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ORIGINAL ARTICLE

Identification and molecular characterization of *Azotobacter chroococcum* and *Azotobacter salinestris* using ARDRA, REP, ERIC, and BOX

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Abstract

Azotobacter chroococcum and *A. salinestris* do not possess significant and distinct morphological and physiological differences and are often mistaken with each other in microbiological research. In this study, 12 isolates of *Azotobacter* isolated by standard protocol from soils were identified morphologically and physiologically as *A. chroococcum*. The isolates were more closely investigated for the molecular differentiation and diversity of *A. chroococcum* and *A. salinestris.* For this purpose, the ARDRA technique including HpaII, RsaI, and AluI restriction enzymes, and REP, ERIC, and BOX markers were used. The *nifD* and *nifH* genes were also utilized to evaluate the molecular identification of these two species. The 16S rDNA evaluation showed that only four out of the 12 isolates were identified as *A. chroococcum* and the rest were *A. salinestris.* The results revealed that HpaII was able to differentiate *A. chroococcum* from *A. salinestris* whereas RsaI and AluI were not able to separate them. Moreover, BOX and REP markers were able to differentiate between *A. chroococcum* and *A. salinestris*. However, ERIC marker and *nifD* and *nifH* genes were unable to separate these species. According to the results, HpaII restriction enzyme is suggested to save time and cost. BOX and REP markers are recommended for differentiation and clear discrimination not only between *A. chroococcum* and *A. salinestris* but also among their strains.

Keywords Diversity · Rep-PCR · Restriction enzyme · rRNA

Introduction

Azotobacter is a Gram-negative, aerobe, heterotrophic, non-symbiotically nitrogen fixer, and polymorphism from the rod, cocci to ovoid, which is capable of cyst formation [1]. *Azotobacter* belongs to the Pseudomonadaceae family and seven species of this genus have been identified so far, including *Azotobacter chroococcum*, *A. vinelandii*, *A. Beijerinckii*, *A. nigricans*, *A. paspali*, *A. salinestris* and *A. armeniacus* [1]. Laboratory identification of *Azotobacter* species is difficult because of their morphological similarity, especially between *A. chroococcum* and *A. salinestris*. *A. chroococcum* is the most famous species of *Azotobacter, which* has considerable variations among its strains in terms of cell shape, colony size, and pigmentation [2]. Page [3] reported that some strains of *A. chroococcum* isolated from slightly saline soils of western Canada were dependent on sodium (Na^+) for growth. Page and Shivprasad [4] proposed a new species named *A. salinestris* for the Na^+-dependent strains of *A. chroococcum*. These two bacterial species have many physiological and morphological similarities and, in many cases, are mistaken for each other. Therefore, it is important to develop a simple and quick molecular identification method to differentiate between the two species. The sequence of 16S rDNA as a molecular tool has been widely used to identify bacteria to the level of genus or species. For more accurate identification and differentiation between these two species, other molecular techniques are required. Amplified ribosomal DNA restriction analysis (ARDRA) is commonly used for the microbial identification and classification at the level of genus and species [5, 6]. The 16S-ARDRA is based on amplified ribosomal DNA restriction analysis of 16S rDNA, which is a simple method routinely used in laboratories owing to not requiring specialized

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equipment and being cheaper than 16S rDNA sequencing. In the ARDRA technique, strains are identified by comparing the gel electrophoresis profiles of restriction fragments of the strains with the profiles of reference strains [7]. There is a small volume of the literature on the molecular differences between *A. chroococcum* and *A. salinestris*. The power of differentiation of ARDRA depends on the type of restriction enzymes. There are various PCR fingerprinting techniques like ERIC, REP, and BOX-PCR for the amplification of repetitive DNA sequences present in the genomes of most Gram-negative and several Gram-positive bacteria. Rep-PCR is based on primers targeting the 35- to 40-bp repetitive extragenic palindromic sequence [8]. ERIC-PCR is based on primers targeting the highly conserved enterobacterial repetitive intergenic consensus (124- to 127-bp). ERIC-PCR is highly sensitive and useful in detecting microorganisms from any environment [9, 10]. BOX-PCR is based on primers targeting the highly conserved repetitive DNA sequences of the BOXA subunit (59 bp) of the BOX element [11]. BOX-PCR is superior to other techniques in creating distinctive fingerprint patterns; however, the other two methods were used as primary methods for genotyping. Most importantly, BOX-PCR does not share any sequence homology with either REP or ERIC-PCR [12, 13]. The advantages of repetitive element-based PCR (Rep-PCR) include the ability to distinguish between closely related strains, as well as being a simple, reliable, cheap, and quick method [14].

Therefore, in this study, some types of enzymes were examined. Moreover, different Rep-PCR techniques including REP, ERIC, and BOX were compared for the molecular differentiation of native *A. chroococcum* and *A. salinestris*, and their strain diversities were also evaluated. In addition, *nifD* and *nifH* genes were used to evaluate the molecular identification of the two species.

