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Diversity of actinobacteria in the marshes of Ezzemoul and Djendli in northeastern Algeria

Maria Smati[1]*, Mahmoud Kitouni[1]

[1] *Biotechnologie et valorisation microbienne des écosystèmes extrêmes, Laboratoire de génie microbiologique et applications, Université des Frères Mentouri Constantine 1, Campus Chaâbat Erssas, Route Ain El Bey, 25000 Constantine, Algeria Corresponding author, Tel.: +213556210363, E-mail:mariasmati87@gmail.com*

ABSTRACT

The main purpose of this research is to study the microbial diversity of actinobacteria, living in "Ezzemoul" and "Djendli" sebkhas soils. These salt lakes are situated in the east of Algeria and they are microbiologically underexploited. Such unexplored ecological niches have been considered by many authors as sources of novel actinobacteria and bioactive molecules. Actinobacteria play an important role in safeguarding the environment by improving plant growth through nitrogen fixation, biodegradation, and bioremediation. Therefore, studying the diversity and distribution of actinobacteria in such special environments is important for determining the ecological and biotechnological roles of these microorganisms. In this article, we focused on the occurrence and the diversity of actinobacteria from sebkhas using two techniques: cultural and culture-independent (molecular cloning). The latter are based on phylogenetic analysis of the 16S rDNA gene. Thus, the cultural method allowed us to obtain 62 isolates: 40 from the "Ezzemoul" site and 22 from the "Djendli" site. These isolates tolerate mainly 2, 5, and 10% sodium chloride (NaCl) and belong to the genera *Nocardiopsis, Streptomyces,* and *Rhodococcus*. Moreover, the molecular cloning gave us 39 clones. Twenty-four clone sequences from "Ezzemoul" site are affiliated to the genera *Demequina, Plantactinospora, Friedmanniella,* and *Mycobacterium*. Also, 15 clone sequences from "Djendli" site are related to the genera *Marmoricola, Phytoactinopolyspora, Streptomyces,* and to an unclassified actinobacterial clone. Some sequences from both sites are related to uncultured clones. In addition to the data provided by the cultural method, molecular cloning allowed us to have additional information about the unknown actinobacteria, uncultured ones as well as on the genera that exist in both sites. So, the cultural method is complementary to the culture-independent one, and their combination revealed an important diversity in targeted saline environments. Furthermore, all new isolated strains that tolerate 10% NaCl may have a very interesting biotechnological potential in the future.

KEYWORDS

Actinobacteria; sebkha; culture; cloning; 16S rRNA gene; phylogenetic biodiversity

INTRODUCTION

Actinobacteria especially actinomycetes are ubiquitous microorganisms. They are mainly prevalent in the soil (Oskay et al. 2004). These bacteria are responsible for the degradation of organic matter and produce many bioactive compounds (Naikpatil and Rathod 2011) such as vitamins, enzymes, antiparasitics, antivirals, immunostimulants, immunosuppressants, nutrients, and cosmetic products. They are well known for the production of antibiotics. Around 80% of the latter in the world come from actinomycetes mainly from the genus *Streptomyces* (Pandey et al. 2004). Several recent studies in Algeria have proved the importance of certain *Actinobacteria* in aflatoxin B1 reduction (Lahoum et al. 2017), enzyme production (Gasmi and Kitouni 2017), and fungicide degradation (Hocinat and Boudemagh 2015). Among the *Actinobacteria*, those that are halophilic or halotolerant create interest in both taxonomic and biotechnological point of view.

Extreme ecosystems, not or a little bit exploited, represent a coveted field of research where rare or new microbial species that may have an interesting production potential are targeted. Wetlands are biologically one of the most important and the most productive ecosystems in the world (Bedford et al. 1999). There are several important wetlands in Algeria like chotts, sebkhas, or salt lakes. Among them, 50 have an international importance and they are classified as Ramsar sites. Most of them are endorheic and are qualified for their wide variety of species (Balla 2012).

In this research, we studied *Actinobacteria* in two sites: the Ezzemoul and Djendli sebkhas, which are parts of the "Hauts Plateaux" region. It is located in the northeast of the country. This region is characterized by a semiarid climate. So,

this type of climate causes the salinization of soil (Rengasamy 2006).

The wetlands that have been chosen for this research are a bit studied and not still very detailed, except some pedological studies (Chenchouni 2009; Aliat et al. 2016) and several others studying the diversity of fauna (Aberkane 2014; Bellagoune 2015), flora (Chenchouni 2009; Neffar et al. 2016), and certain halophilic microorganisms (Kharroub 2007), including actinomycetes (Kitouni et al. 2005; Boughachiche et al. 2016).

Until now, there is little or no research on the taxonomy of *Actinobacteria* in the ecosystems chosen for this study. According to Vartoukian et al. (2010), most of the microorganisms that can be observed in the environment are generally not cultivated by using traditional culture techniques; and thus, the majority of the microflora remains undetected. This limitation can be solved by the application of culture-independent methods to reveal the presence of non-culturable populations and estimate microbial diversity in nature (Cocolin et al. 2013; Hozzein 2015). Nevertheless, culture remains useful for understanding the metabolism and functions of microorganisms (Pham et Kim, 2012). Therefore, the main aim of this article is to explore the actinobacterial biodiversity in the soil of the Ezzemoul and Djendli sebkhas, using cultural and culture-independent (the culture-independent approach is molecular cloning) approaches. These two approaches are based on phylogenetic analysis of the 16S rDNA gene.

1. MATERIALS AND METHODS

1.1. Sampling

According to the Pochon and Tardieux (1962) method, six soil samples were taken in the northeastern region of Algeria from two different sites: the Ezzemoul sebkha (latitude: 35° 53′ 14″ North, longitude: 06° 30′ 20″ East) and the Djendli sebkha (latitude: 35° 43′ 15″ North, longitude: 06° 32′ 23″ East). Samples were frozen in the field and stored during transport on dry ice for molecular work. Those intended for the other works were kept at 4°C.

1.2. Physicochemical analysis of samples

According to referenced methods, three samples of soil from each site were subjected to physicochemical analyses of pH (Pochon and Tardieux 1962), of electrical conductivity (EC) (Richards 1954), of moisture and organic matter content (Lee and Hwang 2002), and of particle size (Dupain et al. 2000).

1.3. Isolation, enumeration, and conservation of *Actinobacteria*

After drying, 2 g of soil of each sample were diluted four times in sterile physiological water (NaCl 9 g/L) and homogenized. Each dilution (100 µL) was spread on the surface of ISP5 medium (Shirling and Gottlieb 1966) supplemented with 2, 5, 10, and 15% NaCl in order to target the slight, moderate, and extreme halophilic actinomycetes; an antifungal agent (nystatin at 50 µg/L) and an anti-Gram-negative antibacterial agent (polymyxin at 20 µg/L). Petri dishes were incubated at 30 °C for 3 weeks. Actinobacterial colonies were enumerated using an automatic counter and estimated in CFU/g. They were purified and stored at −20°C on the same antibiotic-free isolation medium and in the presence of 50% (v/v) glycerol.

1.4. Genomic DNA extraction and 16S rDNA amplification of actinobacterial isolates

From the genomic DNA extracted by heat shock technique (Queipo-Ortuño et al. 2008), the gene 16S rDNA was amplified by polymerase chain reaction (PCR), using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). The PCR was carried out in a thermocycler (Eppendorf) in a final volume of 25 µL containing 16.35 µL of sterile pure water (Sigma Life Science), 2.5 µL of the 10X buffer with 15 mM magnesium chloride ($MgCl_2$) (Roche), 2 µL (2.5 mM) deoxynucleoside triphosphate (dNTP) (Roche), 2 µL (10 µM) of each primer (GATC Biotech), 0.15 µL of AmpliTaq DNA polymerase 5U/µL (Roche), and 2 µL of extracted DNA. The PCR program is as follows: 4 min at 95°C (initial denaturation), followed by 30 cycles of 30 s at 94°C (denaturation), 1 min at 54°C (hybridization), 1 min at 72°C (elongation), and then 10 min at 72°C (final elongation).

