

## Phylogenetic Diversity and Antimicrobial Activities of Culturable Endophytic Actinobacteria Isolated from Different Egyptian Marine Sponges and Soft Corals

<sup>1</sup>Ahmed M. El-Bondkly, <sup>2</sup>Mervat M.A.A. El-Gendy, <sup>3</sup>Jutta Wiese and <sup>3</sup>Johannes F. Imhoff

<sup>1</sup>Genetics and Cytology Department, National Research Center, Dokki, Giza, Egypt

<sup>2</sup>Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Giza, Egypt

<sup>3</sup>Leibniz-Institut für Meereswissenschaften IFM-GEOMAR, Düsternbrooker Weg 20, 24105 Kiel, Germany

---

**Abstract:** A cultivation-based approach was employed to isolate and compare the endophytic culturable actinobacterial diversity associated with different Egyptian marine sponges and soft corals. A total of 13 culturable actinobacteria isolates were obtained, five of which isolated from different sponges, two (AE27 and AE32) and three (AE29, AE41 and AE46) were isolated from *Haliclona* sp. and *Callyspongia* sp. collected from Sharm El-Sheikh and Hurghada, Egypt, respectively. Eight were isolated from different five soft corals, out of them two (AE2 and AE19), two (AE3 and AE15), two (AE20 and AE23), one (AE35) and one (AE13) were isolated from *Rhytisma* sp., *Xenia* sp., *Lobularia* sp., and *Dendronephthya sinaiensis* (collected from Sharm El-Sheikh, Egypt) and the *Dendronephthya hemprichi* (collected from Hurghada, Egypt), respectively. The 16S rRNA genes of the 13 actinobacteria isolates were amplified and sequenced (GenBank Accession numbers JF292923 – JF292930 and JF319146 – JF319150). A BLAST analysis revealed that the 13 actinobacteria isolates belonged to three known families and genera. Six strains isolated from all sponges and soft corals belonged to the genus *Streptomyces* (AE15, AE19, AE20 and AE35, isolated from soft corals; *Xenia* sp., *Rhytisma* sp., *Lobularia* sp., and *Dendronephthya sinaiensis* as well as AE29 and AE41, isolated from sponges; *Callyspongia* sp., respectively). Six strains (AE2, AE3 and AE23, isolated from soft corals; *Rhytisma* sp., *Xenia* sp. and *Lobularia* sp. as well as AE27, AE32 and AE46, isolated from sponges; *Haliclona* sp. and *Callyspongia* sp., respectively) belonged to the genus *Nocardiopsis*. Only one isolate (AE13) derived from *Dendronephthya hemprichi* was belonged to the genus *Pseudonocardia*. The phylogenetic analysis revealed that four culturable actinobacteria strains (AE2, AE27, AE32 and AE46) were more distantly related to other previously published *Nocardiopsis dassonvillei*. Screening for antimicrobial activity revealed that out of the 13 culturable actinobacteria isolates, only five (AE15, AE19, AE20, AE29 and AE41) belonged to the genus *Streptomyces* have anticandida or anti-positive Gram bacteria activity.

**Key words:** Actinobacteria, 16S rDNA sequencing, Marine soft corals and Sponges, Antimicrobial activity.

---

### INTRODUCTION

Marine sponges and soft corals provide classic examples of microbial macro faunal partnerships that have been a productive source for the discovery of bioactive compounds. Marine sponges and soft corals are sessile marine filter feeders that can filter large volumes of surrounding water through a unique aquiferous system (De Vos *et al.*, 1991; Reiswig, 1974; Rohwer *et al.*, 2001 and 2002 and Nithyanand *et al.*, 2011). As a result, marine sponges and soft corals become a rich reservoir of diverse, highly concentrated marine bacteria, some of which may not have been cultured yet. Some sponges and soft corals -derived metabolites reveal striking similarity to known microbial metabolites, and it has been hypothesized that many natural products from marine invertebrates may be of microbial origin (Faulkner, 2002).