Materials and methods

Soil sampling and bacterial isolation

Rhizosphere soils were sampled both from cultivated (under crop plants) and uncultivated lands (under pastures) in arid and semi-arid regions across Tehran, Alborz, Qazvin and Qom Provinces of Iran. For soil sampling, whole plants with roots and surrounding soil were removed from the ground. The samples were transferred to the laboratory and stored at 4 °C for further investigations. Fifty gram of each soil sample were mixed with 0.5 g of pyruvic acid sodium salt and saturated with distilled sterile water. The soil paste was transferred to a sterile 9 cm Petri dish and the surface of the paste was smoothed and leveled by a sterile spatula. After 5–10 days incubation at 30 °C, the soil paste—plates presenting growth of *A. chroococcum* were revealed by the appearance of brown to black colonies [2]. The Winogradsky medium was used for growth of bacteria [1]. The geographical coordinates and some characteristics of the soils from which *Azotobacter* was separated are presented in Table 1. Gram stain, pigmentation, cell morphology, and cyst formation were employed for purification and individual identification of the isolates [1]. Two control strains of *A. salinestris* (strain AS-FE: CCSM-B00469) and *A. chroococcum* (strain AC-SW15: CCSM-B00477) were taken from the culture collection of soil microorganism (CCSM, WDCM 891) of the Soil and Water Research Institute, Iran.

16S rDNA amplification and ARDRA analysis

Genomic 16S rDNA was amplified using 27F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTAC CTTGTTACGACTT-3′) primers [15]. PCR was performed

Table 1 Characteristics of soil samples

Isolates	Geographical coordinates of soil samples	pH_e^a	EC_e^a (dS m^{-1})	Type of plant	Bacterial isolates	Geographical coordinates of soil samples	pH_e^a	EC_e^a (dS m^{-1})	Type of plant
AS-11	E 49°42′38″ N 35°50′34″	7.58	3.29	Crop	AS-66	E 51°40′48″ N 35°19′46″	7.57	3.34	Pasture
AS-12	E 49°36′32″ N 35°51′23″	7.75	0.789	Pasture	AS-69	E51°40′48″ N 35°19′46″	7.67	1.35	Crop
AC-22	E 51°26′13″ N 35°39′20″	7.59	0.632	Crop	AS-70	E 51°40′48″ N 35°19′46″	7.61	2.92	Crop
AS-26	E 51°21′09″ N 35°23′52″	7.43	2.29	Pasture	AS-71	E 51°40′33″ N 35°19′40″	7.80	1.64	Crop
AC-58	E 51°29′13″ N 35°31′36″	7.92	1.72	Pasture	AS-62	E 51°39′47 N 35°20′21″″	8.29	3.95	Pasture
AC-63	E 51°39′47″ N 35°20′21″	7.62	0.555	Pasture	AC-LAB	E 50°57′22″ N 35°45′36″	7.65	1.14	Crop

[a]Measured in soil saturation extract

with a Techne, Genius FGEN02TP thermocycler in a total volume of 50 µl solution containing 5 µl of PCR buffer (10×), 1 µl of 10 mM dNTP, 1 µl of 50 mM $MgCl_2$, 1 µl of each primer (10 pmol), 0.6 µl of Taq DNA polymerase (Smartaq; 5 U/µl) and 3 µl of DNA template. The amplification condition was 5 min at 94 °C for initial denaturation; 30 cycles of 45 s at 94 °C, 45 s at 52 °C, and 2 min at 72 °C, and the final extension at 72 °C for 7 min [16]. Amplified 16S rDNA products were digested with AluI, HpaII, and RsaI restriction enzymes for 2 h at 37 °C according to the manufacturer's instructions.

Amplification of *nifD* and *nifH* genes

The nitrogen-fixing *nifD* and *nifH* genes were amplified using the primers FdB260 (TCRTTIGCIATRTGRTGNCC)/FdB261 (TGGGGICCIRTIAARGAYATG) and PolF (TGCGAYCCSAARGCBGACTC)/PolR (ATSGCCATCATYTCRCCGGA), respectively [17, 18]. The reaction components for the amplification of *nifD* and *nifH* genes were 5 µl of PCR buffer (10×), 0.5 µl of 20 mM dNTP, 1.5 µl of 50 mM $MgCl_2$, 2 µl of each primer (10 pmol), 0.6 µl of Taq DNA polymerase (Smartaq; 5 U/µl), and 1.5 µl of DNA template. The amplification conditions were 5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturation at 94 °C; 1 min annealing step at 53 °C (nifD) or 57 °C (nifH); 1 min extension at 72 °C, and a final extension for 10 min at 72 °C.