1.5. Environmental DNA extraction, actinobacterial 16S rDNA amplification, and cloning

The whole DNA was extracted from 500 mg of each soil sample using the FastDNA™ Spin Kit for Soil and the FastPrep® instrument (MP Biomedicals) according to the manufacturer's recommendations. The extracted DNA was purified using the Illustra™ MicroSpin™ S-400 HR Columns Kit (GH Healthcare), according to the manufacturer's instructions. The 16S rDNA gene (approximately 640 bp) was amplified by PCR using actinobacterial specific primers S-C-Act-235-a-S-20 (5′-CGCGGCCTATCAGCTTGTTG-3′ forward) and S-C-Act-878-a-A-19 (5′-CCGTACTCCCCAGGCGGGG-3′, reverse) (Stach et al. 2003). The PCR mixture (50 µL) contains 37.7 µL of sterile pure water (Sigma Life Science), 5 µL of the 10X buffer with 15 mM $MgCl_2$ (Roche), 2 µL (500 µg/µL) T4gp32 Bulk (MP Biomedicals), 1 µL (10 mM) of dNTP (Thermo Fisher Scientific), 1 µL (10 µM) of each primer (Eurofins Genomics), 0.3 µL of AmpliTaq DNA polymerase 5U/µL (Roche), and 2 µL of purified extracted DNA. The amplification was carried out using the touchdown protocol: 4 min at 95°C (initial denaturation), 10 cycles (during which the hybridization temperature decreased by 0.5°C/cycle): 45 s at 95°C (denaturation), 45 s at 72°C (hybridization), and 1 min at 72°C (elongation), followed by 20 other cycles with the same preceding steps except that the hybridization temperature was 68° C, and then 5 min at 72°C (final elongation). Two separate PCR amplifications were performed for each sample. Their products were pooled and purified by the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's instructions. The pooled and purified amplicons were first cloned using the pGEM®-T and pGEM®-T Easy Vector Systems

(Promega) using the pGEM®-T Vector and *E. coli* JM109 high-efficiency chemical-competent cells, according to the manufacturer's recommendations. Second, cloning was carried out using TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Life Technologies) with *E. coli* electro-competent cells (One Shot® DH5α™-T1R) according to the manufacturer's instructions. All positive clones were selected and examined by colony PCR using the vector-specific primers.

1.6. Purification, sequencing, and sequences analysis

The PCR products from the cultural and culture-independent methods were purified according to the protocol of the Illustra™ ExoStar™ 1-Step Kit (GE Healthcare, Life Sciences) and sequenced. Sequencing was conducted in the sequencer (AB3730, 48 capillaries, Applied Biosystems) according to the Big Dye Terminator protocol Kit ver.3.1 (Applied Biosystems).

All sequences were analyzed by ChromasPro software ver.1.5 (Technelsium Pty Ltd). Clone sequences were analyzed and corrected manually. Primers and vector sequences were eliminated, and the chimeric sequences were verified by DECIPHER's Find Chimeras online tool (Wright et al. 2012). Non-chimeric sequences were compared to other sequences in the EzBioCloud database (Yoon et al. 2017) as well as to GenBank of the NCBI site by the BLAST program (Altschul et al. 1990). Multiple alignment of sequences was achieved by the CLUSTAL X 2.0.12 program (Larkin et al. 2007). The results of multiple alignment by the CLUSTAL X program of the 16S rDNA gene sequences showed that the sequences did not have the same length. This required manual corrections before their use by the MEGA software, which uses the algorithm of progressive multiple alignment CLUSTAL W. It has been proved that the quality of the alignment could have an impact on the final tree. So, it is as important as the method of construction used and it can be more important (Ebihara et al. 2006). The preparation of an alignment of quality is, therefore, a critical step in any phylogenetic analysis. The rooted phylogenetic trees were constructed with the MEGA ver.6.0.6 program (Tamura et al. 2013). According to the neighbor-joining method (Saitou and Nei 1987), the distance matrix was computed according to the Kimura model with two parameters (Kimura 1980). Topology of trees was evaluated by bootstrap analysis with 1000 replicates (Felsenstein 1985).

All actinobacterial sequences appear in GenBank with accession numbers MG597500 to MG597561 for cultivated isolates and MG601182 to MG601220 for uncultured clones.

2. RESULTS

2.1. Physicochemical analyses of soil samples

The average values of the physicochemical characteristics of the soil samples of the Ezzemoul and Djendli sebkhas are presented in Table 1.

Referring to the pH interpretation scale (Gagnard et al. 1988) and EC values (Richards 1954), the soils of the Ezzemoul and Djendli sebkhas are alkaline and extremely salty. According to the Lee and Hwang (2002) classification, our soils are characterized by low rates of moisture and organic matter. According to the triangle of mineral textures (Eswaran et al. 2002), the texture of these soils is silty-clayey-sandy.

2.2. Enumeration of actinobacterial isolates

The average values of the number of *Actinobacteria* that developed on the ISP5 culture medium containing different concentrations of salt (Table 2) vary from 0 to 34.7×10^4 (CFU/g) in "Djendli" site and are higher than those of "Ezzemoul" site, which vary from 0 to 5.65×10^4 (CFU/g). However, the total number of colonies that could have been purified and stored for this study is higher in the Ezzemoul site (40) than that in the Djendli site (22). In both sites, no colony was counted at 15% NaCl concentration. The greatest number of *Actinobacteria* was observed at 2% NaCl concentration.

2.3. Phylogenetic analysis of actinobacterial isolates

The 16S rRNA genes of 62 actinobacterial isolates were sequenced: 40 of "Ezzemoul" sebkha and 22 of "Djendli" sebkha. Sequence analysis of "Ezzemoul" site (Figs 1 & A1) revealed the presence of three genera: *Nocardiopsis, Streptomyces*, and *Rhodococcus*. The genus *Nocardiopsis* dominates with 95% of isolates, which belong to four different species.

Thus, almost half of the isolates are affiliated to the *N. dassonvillei* species with identity percentages of 98.38–99.93%, followed by 35% of *N. lucentensis* with 99.35–100% identity, 10% of *N. aegyptica* with 99.07–99.57% identity, and 2.5% (one isolate) of *N. synnemataformans* (with 100% identity).

The identity percentage 98.38% of isolate ED43 with *N. dassonvillei* is below the threshold 98.7% that separates the new species (Stackebrandt and Ebers 2006). Thus, this isolate could represent a new taxon of the *Nocardiopsis* genus.

Single isolates ES72 and ES42 are related respectively to *R. corynebacterioides* species of the genus *Rhodococcus* (with 99.49% identity) and *S. cavourensis* species of the genus *Streptomyces* (with 99.42% identity).

Table 1. Physicochemical characteristics of soil samples

Study sites	pH	Electrical Conductivity (EC) (dS/m)	M* (%)	O.M† (%)	Sand (%)	Silt (%)	Clay (%)
Ezzemoul sebkha	8.56	16.26	6.01	6.03	52.76	22.39	24.85
Djendli sebkha	8.03	11.57	6.16	5.82	30.97	32.7	36.33

**M: moisture; †O.M: organic matter*

Table 2. Enumeration of the Actinobacteria isolated from "Ezzemmoul" and "Djendli" sebkhas depending on the concentrations of NaCl added to the ISP5 medium

Study sites	ISP5 supplemented with NaCl in %	Number of *Actinobacteria* × 10^4 (CFU/g)
Ezzemoul sebkha	2	5.65 (16)*
	5	2.62 (4)*
	10	3.37 (20)*
	15	0
Djendli sebkha	2	34.7 (11)*
	5	24.4 (11)*
	10	3.33 (0)
	15	0

*Values in brackets represent the number of purified and preserved colonies

The sequences' analysis of the Djendli site (Figs 1 & A2) detected the same genera *Nocardiopsis* and *Streptomyces* already found in the Ezzemoul site, except that in the Djendli site it is the genus *Streptomyces* that dominates with 81.82% of the isolates. The latter are divided into six different species: *S. xantholiticus* (27.27%), *S. albidoflavus* (18.18%), *S. thinghirensis* (18.18%), *S. marokkensis* (9.1%), *S. philanthi* (4.54%), and *S. violascens* (4.54%). Identity percentages of isolates with these species are respectively 99.21–99.43%, 99.71%, 99.55–100%, 98.98–99.78%, 99.54%, and 99.5%.