In recent years, there has been an interest in the bacteria associated with marine sponges and soft corals as sources of bioactive natural products. This interest has been mainly driven by the increasing number of bioactive metabolites isolated from sponges and soft corals -associated bacteria as well as evidence supporting these bacteria as the real producers of bioactive metabolites originally isolated from their host sponges and soft corals (Schmidt *et al.*, 2000 and Stierle and Stierle, 1992). Among all sponges and corals-associated bacteria, actinobacteria are particular interest in producing antibiotics and other therapeutically significant compounds (Takahashi and Omura, 2003). Nevertheless, we believe that expanding the diversity of isolates will lead to renewed discovery. Recent studies showed a novel and abundant actinobacteria assemblage in the marine sponge *Rhopaloeides odorabile* and coral *Acropora digitifera*, assessed by both a culture-independent molecular

---

**Corresponding Author:** Ahmed M. El-Bondkly, Genetics and Cytology Department, National Research Center, Dokki, Giza, Egypt  
E-mail: ahmed\_bondkly@yahoo.com

approach and a culture-based method (Webster *et al.*, 2001 and Nithyanand *et al.*, 2011). Several earlier studies also isolated single strains of actinobacteria from marine sponges (Lee *et al.*, 1998; Imamura *et al.*, 1993). However, our understanding of the sponges and corals-associated-actinobacteria community is still inadequate as isolation and exploitation efforts are just beginning.

To date, for handling of numerous strains, bacterial taxonomic position has primarily been determined by morphological observation, sometimes augmented by chemotaxonomical method. However, these methods have weaknesses in both accuracy and objectivity. For bacterial taxonomy, phylogenetic analysis by 16S rDNA sequences has become the standard approach. However, for the treatment of many isolates, DNA sequence analysis is costly in terms of money, time and effort. The class actinobacteria consists of a diverse range of Gram-positive bacteria with high G + C DNA content and analysis of the 16S rDNA begins by isolating DNA and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample and a computer is then used for studying the sequence for identification using phylogenetic analysis procedures. 16S rDNA analysis has been widely applied for the study of the diversity of microbial communities and for strain identification. Significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and actinomycetes (Yokota, 1997 and Labeda *et al.*, 2012). Sequences of 16S ribosomal DNA have provided actinomycetologists with a phylogenetic tree that allows the investigation of evolution of actinomycetes and also provides the basis for identification. It has been shown from molecular diversity analysis of 16S rDNA sequences that actinobacteria occur abundantly in marine sponges in several studies (Hentschel *et al.*, 2001; Imhoff and Stöhr, 2003; Montalvo *et al.*, 2005 and Webster *et al.*, 2001). It was found that 30% of the clone sequences obtained from *R. odorabile* (Webster *et al.* 2001) and more than 70% of the clones from specimens of *Halichondria panacea* (Imhoff and Stöhr, 2003) were related to actinobacteria. Given the important role of actinobacteria in the production of novel bioactive metabolites, it is important to understand if the abundance of actinobacteria is a general feature across many sponge species, as well as the sponge species-specific associations and diversity.

In the current study, the questions that we addressed are whether it is a general feature that many marine sponges and soft corals host novel actinobacteria and what the differences are in the diversity of the actinobacterial community among different Egyptian marine sponges and soft corals. As a first step to approach these questions, a cultivation-based method was used to isolate actinobacteria from two different sponges and five soft corals. These sponges and soft corals were collected from Sharm El-Sheikh and Hurghada, Egypt and the number of actinobacteria isolates and diversity of the culturable actinobacteria community from these sponges and soft corals were directly identified and compared by 16S rDNA analysis.

## MATERIALS AND METHODS

### **Collection and Processing of Different Sponges and Soft Corals:**

The marine sponges, *Haliclona* sp. and *Callyspongia* sp. were collected from Red Sea, Sharm El-Sheikh and Hurghada, Egypt, respectively. Five soft corals (*Rhytisma* sp., *Xenia* sp., *Lobularia* sp., *Dendronephthya sinaiensis*) collected from Red Sea, Sharm El-Sheikh, Egypt and the *Dendronephthya hemprichi* collected from Red Sea, Hurghada, Egypt. Taxonomic identification of the sponges and soft corals were performed by the National Institute of Oceanography and Fisheries Research Station, Hurghada, Egypt. Sponge and soft coral samples were cut from different sponges and soft corals with a dive knife while wearing latex gloves and individual pieces were put into sterile separate plastic sample collection bags, brought to the surface, maintained at ambient seawater temperature and transported to a land-based laboratory for processing. Immediately after collection, healthy Egyptian sponge and soft coral specimens were rinsed many times with sterile seawater to remove transient and loosely attached bacteria.