Rep-PCR fingerprinting and profile analyses

Genomic fingerprints were generated using a single primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and the primer pairs of ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′)/ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′); and REP1R (5′-IIIICGICGICATCIGGC-3′)/REP2I (5′-ICGICTTATCIGGCCTAC-3′) [19, 20]. The optimized Rep-PCR reactions were performed in a total volume of 30 µl solution containing 2 µl of DNA template, 3 µl of PCR buffer (10×), 0.24 µl of 20 mM dNTP, 1.2 µl of 50 mM $MgCl_2$, 1.8 µl of each primer (10 pmol), 0.48 µl of Taq DNA polymerase (Smartaq; 5 U/µl), and 1 µl of each primer (10 pmol). The amplification program comprised 1 cycle of 6 min at 95 °C; 35 cycles of 1 min at 94 °C; then 1 min at 40 °C (REP) or 50 °C (BOX) or 53 °C (ERIC); then 4 min at 72 °C, and finally, 1 cycle of 10 min at 72 °C.

The 16S rDNA, nifD and nifH products were analyzed by gel electrophoresis on 1% agarose gel stained with 5 µl of SimplySafe™ (EUR_X) for 1.5 h at a constant voltage (100 V). The digested products were electrophoresed on 2% (w/v) agarose gels stained with 5 µl of SimplySafe™ (EUR_X) for 2 h at 90 V. For Rep-PCR, the gels were run on 1.5% agarose gel for 2 h at 100 V. A 100-bp DNA ladder (CinnaGene Inc., Iran) was used as a DNA size marker.

The 16S rDNA, nifD and nifH products were purified and sequenced using the mentioned primers by the Bioneer Company, South Korea. The sequences were compared with those of the most closely related bacterial species using the BLAST program on the NCBI and EzTaxon servers [21, 22]. Finally, the sequence data were deposited in the GenBank database.

A maximum parsimony tree was constructed using the 16S rDNA region of the 12 isolates of *Azotobacter*, AS-FE and AC-SW15 control strains, four reference sequences from NCBI (Accession Numbers: NR_114165, NR_041038, MF805703, MH763851), and one out-group from NCBI (Accession numbers: DQ133506) using the MEGA 6.06 software with bootstrap values calculated from 1000 replicates [23].

The PCR products from REP, ERIC, and BOX analyses were scored according to either presence (1) or absence (0) to construct the binary data matrices. In order to ensure the repeatability of the bands, the experiments were performed in three replications. The Jaccard similarity coefficient (J) was used to estimate the genetic similarities among the isolates, and the dendrograms were generated according to the unweighted pair-group mean arithmetic method (UPGMA) using NTSYSpc (numerical taxonomy and multivariate analysis system) software, Version 2.0 [24].

Results

Bacterial isolation

Twelve isolates of *Azotobacter* were isolated from rhizosphere soil samples of pastures and crops grown in arid and semi-arid regions of Iran. Young cells of the isolates showed polymorphism, but all the isolates were Gram-negative, produced light to dark brown water-insoluble pigments, and formed cysts. Moreover, all the isolates grew on the nitrogen-free medium but did not produce water-soluble pigments. Some morphological and physiological characteristics of the isolates are given in Table 2. The results revealed that the morphological and physiological characteristics of all the isolates were similar to those of *A. chroococcum.*

Molecular identification

The 16S rDNA, *nifD* and *nifH* gene sequences were compared to those of the most closely related bacterial species using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The lengths of 16S rDNA, *nifD* and *nifH* gene sequences for each strain are shown in Table 3. The above-mentioned sequences were submitted to the GeneBank/NCBI database under the accession numbers provided in Table 3.

Table 2 Physiological and morphological characteristics of isolates

Isolates	Young cell morphology	Colony appearance	Water insoluble pigment
AS-11	Coccoid in the form of pairs	Opaque, convex, mucoid, glistening and smooth	Light brown
AS-12	Ellipsoidal in the form of single and pairs	Opaque, convex, mucoid, glistening and smooth	Light brown
AC-22	Ellipsoidal in the form of pairs	Opaque, convex, glistening and smooth	Light brown
AS-26	Coccoid in the form of single pairs and multiple	Opaque, convex, glistening and smooth	Light brown
AC-58	Coccoid in the form of single pairs and multiple	Opaque, convex, mucoid, glistening and smooth	Light brown
AS-62	Ellipsoidal in the form of single and pairs	Opaque, convex, mucoid and wrinkled	Brown
AC-63	Coccoid in the form of single and pairs	Opaque, convex, mucoid, glistening and smooth	Brown
AS-66	Ellipsoidal in the form of single and pairs	Opaque, convex, mucoid, glistening and wrinkled	Light brown
AS-69	Coccoid in the form of single and pairs	Opaque, convex, mucoid, glistening and wrinkled	Brown
AS-70	Coccoid in the form of single pairs and multiple	Opaque, convex, mucoid, glistening and smooth	Brown
AS-71	Coccoid in the form of single pairs and multiple	Opaque, convex, mucoid, glistening and smooth	Light brown
AC-LAB	Coccoid in the form of single pairs and multiple	Opaque, convex, glistening and smooth	Light brown

All the isolates were Gram-negative, grown on N-free medium and formed cyst. None of the isolates produced water-soluble pigments

Table 3 Identification of bacterial isolates based on 16S rDNA, *NifD* and *NifH* gene sequences