In addition to its presence in the Ezzemoul site, the species *N. aegyptica* of the genus *Nocardiopsis* is also detected in the Djendli site and is related to 18.18% of the isolates with 99.71–99.79% identity.

2.4. Phylogenetic analysis of actinobacterial clones

Due to an insufficient number of recombinant colonies, we have used two methods of cloning. Twelve clones are from the first method (using the pGEM®-T vector) and 48 are from the second method (using the pCR®4Blunt-TOPO® vector). The total of 60 clones was sequenced and subjected to phylogenetic analysis, based on 16S rDNA. Twenty-one chimeric sequences were removed from the study. All sequences could be regrouped into seven families of *Actinobacteria*: *Demequinaceae*, *Streptomycetaceae*, *Micromonosporaceae*, *Jiangellaceae*, *Mycobacteriaceae*, *Propionibacteriaceae*, and *Nocardioidaceae*. Adding to that, there is a group of unclassified *Actinobacteria* and two clusters composed of members do not belong to the class *Actinobacteria* (Figs 2 & A3).

The 24 clones derived from the soil of Ezzemoul (EZ) site are affiliated to uncultured clones (20.83%) and the following four genera: *Demequina* (dominant with 62.5%), *Friedmanniella* (4.16%), *Mycobacterium* (4.16%), and *Plantactinospora* with 12.5%. The latter corresponds to clones EZ1, EZ10, and EZ16, which are related to the clone obtained from the alkaline saline soil of Lake Texcoco in Mexico (Valenzuela-Encinas et al. 2009). These genera belong respectively to the different families: *Demequinaceae*, *Propionibacteriaceae*, *Mycobacteriaceae*, and *Micromonosporaceae*. 16.16% do not belong to the class of *Actinobacteria* (cluster I and II) (Figs 2 & A3).

Concerning the soil of the Djendli (DJ) site and from 15 clones, three genera were detected: *Marmoricola* (13.33%), *Phytoactinopolyspora* (6.66%), and *Streptomyces* (6.66%). They belong respectively to the families: *Nocardioidaceae*, *Jiangellaceae,* and *Streptomycetaceae*. The clone DJ77 (6.66%) is an unclassified actinobacterium. These results, and in particular those of genera, are very different from those found in the Ezzemoul site. Contrary to the latter, more than half of the clones (66.66%) of the Djendli site do not belong to the class *Actinobacteria* (cluster I and II) and the majority of them (73.33%) are linked to uncultured clones (Figs 2 & A3).

3. DISCUSSION

The physicochemical analysis (Table 1) showed that the soils of "Ezzemoul" and "Djendli" sebkhas are mainly characterized by their very high salinity and their alkaline pH. The phenomenon of salinization is not only due, chiefly, to the rarity of the rain that is used to transport the salts, but also to the high rates of evaporation that are characteristic of regions with arid and semiarid climate. These soils are also silty-clayey-sandy and have low rates of moisture and organic matter. In general, our results are consistent with those reported by Chenchouni (2009) and Aliat et al. (2016). The physicochemical characteristics may act as potential factors influencing the number, growth, and composition of the actinobacterial community in the studied soils.

After isolation and counting of actinobacteria (Table 2), it is 2% NaCl concentration that allowed us to obtain the greatest number of bacteria in "Djendli" (34.7 × 10^4 CFU/g) and "Ezzemoul" (5.65 × 10^4 CFU/g) sebkhas. Forty viable actinobacteria were recovered from the salty soils of "Ezzemoul" and 22 from those of "Djendli." Our results are higher than those found by Okoro et al. (2009) in salty soils of Chile, who used different recovery media. Also, our isolates tolerate only 2, 5, and 10% NaCl. It can therefore be concluded that the bacteria in this study are slight and moderate halophiles or halotolerants.

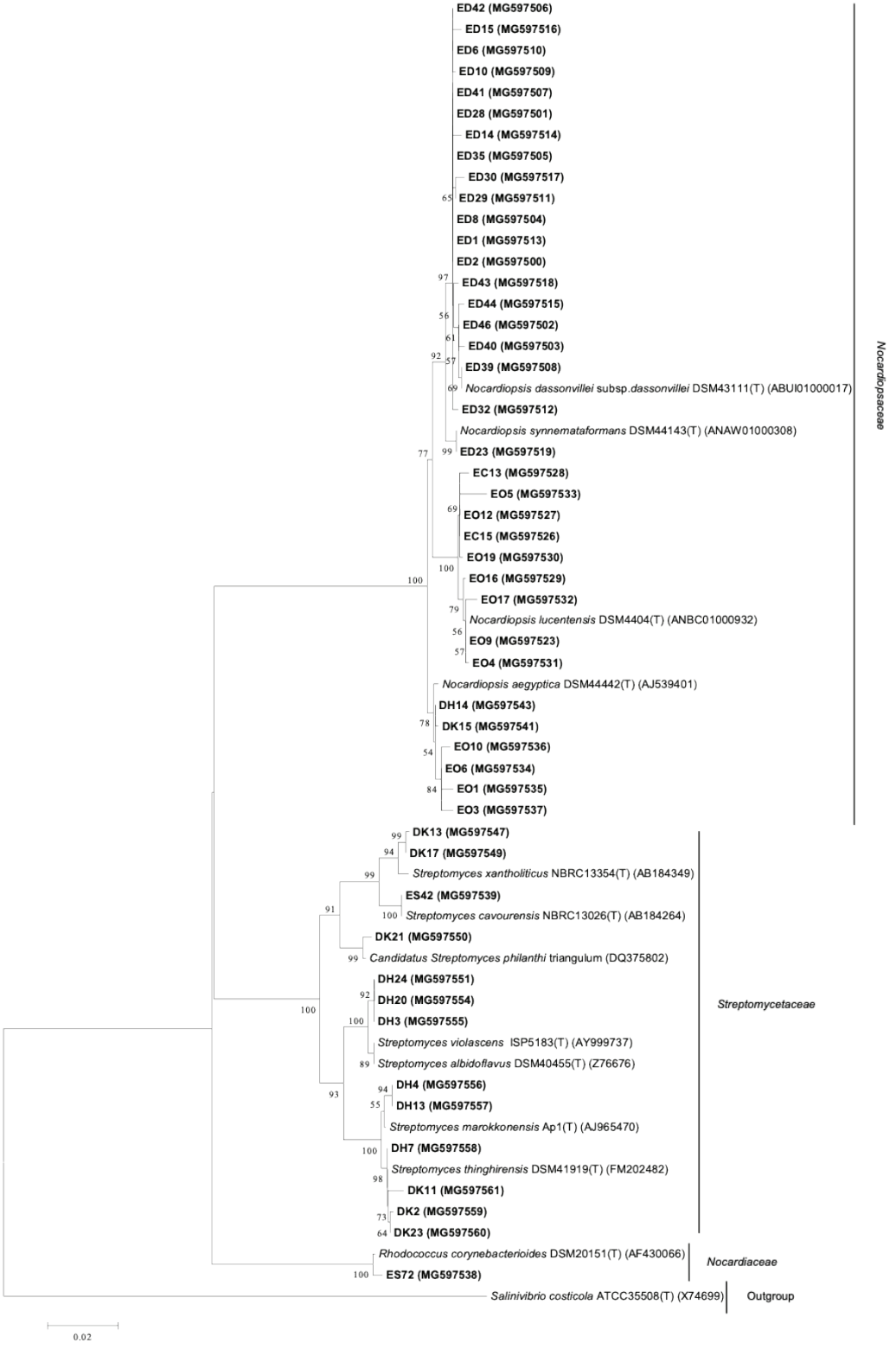

Figure 1. Neighbor-joining tree showing the phylogenetic relationships between the nearly complete 16S rRNA gene sequences isolated from "Ezzemoul" (EO, EC, ES, ED) and "Djendli" (DK, DH) sebkhas and their closely related sequences from the EzBioCloud database. The very close sequences are not represented on the tree. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position. (EO, DK), (EC, ES, DH), and ED are isolates from 2%, 5%, and 10% of NaCl concentrations, respectively. Salinivibrio costicola (X74699) is used as an outgroup.