### **Isolation of Actinobacteria:**

The endophytic actinobacteria strains were separated from the inner healthy tissue of sponges and soft corals according to the procedure described by Taechowisan and Lumyong, 2003 and Taechowisan *et al.*, 2003 and slight modified. Tissue were rinsed in 0.1% Tween 20 for 1 min, and then in 2.5% sodium hypochlorite for 15 min followed by washing in sterile seawater for 5 min. The surface was sterilized with 75% ethanol for 5 min then rinsed in sterile seawater three times. Finally the samples cut into small pieces of ca. 4 x 4 mm<sup>2</sup> and the pieces were transferred to dishes of specialized medium supplemented with a final concentration of 50 µg/ml of K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, 100 µg/ml nystatin and cycloheximide to inhibit fungal growth (Yang *et al.*, 1995) as well as 15 µg/ml of nalidixic acid to inhibit many fast-growing gram-negative bacteria so as to allow the isolation of slow-growing actinobacteria (Webster *et al.*, 2001) from the injury surface. The medium (CM) compositions were (10 g of soluble starch, 4 g of yeast extract, 2 g of peptone, 20 g of Agar and 1 L of seawater, finally adjusted to pH 7.0. The plates were then incubated at 30°C for 4 weeks. Single colonies were transferred periodically to the

same medium, and after 7 days, pure cultures of actinobacteria isolates were obtained and examined for antimicrobial substance production.

#### **Screening for Antimicrobial Activities of Endophytic Marine Actinobacteria:**

Single colonies of actinobacteria that grew on the plates transferred into a 250 ml Erlenmeyer flask with 50 ml fermentation medium. The medium contained (g/L) starch, 24; glucose, 8; peptone, 3; meat extract, 3; yeast extract, 5 and CaCO<sub>3</sub>, 4 in artificial seawater (according to Li and Liu, 2006 with increasing NaCl concentration to 36 g/L), and the pH was adjusted to 7.2 before autoclaving. The culture was incubated at 30 °C for 7 days on a rotary shaker (180 rpm). After fermentation, antimicrobial activity yield of each colony was analyzed by a diffusion bioassay test using various Gram-positive bacteria (*Bacillus subtilis* DSM 347 and *Staphylococcus lentus* DSM 6672), Gram-negative bacteria (*Escherichia coli* K 12 DSM 498 and *Pseudomonas fluorescens* NCIMB 10586) and fungal (*Candida glabrata* DSM 6425) target strains. Indicator bacteria were grown in Luria Broth (LB) medium contains (g/L) tryptone, 10; yeast extract, 5; NaCl, 10; Agar, 15 with the pH adjusted to 7.0 as well as *Candida glabrata* DSM 6425 was grown in PDA medium. Antibiotic assay discs (Whatman product No. 484000, 6 mm diameter) were saturated with 25 µL of each actinobacteria isolate supernatant and placed on the surface of indicator test plates then incubated overnight at 37 °C.

#### **Strain Culture, Genomic DNA Extraction and 16S rDNA Sequencing:**

All the actinobacterial isolates were cultured on broth CM medium. Genomic DNA was extracted and purified using the QIAGEN DNeasy Tissue Kit following the manufacturer's protocol for Gram-positive, bacteria. Amplification of ribosomal DNA was performed using puReTaq™ Ready-To-Go™ PCR beads (GE Healthcare). For amplification of the nearly complete 16S rRNA gene (approximately 1500 bp, Figure 1), the eubacterial primers 27f and 1492r were used (Lane, 1991). The conditions for this PCR were initial denaturation (5 min at 94 °C) followed by 45 cycles of primer annealing (1 min at 55 °C), primer extension (2 min at 72 °C) and denaturation (1 min at 94 °C), a final primer annealing (1 min at 42 °C) and a final extension phase (5 min at 72 °C) and then cooled to 4 °C. PCR products were checked for correct length on a 1% Tris–borate-EDTA (TBE) agarose gel (1% agarose, 8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA), stained with ethidium bromide and visualized under UV illumination (Figure 1). Purification of PCR products and determination of sequences using the 16S rDNA-specific primers 342f, 534r, 790f and 1492r were done (Vera *et al.*, 2006 and Imhoff and Stöhr, 2003) at the Institute for Clinical Molecular Biology (Universitätskliniken Schleswig-Holstein in Kiel).