Isolates	Accession no.			Length (bp)			Blast hit (% similarity), respectively	Bacterial species
	16S rDNA	Nif D	Nif H	16S DNA	Nif D	Nif H		
AS-12	MG386285	MG581327	MG581341	1400	350	326	NR_114165 (99), CP011835 (100), CP010415 (98)	*A. salinestris*
AS-62	MG386286	MG581329	MG581343	1402	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-66	MG386292	MG581330	MG581344	1397	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-26	MG386294	MG581328	MG581342	1371	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-11	KY404165	MG581326	MG581340	1396	350	326	NR_114165 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-71	MG386290	MG581333	MG581347	1393	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-70	KY404168	MG581332	MG581346	1396	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AS-69	KY404167	MG581331	MG581345	1395	350	326	FJ032010 (99), CP011835 (98), CP010415 (99)	*A. salinestris*
AC-22	MG386293	MG581321	MG581335	1400	350	326	EF620428 (99), CP010415 (100), CP010415 (99)	*A. chroococcum*
AC-LAB	MG386287	MG581324	MG581338	1393	350	326	EU930421 (99), CP011835 (99), CP010415 (99)	*A. chroococcum*
AC-63	KY404166	MG581323	MG581337	1387	350	326	CP010415 (99), CP010415 (100), CP010415 (99)	*A. chroococcum*
AC-58	MG386288	MG581322	MG581336	1394	350	326	JQ692178 (99), CP011835 (99), CP010415 (99)	*A. chroococcum*

The analyses of the 16S rDNA regions revealed that the AC-63, AC-22, AC-58, and AC-LAB isolates were *A. chroococcum*, whereas the other eight isolates were identified as *A. salinestris* (Table 3). The maximum parsimony tree based on the 16S rDNA region sequence showed two groups. One group consisted of eight isolates of *A. salinestris* (AS-12, AS-71, AS-62, AS-26, AS-70, AS-66, AS-11, and AS-69), AS-FE control strain, and two reference sequences (AS-NBRC102611, AS-ATCC49674). The other group included AC-SW15 control strain, four isolates of *A. chroococcum* (AC-63, AC-22, AC-58, and AC-LAB), and two reference sequences (AC-AzXU1, AC-DC4) (Fig. 1). The *nifD* and *nifH* genes were unable to separate the species based on GeneBank/NCBI database.

ARDRA profile of isolates

The results of ARDRA showed that HpaII was able to differentiate *A. chroococcum* from *A. salinestris* whereas RsaI and AluI were unable to separate these species. The ARDRA profiles of the *Azotobacter* isolates are shown in Fig. 2.

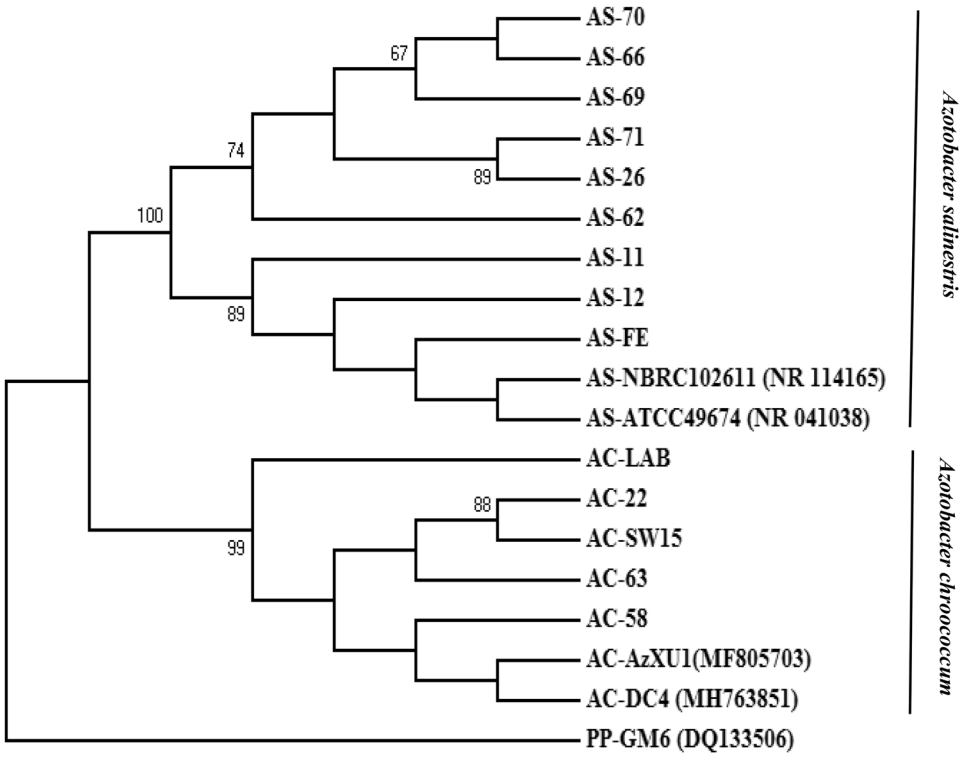

Fig. 1 A maximum parsimony tree based on the 16S rDNA region sequences (1390 bp) of the 12 isolates of *Azotobacter*, AS-FE and AC-SW15 control strains, four reference sequences from Genbank (Accession Numbers: NR_114165, NR_041038, MF805703, MH763851).The tree rooted with *Pseudomonas putida* PP-GM6 (DQ133506). Only bootstrap values > 60% (1000 replications) are shown at the branches

