. Analysis of the *nodC* gene showed that the strains belong to two groups within the symbiovar trifolii which was identified in Egypt linked to the species *R. bangladeshense*. Based on the genotypic and phenotypic characteristics, the group I strains belong to a new species for which the name *Rhizobium aegyptiacum* sp. nov. (sv. trifolii) is proposed, with strain 1010^T being designated as the type strain (= USDA 7124^T = LMG 29296^T = CECT 9098^T).

Several fast growing *Rhizobium* strains isolated from *Trifolium alexandrinum* in Egypt were previously classified into the species *Rhizobium etli* because they were phylogenetically related with the type strain of this species (CFN42^T) on the basis of 16S rRNA gene analysis [18]. Nevertheless, in the past year four new species have been described within the phylogenetic group of *R. etli* with very close or even identical 16S rRNA gene sequences and phylogenetically divergent housekeeping genes, including *recA* and *atpD* [5,11]. From them, *Rhizobium bangladeshense* and *Rhizobium binae* have identical 16S rRNA genes and were very closely related to *Rhizobium lentis* and *R. etli*, also with identical 16S rRNA genes [11].

In the present work we analyzed strains closely related to *R. etli* that were previously isolated from nodules of *Trifolium alexandrinum* L. plants growing in different regions in Egypt. We found that these strains have identical 16S rRNA genes than the type strains of the recently described species *R. bangladeshense* and *R. binae* [11]. Nevertheless, the Egyptian strains could be divided into two clusters based on the analyses of *recA* and *atpD* genes. Cluster II strains were identified as *R. bangladeshense*, but cluster I strains constitute a new species in the genus *Rhizobium* from which we propose the name *Rhizobium aegyptiacum* sp. nov. All these strains carry *nodC* genes typical of members from the symbiovar trifolii, reported in this work for the first time in the species *R. bangladeshense*.

The genetic diversity of the Egyptian strains was assessed by RAPD fingerprinting using the M13 primer as was previously described [12] and the results showed that these strains have six different RAPD patterns (Fig. S1). The strains 950, 935, 996, 1017,

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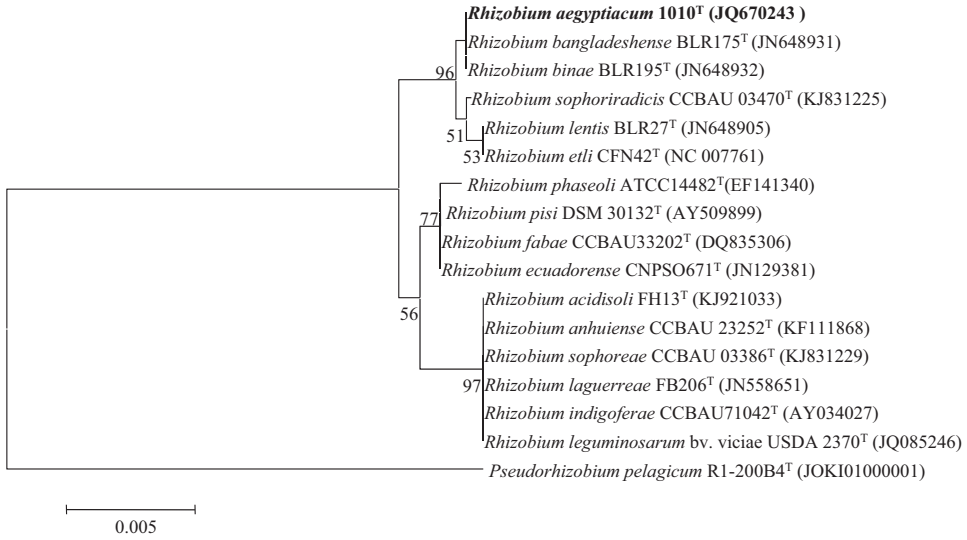

Fig. 1. Neighbour-joining plylogenetic tree based on 16S rRNA gene sequences (1434 positions) showing the relationships among *Rhizobium aegyptiacum* sp. nov. and closely related species in the genus *Rhizobium*. The significance of each branch is indicated by a bootstrap value (in percentage) calculated for 1000 subsets (only values higher than 50% are indicated). Bar, 0.5 substitution per 100 nucleotide position.

1010^T, and 1024 representing RAPD patterns types I, II, III, IV, V and VI, respectively, were selected for gene sequencing.