#### **Sequencing Alignment and Phylogenetic Analysis:**

Sequence data were edited with Lasergene Software SeqMan (DNASTar Inc.). Next relatives were determined by comparison to 16S rRNA genes in the National Center for Biotechnology Information NCBI GenBank database using BLAST (Basic Local Alignment Search Tool, <http://www.ncbi.nlm.nih.gov> website) and Ribosomal Database Project (RDP) searches (Altschul *et al.*, 1990) to create a matrix using MEGA5 and ClustalW programs (Tamura *et al.*, 2011). The tree topologies were evaluated by bootstrap analyses based on 1,000 replications with MEGA5 and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei, 1987). The complete 16S rDNA sequences of 13 representative strains have been deposited in GenBank database and assigned under the following Accession numbers: JF292923 – JF292930 (AE3, AE13, AE15, AE19, AE20, AE23, AE35 and AE2, respectively) and JF319146 – JF319150 (AE27, AE29, AE32, AE41 and AE46, respectively).

## **RESULTS AND DISCUSSION**

#### **Diversity of Culturable Actinobacteria Isolates:**

After the removal of loosely attached microorganisms by carefully washing the sponge and soft coral materials with sterile seawater, the endophytic actinobacteria isolates were separated from the inner healthy tissue of sponges and soft corals by using of CM medium and tested for antimicrobial activity. Samples from the two different marine sponges, *Haliclona* sp. and *Callyspongia* sp. collected from Sharm El-Sheikh and Hurghada, respectively as well as five soft corals (*Rhytisma* sp., *Xenia* sp., *Lobularia* sp., *Dendronephthya sinaiensis*) collected from Sharm El-Sheikh, and the *Dendronephthya hemprichi* collected from Hurghada were plated on CM medium for isolation of actinobacteria culturable isolates. A total of 13 culturable actinobacteria isolates were obtained from different sponge and soft coral samples. The isolates were initially identified based on the morphology and cultural characteristic. Five isolated from different sponges, out of which, two (AE27 and AE32) and three (AE29, AE41 and AE46) were isolated from *Haliclona* sp. and *Callyspongia* sp. collected from Sharm El-Sheikh and Hurghada, Egypt, respectively. Eight isolated from different five soft corals, out of which, two (AE2 and AE19), two (AE3 and AE15), two (AE20 and AE23), one (AE35) and one (AE13) were isolated from *Rhytisma* sp., *Xenia* sp., *Lobularia* sp., and *Dendronephthya sinaiensis* (collected from Sharm El-Sheikh, Egypt) and the *Dendronephthya hemprichi* (collected from Hurghada, Egypt), respectively (Table 1).

Cultivation-based methods are always highly selective due to the choice of media and culture conditions (Webster *et al.*, 2001; Imhoff and Stöhr, 2003 and Nithyanand *et al.*, 2011). It should be noted that cultivation-based approaches are limited since the high selectivity of isolation media and culture conditions usually allow only a small fraction of the bacteria present within a sponge and coral specimens to be isolated (Webster *et al.*, 2001; Imhoff and Stöhr, 2003 and Nithyanand *et al.*, 2011). In the case of *R. odorabile*, the most abundant bacteria isolated on standard media from 40 sponge specimens collected from different regions of Great Barrier Reef was an  $\delta$ -Proteobacterium (Webster and Hill, 2001).