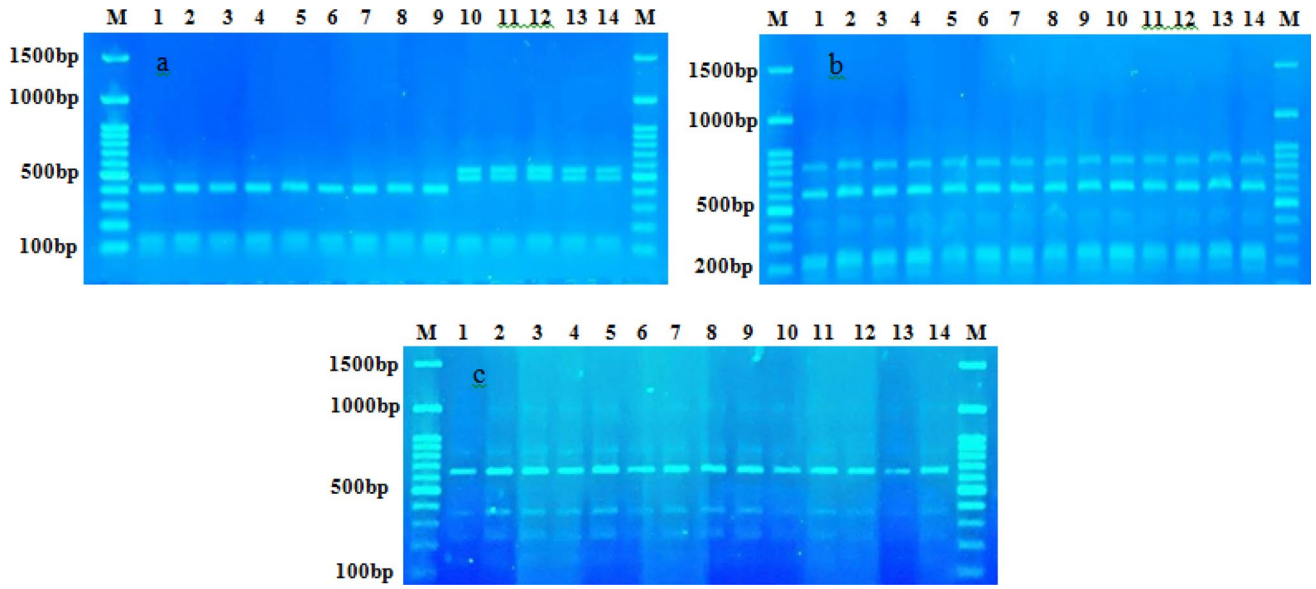

Fig. 2 ARDRA profiles of the 12 *Azotobacter* isolates obtained by digestion of the 16S rDNA region by **a** HpaII, **b** RsaI, and **c** AluI. M: 100 bp DNA ladder. Lane 1 and 10 (controls): *Azotobacter salinestris* AS-FE and *Azotobacter chroococcum* AC-SW15, respectively. Lane 2–9: *Azotobacter salinestris* isolates; AS-12, AS-62, AS-66, AS-26, AS-11, AS-71, AS-70, and AS-69, respectively. Lane 11–14: *Azotobacter chroococcum* isolates; AC-22, AC-LAB, AC-63, and AC-58, respectively

Rep-PCR fingerprinting and profile analyses

The profiles of genomic DNA fingerprinting of BOX, ERIC, and REP of the *Azotobacter* isolates and their dendrogram are shown in Figs. 3, 4, and 5, respectively. The number of amplified bands was between 6 and 16 and the band sizes ranged from 340 to 4000 bp with BOX primer. The isolates were divided into two groups based on BOX patterns.