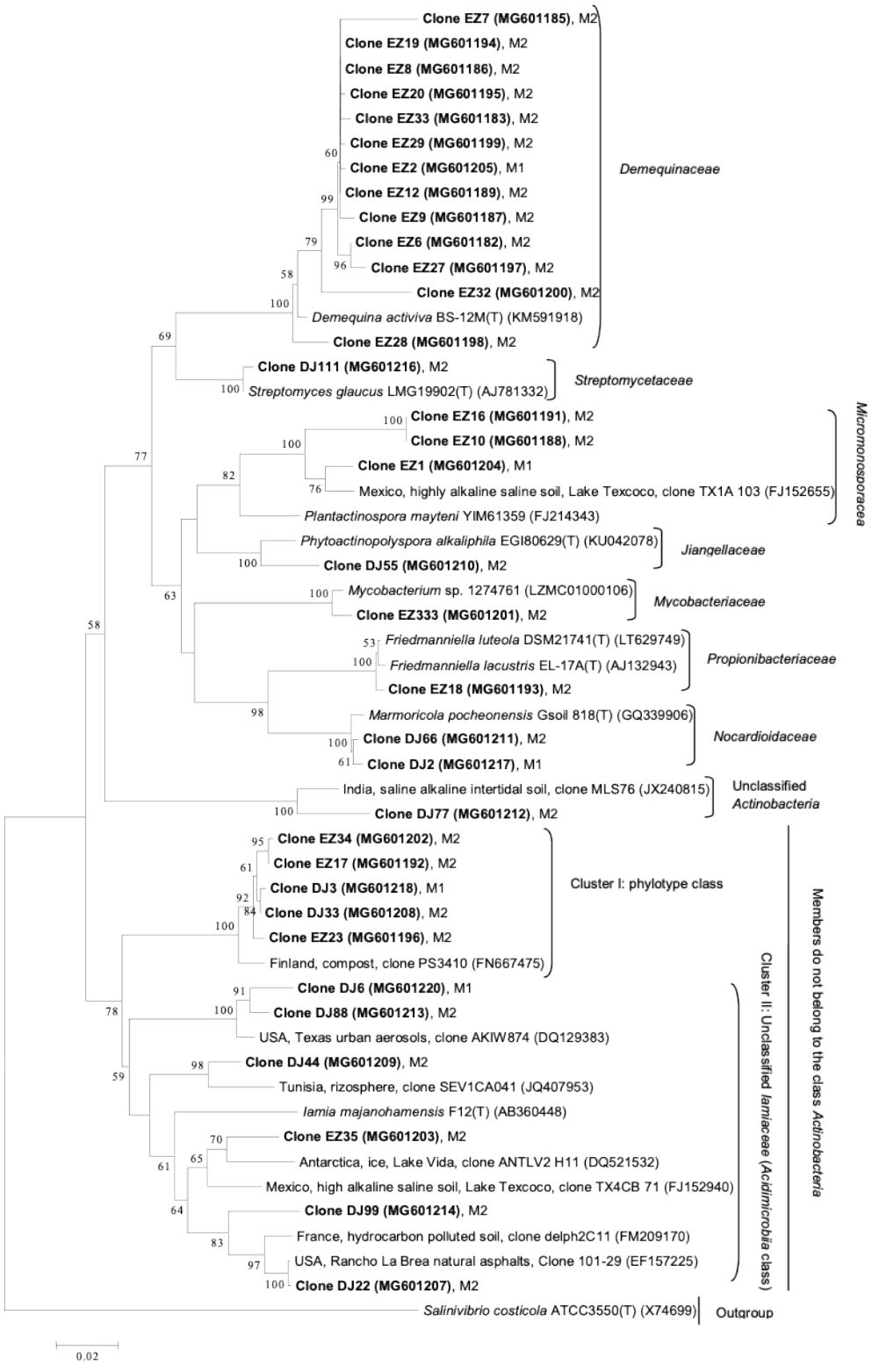

Figure 2. Neighbor-joining tree showing the phylogenetic relationships between the partial actinobacterial 16S rRNA gene sequences cloned from "Ezzemoul" (EZ) and "Djendli" (DJ) sebkhas and their closely related sequences from the EzBioCloud database. The very close sequences are not represented on the tree. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position. M1 and M2 respectively represent the first and second cloning methods whose clones are derived. Salinivibrio costicola (X74699) is used as an outgroup.

The results of the cultural method and molecular cloning revealed the presence of several genera and groups of actinobacteria in the sites of Ezzemoul and Djendli. It should be noted that the species *Streptomyces thinghirensis*, *Candidatus Streptomyces philanthi*, and *Marmoricola pocheonensis*, identified from both methods, were detected for the first time in the extremely salty soils. After culture, phylogenetic analysis of the 16S rDNA sequences of "Ezzemoul" and "Djendli" isolates (40 and 22, respectively) showed the existence of three genera: *Nocardiopsis*, *Streptomyces*, and *Rhodococcus* (Figs 1, A1, & A2). In the salty soil of Ezzemoul (Figs 1 & A1) it is the genus *Nocardiopsis* that dominates with more than 90% of sequences. These sequences are represented by four species: *N. dassonvillei (*50% of sequences), followed by *N. lucentensis*, *N. aegyptica*, and *N. synnemataformans* with identity percentages ranging from 98.7 to 100%. The genus *Rhodococcus* represents a minority and is detected only in "Ezzemoul" site. On the other hand, in the salty soil of Djendli (Figs 1 & A2), it is the genus *Streptomyces* that dominates with more than 80% of the sequences. These sequences belong to six species: *S. xantholiticus*, *S. albidoflavus*, *S. thinghirensis*, *S. marokkensis*, *S. philanthi*, and *S. violascens* with identity percentages ranging from 99.21% up to 100%. Many species of *Nocardiopsis* are of a halotolerant nature, and they are isolated from desert soils and marine environments (Bennur et al. 2016). Those of *Streptomyces* can adapt to high salt concentrations in soils (Sudnitsyn 2009). Studies have reported that members of the genus *Nocardiopsis* were predominant in some desert soils in Algeria (Meklat et al. 2011) and in saline soil in China (Lv et al. 2006). Those of the genus *Streptomyces* were predominant in other saline soils in China (Cai et al. 2009) and in India (Jose and Jebakumar 2013). *Rhodococcus* species can occupy several habitats such as soil, water, air, plants, and animals. Some have been found in the marine environment (Li et al. 2012) and very few have been described in saline soils (Táncsics et al. 2017).

Regarding the culture-independent method, phylogenetic analysis allowed us to retain 39 clone sequences. In "Ezzemoul" sebkha (Figs 2 & A3), there are nearly 21% of uncultured clones and four genera: *Demequina*, *Plantactinospora*, *Friedmanniella*, and *Mycobacterium.* The genus *Demequina* has already been found by cultivation methods in the marine environments of South Korea (Park et al. 2016) and Japan (Hamada et al. 2013), in the saline soil of the Japanese mangrove (Matsumoto et al. 2010), in a salt marsh plant in Portugal (Fidalgo et al. 2016), and even in the permafrost of the Arctic Highlands of Norway (Finster et al. 2009). *Plantactinospora* species have been mainly isolated from Chinese plants (Guo et al. 2016). The genus *Friedmanniella* has been isolated in China from an unsalted soil (Zhang et al. 2013) and from various habitats in the world such as plants (Tuo et al. 2016), activated mud moss (Maszenan et al. 1999), sandstone (Schumann et al. 1997), air (Kim et al. 2016), hypersaline lake water (Lawson et al. 2000), and spiders and their webs (Iwai et al. 2010). The genus *Mycobacterium* is known for its pathogenicity in human beings and animals. It is mostly isolated from clinical specimens (Vasireddy et al. 2016). Some are isolated from soil and water (Peeters et al. 2016). The species *M. algericum* has been discovered for the first time in Algeria in goat lung lesions (Sahraoui et al. 2011). In "Djendli" sebkha, there are three genera: *Marmoricola*, *Phytoactinopolyspora*, *Streptomyces*, and an unclassified actinobacterium (DJ77). Most clones are uncultured (over 70%).The genus *Marmoricola* comes from several places in the world such as the agricultural (*M. pocheoensis*) and forest soils (Dastager et al. 2008; Lee et al. 2016), the marine environment (Maszenan et al. 1999), marble (Urzì et al. 2000), and volcanic ashes (Lee et al. 2011). The genus *Phytoactinopolyspora* was recently discovered in China. The known species are halotolerant. Some are isolated from saline soils (Ji et al. 2017) and others are derived from plants (Li et al. 2015). Several species of the genus *Streptomyces* were detected by the cultural method and only one (*S. glaucus*) was found by molecular cloning. Several clones in both sebkhas do not belong to the class *Actinobacteria* and are represented by two Clusters (Figs 2 & A3). Cluster I represents a phylotype that does not have a valid name yet. Ghai et al. (2013) suggested a new subclass "*Candidatus Actinomarinidae*" with an *Actinomarinales* order, which includes uncultured marine *Actinobacteria* with low GC%. However, this division does not still exist in the classification. Cluster II represents unclassified members of the family *Iamiaceae* of the class *Acidimicrobiia* belonging to the phylum *Actinobacteria* (Norris 2012).The members linked to these two clusters are not a part of the class *Actinobacteria*, whereas the primers used in this study are supposed to be specific to this class (Stach et al. 2003). This could be explained by the lack of specificity of these primers. This point has already been reported by Song et al. (2009), while other researchers (Piao et al. 2008) have found the efficiency of these primers.