**Table 1:** Isolation sources, regions and antimicrobial activities of endophytic marine actinobacterial isolates.

	Isolation source	Isolation place	Antimicrobial activity (mm)				
			<i>B. subtilis</i>	<i>S. lentus</i>	<i>E. coli</i>	<i>P. fluorescens</i>	<i>C. glabrata</i>
AE2	<i>Rhytisma fulvum</i>	Sharm El-Sheikh	0	0	0	0	0
AE3	<i>Xenia</i> sp.	Sharm El-Sheikh	0	0	0	0	0
AE13	<i>Dendronephthya hemprichi</i>	Hurghada	0	0	0	0	0
AE15	<i>Xenia</i> sp.	Sharm El-Sheikh	0	0	0	0	15
AE19	<i>Rhytisma fulvum</i>	Sharm El-Sheikh	0	0	0	0	16
AE20	<i>Lobularia</i> sp.	Sharm El-Sheikh	0	0	0	0	17
AE23	<i>Lobularia</i> sp.	Sharm El-Sheikh	0	0	0	0	0
AE27	<i>Haliclona</i> sp.	Sharm El-Sheikh	0	0	0	0	0
AE29	<i>Callyspongia</i> sp.	Hurghada	0	0	0	0	19
AE32	<i>Haliclona</i> sp.	Sharm El-Sheikh	0	0	0	0	0
AE35	<i>Dendronephthya sinaiensis</i>	Sharm El-Sheikh	0	0	0	0	0
AE41	<i>Callyspongia</i> sp.	Hurghada	20	15	0	0	0
AE46	<i>Callyspongia</i> sp.	Hurghada	0	0	0	0	0

#### Antimicrobial Potential Screening:

The tests of 13 actinobacteria isolates against five microbial indicators (*Bacillus subtilis* DSM 347, *Staphylococcus lentus* DSM 6672, *Escherichia coli* K 12 DSM 498, *Pseudomonas fluorescens* NCIMB 10586 and *Candida glabrata* DSM 6425), showed that 5 out of 13 which have antimicrobial potential against *Candida glabrata* DSM 6425 and positive Gram bacteria (Table 1). Actinobacteria isolates AE15, AE19, AE20 and AE29 exhibited evident inhibition zone against *Candida glabrata* DSM 6425 strain (Figure 2). One actinobacteria isolate AE41 exhibited inhibition zone against positive Gram bacteria (*Bacillus subtilis* DSM 347 and *Staphylococcus lentus* DSM 6672, Figure 2).

**Table 2:** Similarity to the closest relative in GenBank and accession numbers of 16S rDNA sequences of endophytic marine actinobacterial isolated from different marine sponges and soft corals.

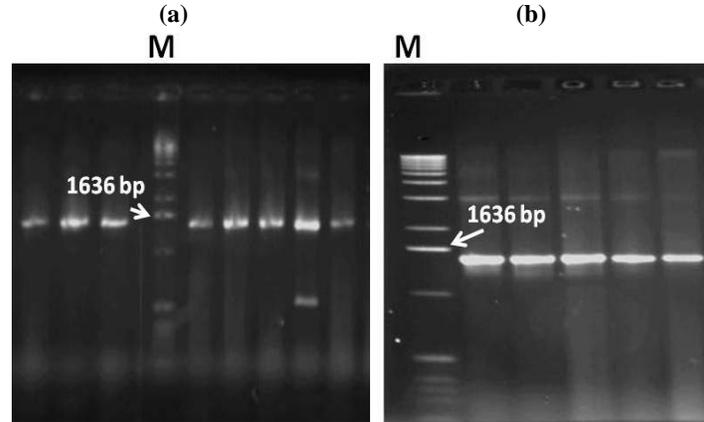
Isolate code	Accession number	Length (bp)	Family	Genus	The most similar species (Accession No.)	Identity %
AE2	JF292930	1492	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis dassonvillei</i> DSM 43111 (X97886)	99.73
AE3	JF292923	1488	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis composta</i> KS9 (AF360734)	97.33
AE13	JF292924	1482	Pseudonocardiaceae	Pseudonocardia	<i>Pseudonocardia carboxydivorans</i> Y8 (EF114314)	99.39
AE15	JF292925	1330	Streptomycetaceae	Streptomyces	<i>Streptomyces macrosporus</i> DSM 41449 (Z68099)	97.23
AE19	JF292926	1487	Streptomycetaceae	Streptomyces	<i>Streptomyces intermedius</i> NBRC 13049 (AB184277)	99.59
AE20	JF292927	1485	Streptomycetaceae	Streptomyces	<i>Streptomyces champavatii</i> NRRL B-5682 (DQ026642)	99.26
AE23	JF292928	1490	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis aegyptia</i> DSM 44442 (AJ539401)	98.36
AE27	JF319146	1480	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis dassonvillei</i> DSM 43111 (X97886)	99.80
AE29	JF319147	1487	Streptomycetaceae	Streptomyces	<i>Streptomyces albidoflavus</i> strain HD-109 (EF620361)	99.33
AE32	JF319148	1468	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis dassonvillei</i> DSM 43111 (X97886)	99.73
AE35	JF292929	1478	Streptomycetaceae	Streptomyces	<i>Streptomyces pilosus</i> NBRC 12807 (AB184161)	99.65
AE41	JF319149	1486	Streptomycetaceae	Streptomyces	<i>Streptomyces griseoincarnatus</i> LMG 19316 (AJ781321)	99.93
AE46	JF319150	1435	Nocardiopsaceae	Nocardiopsis	<i>Nocardiopsis dassonvillei</i> DSM 40465 (X97882)	99.93