The 16S rRNA gene sequences were again obtained in this study in order to enlarge them according to Rivas et al. [13], sequences of *recA* and *atpD* genes were obtained as described by Gaunt et al. [4] and that of *nodC* gene as described by Laguerre et al. [7]. The sequences obtained were compared with those from GenBank using the BLASTN algorithm [1]. Sequences were aligned using the ClustalX software [20] and distances were calculated according to Kimura's two-parameter model [6]. Phylogenetic trees were inferred using the neighbour-joining (NJ) [16] and maximum likelihood (ML) [14] analyses. MEGA5 software [19] was used for all analyses.

The 16S rRNA gene was identical in all strains from the new species *R. aegyptiacum* and therefore only the type strain 1010^T was included in the phylogenetic analyses. They showed that the sequence of this gene was also identical to that of *R. bangladeshense* BLR175^T (LMG 28442^T) and *R. binae* BLR195^T (LMG 28443^T), which formed a wide cluster including *R. lentis* BLR27^T (LMG 28441^T), *R. etli* CFN 42^T and *Rhizobium sophoradicis* CCBAU 03470^T after ML (Fig. 1) and NJ (Fig. S2) analyses. All these species are distinguishable by housekeeping gene analysis, which complements that of 16S rRNA gene analysis in taxonomic studies at the species level [21].

The *recA* and *atpD* housekeeping genes have been sequenced in all members of genus *Rhizobium* and they are useful to differentiate among closely related species within the phylogenetic group encompassing the strains from this study [5,11]. The concatenated *recA* and *atpD* gene sequence analyses placed these strains in two clusters related to the species *R. bangladeshense* and *R. etli* (Figs. 2 and S3). The strains 1017 and 1024 (cluster II) have *recA* and *atpD* gene closely related to those from *R. bangladeshense* BLR175^T (>98% similarity in both genes) and then they should be classified within this species. However, the strains 1010^T, 935, 950 and 996 (cluster I) were phylogenetically divergent to both *R. bangladeshense* BLR175^T and *R. etli* CFN42^T constituting a new *Rhizobium* species named *R. aegyptiacum* with internal similarities >99% in both the *recA* and *atpD* genes. The closest related strains to *R. aegyptiacum* were *R. etli* CFN42^T and *R. bangladeshense* BLR175^T with <94% similarity values in both *recA* and *atpD* genes. These values are lower than those found between several *Rhizobium* species, including *R. bangladeshense* and *R. etli* or *R. bangladeshense* and *R. binae* (Table S1).

DNA–DNA hybridization experiments were done as was previously reported [3,23]. Strains 1010^T and 950 (cluster I) that present different RAPD patterns and have different *recA* and *atpD* gene sequences showed an average of 81% (± 2%) DNA–DNA relatedness confirming they belong to the same species. The strain 1010^T shows 42% (± 4%) and 48% (± 6%) with respect to *R. bangladeshense* LMG 28442^T and *R. etli* CFN42^T, respectively. These values are below the 70% threshold value of DNA–DNA similarity for definition of bacterial species [22], confirming the classification of strains 1010^T, 935, 950 and 996 into a new species.

DNA for base composition analysis was prepared according to Chun and Goodfellow [2]. The mol% G+C content of DNA was determined using the thermal denaturation method [8]. The G+C content of strain 1010^T was 61.6%, which is within the range reported for its closest related *Rhizobium* species [11].

The phenotypic characterization was performed using the same tests and methodologies previously reported [10,15], including API ID32GN (with the addition of $MgSO_4$ up to a final concentration of 0.02 g l^{-1}), API 20NE systems and 24 PNP (paranitrophenyl)-substrates. Tolerance to NaCl, temperature and pH, and antibiotic sensitivity were determined following the protocols described in our previous work [15]. The type strains of the most closely related *Rhizobium* species were included in the phenotypic study as reference. Phenotypic characteristics of the new species are reported below in the species description and the differential features with respect to the closest species of *Rhizobium* are recorded in Table 1.

The analysis of core genes allowed the classification at genus and species level in rhizobia, but these bacteria also carry genes involved in the interactions with plants, from which the *nodC* gene has been most used to define symbiovars [9]. The closest related species to the Egyptian strains were *R. bangladeshense* isolated from nodules of *Lens culinaris* and *R. etli* isolated from nodules of *Phaseolus vulgaris* [11,17]. The type strains of these species belong to the symbiovars viciae and phaseoli, respectively (Fig. 3). The analysis of the *nodC* gene showed that, as expected, the strains from the two species isolated from Egyptian beersem clover, *R. aegyptiacum* and *R. bangladeshense*, belong to the symbiovar trifolii (Fig. 3). This result is particularly interesting in the case of strains identified as *R. bangladeshense* since the strains of this species belong to the symbiovar viciae [11], but here we report for the first time the existence of the symbiovar trifolii in this species (Fig. 3). A comparison of the *nodC* genes of the Egyptian clover isolates with