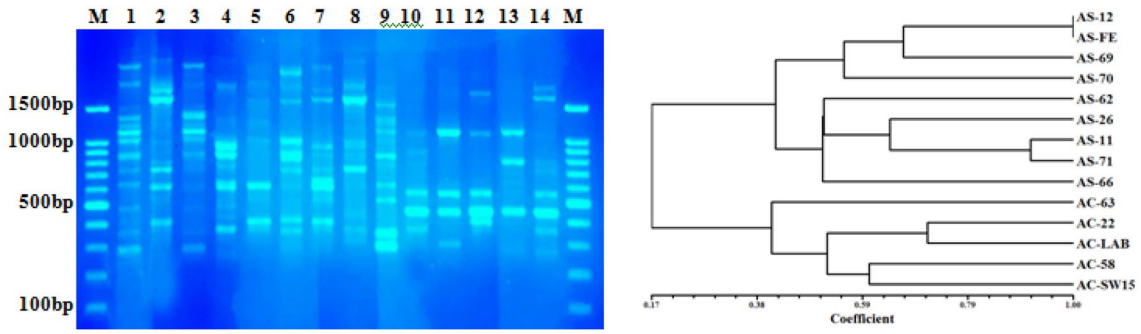

Fig. 3 BOX profiles of the 12 *Azotobacter* isolates. M: 100 bp DNA ladder. Lane 1 and 10 (controls): *Azotobacter salinestris* AS-FE and *Azotobacter chroococcum* AC-SW15, respectively. Lane 2–9: *Azotobacter salinestris* isolates; AS-12, AS-62, AS-66, AS-26, AS-11, AS-71, AS-70, and AS-69, respectively. Lane 11–14: *Azotobacter chroococcum* isolates; AC-22, AC-LAB, AC-63, and AC-58, respectively. Dendrogram was constructed based on BOX profiles by UPGMA method

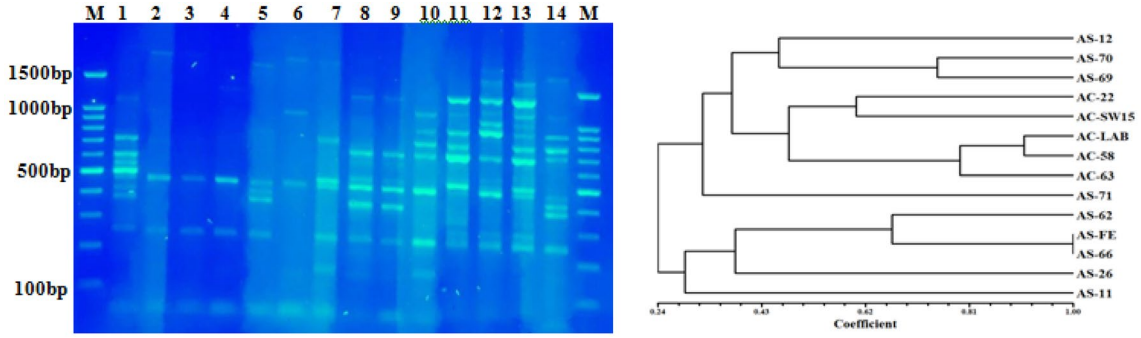

Fig. 4 ERIC profiles of the 12 *Azotobacter* isolates. M: 100 bp DNA ladder. Lane 1 and 10 (controls): *Azotobacter salinestris* AS-FE and *Azotobacter chroococcum* AC-SW15, respectively. Lane 2–9: *Azotobacter salinestris* isolates; AS-12, AS-62, AS-66, AS-26, AS-11, AS-71, AS-70, and AS-69, respectively. Lane 11–14: *Azotobacter chroococcum* isolates; AC-22, AC-LAB, AC-63, and AC-58, respectively. Dendrogram was constructed based on ERIC profiles by UPGMA method

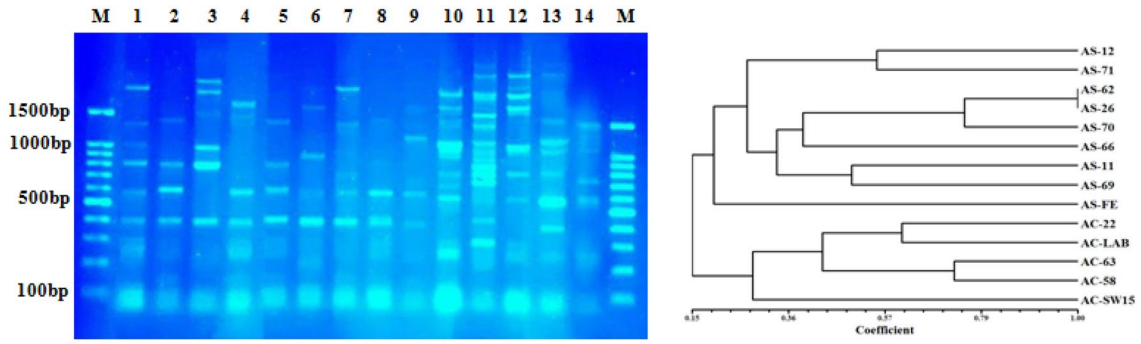

Fig. 5 REP profiles of the 12 *Azotobacter* isolates. M: 100 bp DNA ladder. Lane 1 and 10 (controls): *Azotobacter salinestris* AS-FE and *Azotobacter chroococcum* AC-SW15, respectively. Lane 2–9: *Azotobacter salinestris* isolates; AS-12, AS-62, AS-66, AS-26, AS-11, AS-71, AS-70, and AS-69, respectively. Lane 11–14: *Azotobacter chroococcum* isolates; AC-22, AC-LAB, AC-63, and AC-58, respectively. Dendrogram was constructed based on REP profiles by UPGMA method

The dendrogram showed that one group consisted of AS-FE and eight isolates of *A. salinestris* (AS-12, AS-71, AS-62, AS-26, AS-70, AS-66, AS-11, and AS-69) and the other group included AC-SW15 and four isolates of *A. chroococcum* (AC-63, AC-22, AC-58, and AC-LAB). The BOX marker could split the two *A. chroococcum* and *A. salinestris* species. In addition, the number of polymorphic bands was 25, which showed 100% polymorphism (Fig. 3).