The results obtained by the cultural method are clearly different from those achieved by the culture-independent method (molecular cloning) in the two study sites, except from the genus *Streptomyces* that was reported simultaneously by both methods in the Djendli site. Therefore, the cultural method is complementary to the culture-independent method and their combination can give a good description of the actinobacterial diversity in the salty soils of sebkhas. Based on the comparison of the two methods, our results may agree with those of other researchers (Borsodi et al. 2013) who studied the diversity of *Bacteria* and *Archaea* in saline environments.

4. CONCLUSIONS

This research was the first to study the diversity of *Actinobacteria* in "Ezzemoul" and "Djendli" sebkhas soils in Algeria by combining two methods: one cultural and the other by molecular cloning. Based on the 16S rRNA gene, our primary data indicated that the actinobacterial community is very diverse. Besides the genera *Streptomyces* and *Nocardiopsis* found in the two studied sites (with the probability of the presence of a new species of the genus *Nocardiopsis* in the Ezzemoul sebkha), many other different genera were detected with some

unknown members and not belonging to the class *Actinobacteria*. In addition, all our new isolates that tolerate 10% NaCl may be important candidates for biotechnological applications.

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References

Aberkane M (2014) Ecologie de la Sarcelle marbrée *Marmaronetta angustirostris* dans les zones humides de l'Est algérien. PhD thesis, Badji Mokhtar University, Annaba, Algeria, (in French).

Aliat T, Kaabeche M, Khomri H, et al (2016) A Pedological Characterisation of Some Inland Wetlands and Ramsar Sites in Algeria. Land Degrad Dev 27:693–705. doi: 10.1002/ldr.2467

Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Balla A (2012) Synthèse écologique sur les zones humides algériennes d'importance internationale be "Sites Ramsar". PhD thesis, Batna University, Algeria, (in French).

Bedford BL, Walbridge MR, Aldous A (1999) Patterns in Nutrient Availability and Plant Diversity of Temperate North American Wetlands. Ecology 80:2151–2169. doi: 10.2307/176900

Bellagoune S (2015) Hivernage du Tadorne de Belon *Tadorna tadorna* (Anatidés) dans la sebkha de Djendli (Batna, Est algérien).PhD thesis, Badji Mokhtar University, Annaba, Algeria, (in French).

Bennur T, Ravi Kumar A, Zinjarde SS, Javdekar V (2016) *Nocardiopsis* species: a potential source of bioactive compounds. J Appl Microbiol 120:1–16. doi: 10.1111/jam.12950

Borsodi AK, Felföldi T, Máthé I, et al (2013) Phylogenetic diversity of bacterial and archaeal communities inhabiting the saline Lake Red located in Sovata, Romania. Extremophiles 17:87–98. doi: 10.1007/s00792-012-0496-2

Boughachiche F, Rachedi K, Duran R, et al (2016) Optimization of alkaline protease production by *Streptomyces* sp. strain isolated from saltpan environment. Afr J Biotechnol 15:1401–1412

Cai Y, Xue Q, Chen Z, Zhang R (2009) Classification and salt-tolerance of actinomycetes in the Qinghai lake water and lakeside saline soil. J Sustain Dev 2:107–110

Chenchouni H (2009) Place des argiles dans la caractérisation écopédologique du Chott de Djendli (Batna, Algérie) et mise en évidence de la relation salinité–répartition des halophytes, (in French). In: Proceedings of the 3rd Maghrebin Symposium on Clays 'SMA. pp 23–25

Cocolin L, Alessandria V, Dolci P, et al (2013) Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. International Journal of Food Microbiology 167:29–43. doi: 10.1016/j.ijfoodmicro.2013.05.008

Dastager SG, Lee J-C, Ju Y-J, et al (2008) *Marmoricola bigeumensis* sp. nov., a member of the family *Nocardioidaceae*. Int J Syst Evol Microbiol 58:1060–1063. doi: 10.1099/ijs.0.65576-0

Dupain R, Lanchon R, Saint-Arroman JC (2000) Granulats, sols, ciments et bétons: caractérisation des matériaux de génie civil par les essais de laboratoire, (in French). Paris: Casteilla

Ebihara H, Takada A, Kobasa D, et al (2006) Molecular Determinants of Ebola Virus Virulence in Mice. PLOS Pathog 2:e73. doi: 10.1371/journal.ppat.0020073

Eswaran H, Rice T, Ahrens R, Stewart B. (2002) Soil classification: a global desk reference. Boca Raton: CRC Press

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fidalgo C, Henriques I, Rocha J, et al (2016) Culturable endophytic bacteria from the salt marsh plant Halimione portulacoides: phylogenetic diversity, functional characterization, and influence of metal(loid) contamination. Environ Sci Pollut Res 23:10200–10214. doi: 10.1007/s11356-016-6208-1

Finster KW, Herbert RA, Kjeldsen KU, et al (2009) *Demequina lutea* sp. nov., isolated from a high Arctic permafrost soil. Int J Syst Evol Microbiol 59:649–653. doi: 10.1099/ijs.0.004929-0

Gagnard J, Huguet C, Ryser J-P (1988) L'analyse du sol et du végétal dans la conduite de la fertilisation. Le controle de la qualite des fruits, (in French).

Gasmi M, Kitouni M (2017) Optimization of chitinase production by a new *Streptomyces* griseorubens C9 isolate using response surface methodology. Ann Microbiol 67:175–183. doi: 10.1007/s13213-016-1249-8

Ghai R, Mizuno CM, Picazo A, et al (2013) Metagenomics uncovers a new group of low GC and ultra-small marine Actinobacteria. Sci Rep 3:2471. doi: 10.1038/srep02471

Guo X, Guan X, Liu C, et al (2016) *Plantactinospora soyae* sp. nov., an endophytic actinomycete isolated from soybean root [Glycine max (L.) Merr]. Int J Syst Evol Microbiol 66:2578–2584. doi: 10.1099/ijsem.0.001088

Hamada M, Tamura T, Yamamura H, et al (2013) *Demequina flava* sp. nov. and *Demequina sediminicola* sp. nov., isolated from sea sediment. Int J Syst Evol Microbiol 63:249–253. doi: 10.1099/ijs.0.039297-0

Hocinat A, Boudemagh A (2015) Biodegradation of commercial Ortiva fungicide by isolated actinomycetes from the activated sludge. Desalination Water Treat 57:6091–6097. doi: 10.1080/19443994.2015.1022799

Hozzein WN (2015) Biodiversity of Halophilic and Halotolerant Actinobacteria. In: DK Maheshwari, M Saraf (Eds.), *Halophiles* Sus-

tainable Development and Biodiversity (pp.1-28). Switzerland: Springer International Publishing

Iwai K, Aisaka K, Suzuki M (2010) *Friedmanniella luteola* sp. nov., *Friedmanniella lucida* sp. nov., *Friedmanniella okinawensis* sp. nov. and *Friedmaniella sagamiharensis* sp. nov., isolated from spiders. Int J Syst Evol Microbiol 60:113–120. doi: 10.1099/ijs.0.007815-0

Ji Y, Chunyu W-X, Li E-Y, et al (2017) *Phytoactinopolyspora halotolerans* sp. nov., a halotolerant actinobacterium isolated from a saline soil in Xinjiang, northwest of China. Antonie Van Leeuwenhoek 111:27–34. doi: 10.1007/s10482-017-0923-6

Jose PA, Jebakumar SRD (2013) Phylogenetic appraisal of antagonistic, slow growing actinomycetes isolated from hypersaline inland solar salterns at Sambhar salt Lake, India. Front Microbiol 4:190. doi: 10.3389/fmicb.2013.00190

Kharroub K (2007) Identification et étude moléculaire des bactéries et des archéobactéries aérobies halophiles de la sebkha Ezzemoul (Ain M'Lila). PhD thesis, Mentouri University, Constantine, Algeria, (in French)

Kim S-J, Hamada M, Ahn J-H, et al (2016) *Friedmanniella aerolata* sp. nov., isolated from air. Int J Syst Evol Microbiol 66:1970–1975. doi: 10.1099/ijsem.0.000973

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. doi: 10.1007/BF01731581

Kitouni M, Boudemagh A, Oulmi L, et al (2005) Isolation of actinomycetes producing bioactive substances from water, soil and tree bark samples of the north–east of Algeria. J Mycol Médicale-Journal Med Mycol 15:45–51

Lahoum A, Verheecke-Vaessen C, Bouras N, et al (2017) Taxonomy of mycelial actinobacteria isolated from Saharan soils and their efficiency to reduce aflatoxin B1 content in a solid-based medium. Ann Microbiol 67:231–237. doi: 10.1007/s13213-017-1253-7

Lane DJ (1991) 16S/23S rRNA sequencing. In: E Stackebrandt, M Goodfellow (Eds.), Nucleic acid techniques in bacterial systematics (pp.115–175). New York: John Wiley and Sons

Larkin MA, Blackshields G, Brown NP, et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948. doi: 10.1093/bioinformatics/btm404

Lawson PA, Collins MD, Schumann P, et al (2000) New LL-diaminopimelic acid-containing actinomycetes from hypersaline, heliothermal and meromictic Antarctic Ekho Lake: *Nocardioides aquaticus* sp. nov. and *Friedmanniella* [correction of Friedmannielly] *lacustris* sp. nov. Syst Appl Microbiol 23:219–229

Lee JY, Hwang BK (2002) Diversity of antifungal actinomycetes in various vegetative soils of Korea. Can J Microbiol 48:407–417. doi: 10.1139/w02-025

Lee SD, Lee DW, Ko Y-H (2011) *Marmoricola korecus* sp. nov. Int J Syst Evol Microbiol 61:1628–1631. doi: 10.1099/ijs.0.025460-0

Lee S-Y, Im W-T, Kang M-S, et al (2016) *Marmoricola ginsengisoli* sp. nov. and *Marmoricola pocheonensis* sp. nov. isolated from a ginseng-cultivating field. Int J Syst Evol Microbiol 66:1996–2001. doi: 10.1099/ijsem.0.000977

Li J, Zhao G-Z, Long L-J, et al (2012) *Rhodococcus nanhaiensis* sp. nov., an actinobacterium isolated from marine sediment. Int J Syst Evol Microbiol 62:2517–2521. doi: 10.1099/ijs.0.038067-0

Li L, Ma J-B, Abdalla Mohamad O, et al (2015) *Phytoactinopolyspora endophytica* gen. nov., sp. nov., a halotolerant filamentous actinomycete isolated from the roots of Glycyrrhiza uralensis F. Int J Syst Evol Microbiol 65:2671–2677. doi: 10.1099/ijs.0.000322

Lv Z., Zhang L., Li Y, et al (2006) Biodiversity of halophilic actinomycetes of Cangzhou salty environments. , 26(1):1-6. J Hebei Univ Nat Sci Ed 26:1–6

Maszenan AM, Seviour RJ, Patel BK, et al (1999) *Friedmanniella spumicola* sp. nov. and *Friedmanniella capsulata* sp. nov. from activated sludge foam: gram-positive cocci that grow in aggregates of repeating groups of cocci. Int J Syst Bacteriol 49 Pt 4:1667–1680. doi: 10.1099/00207713-49-4-1667

Matsumoto A, Nakai K, Morisaki K, et al (2010) *Demequina salsinemoris* sp. nov., isolated on agar media supplemented with ascorbic acid or rutin. Int J Syst Evol Microbiol 60:1206–1209. doi: 10.1099/ijs.0.012617-0

Meklat A, Sabaou N, Zitouni A, et al (2011) Isolation, Taxonomy, and Antagonistic Properties of Halophilic Actinomycetes in Saharan Soils of Algeria. Appl Environ Microbiol 77:6710–6714. doi: 10.1128/AEM.00326-11

Naikpatil SV, Rathod JL (2011) Selective isolation and antimicrobial activity of rare actinomycetes from mangrove sediment of Karwar. J Ecobiotechnology 3:48–53

Neffar S, Chenchouni H, Si Bachir A (2016) Floristic composition and analysis of spontaneous vegetation of Sabkha Djendli in northeast Algeria. Plant Biosyst - Int J Deal Asp Plant Biol 150:396–403. doi: 10.1080/11263504.2013.810181

Norris PR (2012) Class Acidimicrobiia. In: M Goodfellow, P Kämpfer, HJ Busse, ME Trujillo, K Suzuki, W Ludwig, WB Whitman (Eds.), Bergey's Manual of Systematic Bacteriology, 2nd edn (pp.1968–1969). New York: Springer

Okoro CK, Brown R, Jones A, et al (2009) Diversity of culturable actinomycetes in hyper-arid soils of the Atacama Desert, Chile. Antonie Van Leeuwenhoek 95:121–133. doi: 10.1007/s10482-008-9295-2

Oskay M, Tamer AU, Azeri C, et al (2004) International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada. Afr J Biotechnol 3:441–446

Pandey B, Ghimire P, Agrawal VP (2004) International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada.

Park S, Jung Y., Won S., Yoon J. (2016) *Demequina litorisediminis* sp. nov., isolated from a tidal flat, and emended description of the genus *Demequina*. Int J Syst Evol Microbiol 66:4197–4203. doi: 10.1099/ijsem.0.001335

Peeters C, Depoorter E, Praet J, Vandamme P (2016) Extensive cultivation of soil and water samples yields various pathogens in

patients with cystic fibrosis but not Burkholderia multivorans. J Cyst Fibros 15:769–775. doi: 10.1016/j.jcf.2016.02.014

Pham VHT, Kim J (2012) Cultivation of unculturable soil bacteria. Trends in Biotechnology 30:475–484. doi: 10.1016/j.tibtech.2012.05.007

Piao Z, Yang L, Zhao L, Yin S (2008) Actinobacterial Community Structure in Soils Receiving Long-Term Organic and Inorganic Amendments. Appl Environ Microbiol 74:526–530. doi: 10.1128/AEM.00843-07

Pochon J, Tardieux P (1962) Techniques d'analyse en microbiologie du sol, (in French)

Queipo-Ortuño MI, Colmenero JDD, Macias M, et al (2008) Preparation of Bacterial DNA Template by Boiling and Effect of Immunoglobulin G as an Inhibitor in Real-Time PCR for Serum Samples from Patients with Brucellosis. Clinical and Vaccine Immunology 15:293–296. doi: doi:10.1128/CVI.00270-07

Rengasamy P (2006) World salinization with emphasis on Australia. Journal of Experimental Botany. 57:1017–1023. doi: DOI:10.1093/jxb/erj108.