The ERIC patterns represented that the number of amplified bands was between 2 and 11 and the band sizes ranged from 240 to 2100 bp. Moreover, the number of polymorphic bands was 18, which showed 94.7% polymorphism (Fig. 4).

The number of amplified bands of REP was between 3 and 15 and the band sizes ranged from 320 and 3500 bp. The analysis of REP profiles revealed that the isolates were divided into two groups. The REP marker could split the two species *A. chroococcum* and *A. salinestris.* Further, the number of polymorphic bands was 22, which showed 100% polymorphism (Fig. 5).

The consensus tree derived from ERIC, BOX, and REP profiles revealed the ability to differentiate between *A. chroococcum* and *A. salinestris* species and their strains (Fig. 6).

Discussion

Azotobacter is used for inoculation of plants as a biofertilizer due to its rapid growth, ability to fix N_2 and production of plant growth substances. It forms cysts that help to cope with stress and grows better in saline conditions such as Iranian soils. Therefore, an accurate study of *Azotobacter* in agriculture and natural resources is particularly important for the production of biological fertilizers. In this study, 20 *Azotobacter* isolates were first isolated from 77 slightly saline soil samples in arid and semiarid regions. Based on the physiological and morphological characteristics, 12 isolates were selected for more investigation. During the sampling, according to the various growth stages of plants, it was not possible to identify all of them, particularly pasture plants. It is necessary to mention that *Azotobacter* is a free-living bacterium and does not need a plant for growth, but due to root exudates, its population is larger in the rhizosphere. However, soil characteristics have a greater impact on the growth, activity, and population of *Azotobacter* than the plant type. Therefore, *Azotobacter* isolates were obtained from soils and not from the roots using the soil paste method. This method was recommended as a reliable technique for the isolation and preliminary identification of *A. chroococcum* from soil samples [2]. These isolates were investigated in terms of young cell morphology, colony appearance, cyst formation, and water-soluble and insoluble pigment production (Table 2). The isolates were identified based on morphological and physiological characteristics as *A. chroococcum.* The analyses of the 16S rDNA regions revealed that only four out of the 12 isolates were identified as *A. chroococcum* and the rest were *A. salinestris.* The 16S rDNA has been used in many types of research for the molecular identification of bacteria such as *Azotobacter*. Chen et al. [25] employed 16S rDNA for identification of *Azotobacter* species including *A. chroococcum*, *A. vinelandii*, *A.beijerinckii*, and *A. tropicalis* from the rice rhizosphere of Taiwan.

In this study, the isolates were further investigated to differentiate between the two species using other molecular methods. It is necessary to mention that some Na^+-dependent isolates of *A. chroococcum* isolated from slightly saline soils were proposed as a new species named *A. salinestris* [4].

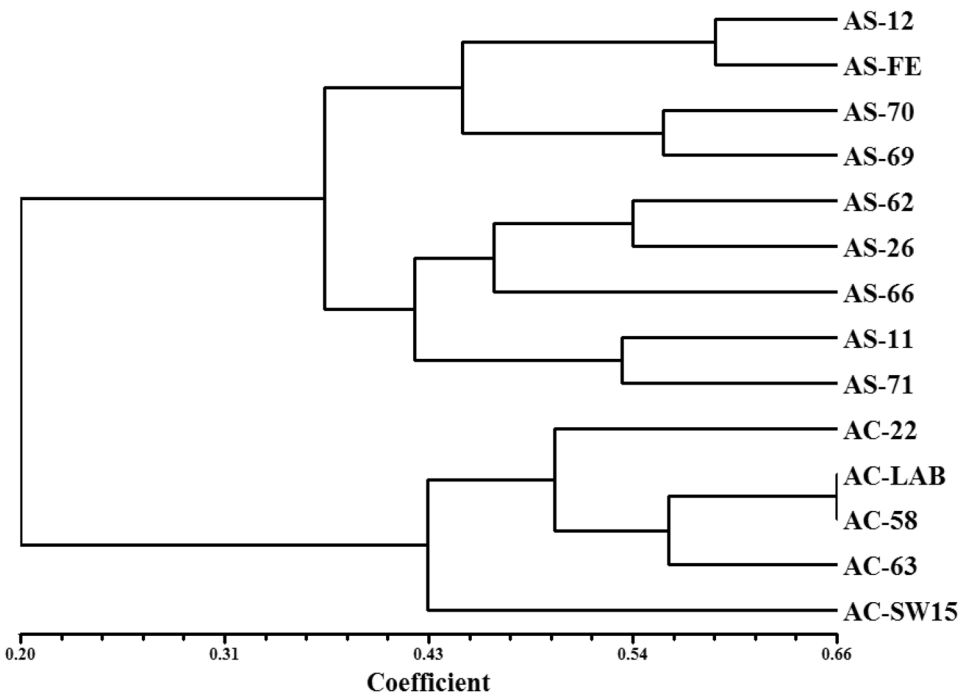

Fig. 6 The consensus dendrogram was constructed based on BOX, REP, and ERIC profiles of the 12 *Azotobacter* isolates, AS-FE and AC-SW15 control strains by UPGMA method