Richards LA (1954) Diagnostic and improvement of saline and alkaline soils. U.S Department of agriculture, Washington D.C

Sahraoui N, Ballif M, Zelleg S, et al (2011) *Mycobacterium algericum* sp. nov., a novel rapidly growing species related to the *Mycobacterium terrae* complex and associated with goat lung lesions. Int J Syst Evol Microbiol 61:1870–1874. doi: 10.1099/ijs.0.024851-0

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. doi: 10.1093/oxfordjournals.molbev.a040454

Schumann P, Prauser H, Rainey FA, et al (1997) *Friedmanniella antarctica* gen. nov., sp. nov., an LL-diaminopimelic acid-containing actinomycete from Antarctic sandstone. Int J Syst Bacteriol 47:278–283. doi: 10.1099/00207713-47-2-278

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 16:313–340

Song Z, Zhi X, Li W, et al (2009) Actinobacterial Diversity in Hot Springs in Tengchong (China), Kamchatka (Russia), and Nevada (USA). Geomicrobiol J 26:256–263. doi: 10.1080/01490450902892373

Stach JEM, Maldonado LA, Ward AC, et al (2003) New primers for the class *Actinobacteria*: application to marine and terrestrial environments. Environ Microbiol 5:828–841. doi: 10.1046/j.1462-2920.2003.00483.x

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–5

Sudnitsyn II (2009) Specificity of actinomycetes in salt-affected soils. Eurasian Soil Sci 42:235–236. doi: 10.1134/S106422930902015X

Tamura K, Stecher G, Peterson D, et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Táncsics A, Máthé I, Benedek T, et al (2017) *Rhodococcus sovatensis* sp. nov., an actinomycete isolated from the hypersaline and heliothermal Lake Ursu. Int J Syst Evol Microbiol 67:190–196. doi: 10.1099/ijsem.0.001514

Tuo L, Pan Z, Li F-N, et al (2016) *Friedmanniella endophytica* sp. nov., an endophytic actinobacterium isolated from bark of Kandelia candel. Int J Syst Evol Microbiol 66:3057–3062. doi: 10.1099/ijsem.0.001146

Urzì C, Salamone P, Schumann P, Stackebrandt E (2000) *Marmoricola aurantiacus* gen. nov., sp. nov., a coccoid member of the family *Nocardioidaceae* isolated from a marble statue. Int J Syst Evol Microbiol 50:529–536

Valenzuela-Encinas C, Neria-González I, Alcántara-Hernández RJ, et al (2009) Changes in the bacterial populations of the highly alkaline saline soil of the former lake Texcoco (Mexico) following flooding. Extremophiles 13:609–621. doi: 10.1007/s00792-009-0244-4

Vartoukian SR, Palmer RM, Wade WG (2010) Strategies for culture of 'unculturable' bacteria. FEMS microbiology letters 309:1–7

Vasireddy R, Vasireddy S, Brown-Elliott BA, et al (2016) *Mycobacterium arupense*, *Mycobacterium heraklionense*, and a Newly Proposed Species, "*Mycobacterium virginiense*" sp. nov., but Not *Mycobacterium nonchromogenicum*, as Species of the *Mycobacterium terrae* Complex Causing Tenosynovitis and Osteomyelitis. J Clin Microbiol 54:1340–1351. doi: 10.1128/JCM.00198-16

Wright ES, Yilmaz LS, Noguera DR (2012) DECIPHER, a Search-Based Approach to Chimera Identification for 16S rRNA Sequences. Appl Environ Microbiol 78:717–725. doi: 10.1128/AEM.06516-11

Yoon S-H, Ha S-M, Kwon S, et al (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67:1613–1617. doi: 10.1099/ijsem.0.001755

Zhang X, Zhang J, Zhang Y, et al (2013) *Friedmanniella flava* sp. nov., a soil actinomycete. Int J Syst Evol Microbiol 63:1771–1775. doi: 10.1099/ijs.0.043984-0

SUPPLEMENTARY MATERIAL

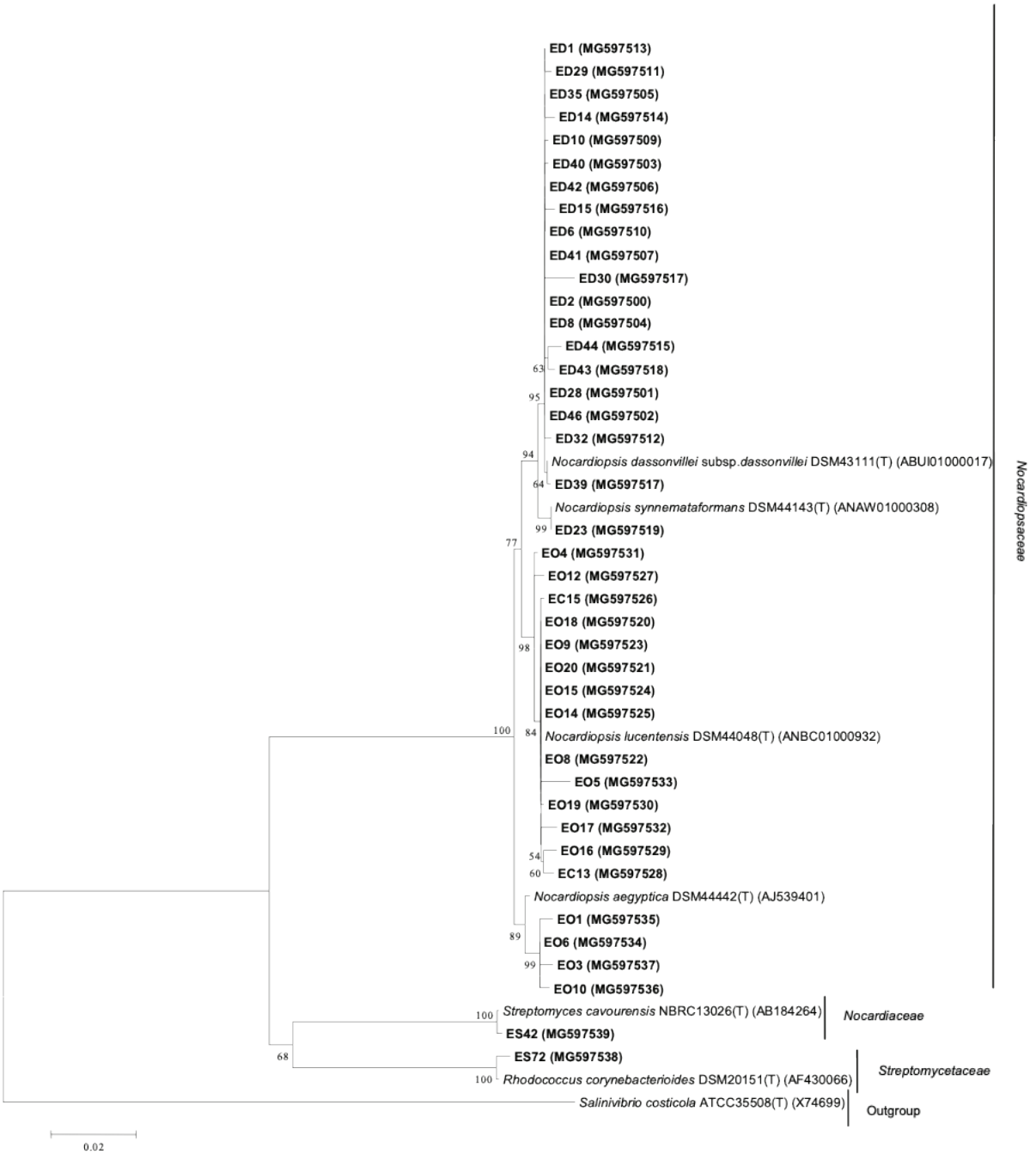

Figure A1. Neighbor-joining tree showing the phylogenetic relationships between the nearly complete 16S rRNA gene sequences isolated from "Ez-zemoul" sebkha and their closely related sequences from the EzBioCloud database. All sequences are represented. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position. EO, (EC, ES), and ED are isolates from 2%, 5%, and 10% of NaCl concentrations, respectively. Salinivibrio costicola (X74699) is used as an outgroup.

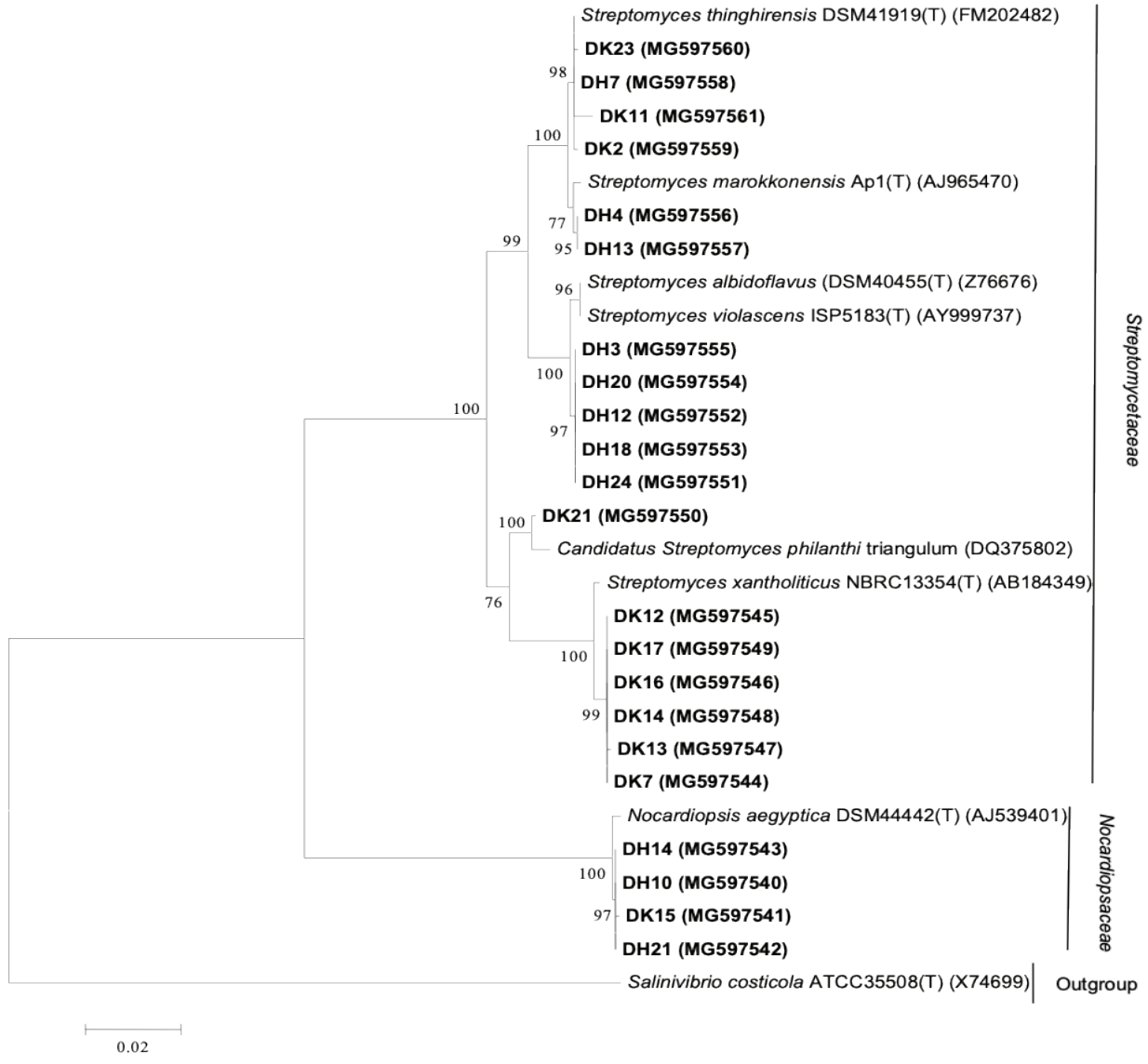

Figure A2. Neighbor-joining tree showing the phylogenetic relationships between the nearly complete 16S rRNA gene sequences isolated from "Djendli" sebkha and their closely related sequences from the EzBioCloud database. All sequences are represented. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position. DK and DH are isolates from 2% and 5% of NaCl concentrations, respectively. Salinivibrio costicola (X74699) is used as an outgroup.

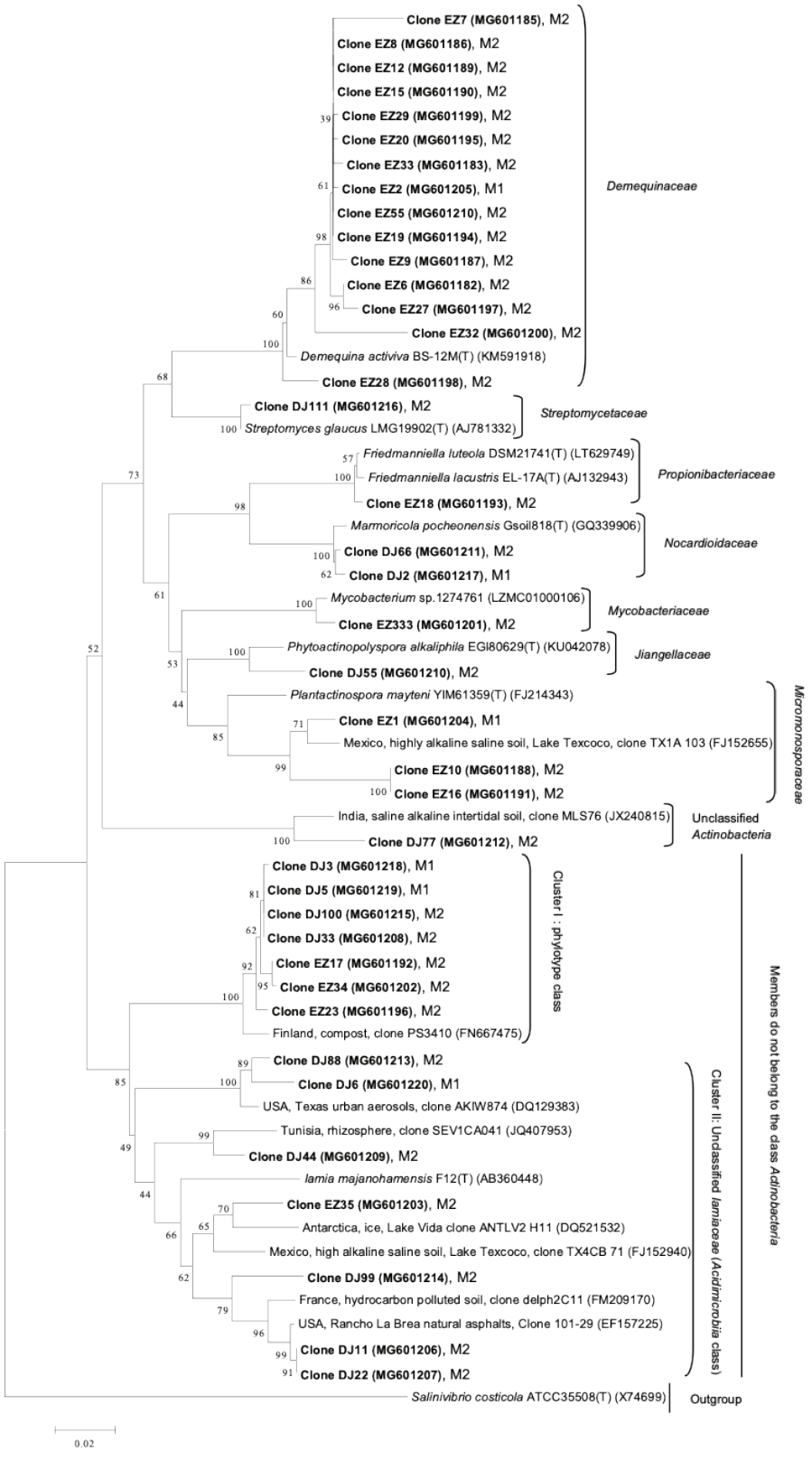

Figure A3. Neighbor-joining tree showing the phylogenetic relationships between the partial actinobacterial 16S rRNA gene sequences cloned from "Ezzemoul" (EZ) and "Djendli" (DJ) sebkhas and their closely related sequences from the EzBioCloud database. All sequences are represented. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position. M1 and M2 respectively represent the first and second cloning methods whose clones are derived. Salinivibrio costicola (X74699) is used as an outgroup.