Based on the previous investigations, AluI, HpaII, and RsaI restriction enzymes have been mainly used for identification of *Azotobacter* genus from other genera [26, 27]. But in our research, these restriction enzymes were used for differentiation at the species level. Aquilanti et al. [26] reported that the 16S rDNA region and ARDRA method with five restriction enzymes including RsaI, HhaI, HpaII, FnuDII, and AluI were useful markers for identification of *Azotobacter* genus from other free nitrogen-fixing bacteria. Jiménez et al. [27] expressed similar results based on morphological characteristics, 16S rDNA, and ARDRA with AluI, HpaII, and RsaI to identify *Azotobacter* species from soil samples. The number of restriction enzymes required for species differentiation varies depending on the species and the presence of the restriction sites. Some studies have revealed that at least four restriction enzymes are essential to resolve the 16S rRNA gene of different species [28, 29]. In contrast, species-level identification has been reported to be achievable even with three or fewer restriction enzymes [7, 30].

In our research, the ARDRA evaluation showed that among three enzymes, the best restriction enzyme for differentiating between *A. salinestris* and *A. chroococcum* was HpaII. This led to the clear discrimination between the two species. AluI and RsaI showed the same pattern characteristic of the genus *Azotobacter*, due to the presence of same restriction sites on the entire rRNA gene copies present in a single genome. Our results were in agreement with those of Rubio et al. [31], which reported that RsaI produced identical bands among *A. chroococcum*, *A. salinestris*, and *A. armeniacus.* The results are in line with results reported by Mazinani and Asgharzadeh [32], who noted that genetic diversity of *A. chroococcum*, *A. vinelandii* and *A. beijerncki* by RsaI was very low and it was because of the same digestion regions in their conserved sites. On the other hand, the use of HpaII and HhaI on strains led to the separation among the *Azotobacter* species. Other studies have employed the ARDRA technique for the diversity evaluation of *Azotobacter* [33, 34].

The findings of Rep-PCR fingerprinting revealed that BOX and REP markers could separate the two species, but the ERIC marker was incapable of fully separating them. The percentages of polymorphic bands obtained with BOX and REP markers were 100%; however, the ERIC marker did not show completely polymorphic bands. Few studies have distinguished between *A. chroococcum* and *A. salinestris* using REP, ERIC, and BOX. Lenart-Boroń et al. [35] reported that the high level of genetic diversity observed using Random Analysis of Polymorphic DNA and BOX markers. These markers could show the genetic diversity of the *A. salinestris*, *A. chroococcum,* and *A. vinelandii*. In addition, similar to our experiment, no monomorphic band was observed using BOX. Rubio et al. [31] reported that Rep-PCR is a useful tool for the taxonomic classification of *Azotobacter* isolates and they found high genetic diversity among *A. chroococcum*, *A. salinestris*, and *A. armeniacus*.

Rep-PCR was reported as a distinguishable marker for differentiation between *A. chroococcum* and *Azospirillum brasilense* strains [36]. Similar to our experiment, Chen et al. [37] proved that Rep-PCR was a remarkable tool for genotyping of bacterial species. They concluded that a combination of Box, Eric and Miniprimer-PCR results was a fast and reliable method for segregation among *P. fluorescens* isolates. In another research, the Box PCR technique was used to discriminate between *P. aeruginosa* isolates [38]. A high degree of clonal diversity among *P. aeruginosa* strains was observed using REP and ERIC markers [39, 40].

The NCBI BLAST of *nifD* and *nifH* gene sequences showed that these genes could not distinguish between the two species.One reason that *nif* genes were unable to differentiate between the two species can be the shorter length and similarity of the sequenced region as well as the lack of sufficient data in the database. However, these sequences were submitted to the GeneBank/NCBI database under the accession numbers provided in Table 3.

Conclusion

Azotobacter chroococcum and *A. salinestris* as beneficial soil bacteria are almost completely similar in terms of morphological and physiological characteristics; thus, the exact diagnosis of these two species requires molecular investigations. The sequence of 16S rDNA was used for the preliminary separation of these two species. In this research, additional molecular techniques including ARDRA and different Rep-PCR were used to study the diversity between these two species more closely. Our findings suggested that HpaII was a suitable restriction enzyme for differentiation and clear discrimination between *A. chroococcum* and *A. salinestris*. Although the results of 16S rDNA and HpaII were similar in separating these two species, the use of this restriction enzyme is suggested because of no need for sequencing and time and cost savings. BOX and REP markers were able to differentiate not only between the two species but also among their strains. Therefore, these two markers are recommended for the diversity studies of these two species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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