#### Blast Search and Phylogenetic Analysis Based on 16S rRNA Gene Sequences:

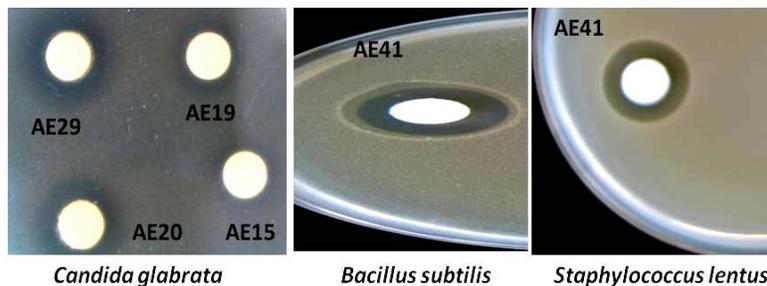
The 16S rRNA genes of the 13 actinobacteria isolates were amplified and sequenced (Table 2; GenBank Accession numbers JF292923 – JF292930 and JF319146 – JF319150). A BLAST analysis was carried out via blastn search through GenBank (<http://www.ncbi.nlm.nih.gov>) and Ribosomal Database Project (RDP) revealed that the 13 actinobacteria isolates belonged to three known families and genera (Table 2, Figure 3 and Figure 4). As shown in Tables 1 and 2 as well as Figures 3 and 4 about six of the strains isolated from all sponges and soft corals are belonged to the family *Streptomycetaceae* and genus *Streptomyces* (AE15, AE19, AE20 and AE35, isolated from soft corals; *Xenia* sp., *Rhytisma* sp., *Lobularia* sp., and *Dendronephthya sinaiensis* collected from Sharm El-Sheikh, Egypt as well as AE29 and AE41, isolated from sponge; *Callyspongia* sp. collected from Hurghada, Egypt, respectively). Six strains (AE2, AE3 and AE23, isolated from soft corals; *Rhytisma* sp., *Xenia* sp. and *Lobularia* sp. collected from Sharm El-Sheikh, Egypt as well as AE27, AE32 and AE46, isolated from sponges; *Haliclona* sp. and *Callyspongia* sp. collected from Sharm El-Sheikh and Hurghada, Egypt, respectively) are belonged to family *Nocardiopsaceae* and genus *Nocardiopsis* (Tables 1, 2 and Figures 3). Only one isolate (AE13) derived from *Dendronephthya hemprichi* (collected from Hurghada, Egypt) was belonged to family *Pseudonocardiaceae* and genus *Pseudonocardia* (Tables 1, 2 and Figures 3, 4).

Further sequence analysis excluded redundant sequences and identified 13 independent isolates. Figure 3 displays the phylogenetic affiliation of the representative endophytic isolates (AE2, AE3, AE13, AE15, AE19, AE20, AE23 and AE35) derived from different soft coral samples (Table 1). 50 % of these isolates (AE15,

AE19, AE20 and AE35) were members of the genus *Streptomyces*, which was the dominant actinobacterial genus within soft corals. Isolates AE15, AE19, AE20 and AE35 showing 97.23 %, 99.59 %, 99.26 % and 99.65 % homology to *Streptomyces macrosporus* DSM 41449, *Streptomyces intermedius* NBRC 13049, *Streptomyces champavatii* NRRL B-5682 and *Streptomyces pilosus* NBRC 12807, respectively (Table 2 and Figure 3). All displayed anticandida activity except, isolate AE35 which displayed no activity against all tested microorganisms (Table 1). Three (37.5 %) isolates (AE2, AE3 and AE23) belonged to genus *Nocardiopsis*, showing 99.73 %, 97.33 % and 98.36 % homology to *Nocardiopsis dassonvillei* DSM 43111, *Nocardiopsis composta* KS9 and *Nocardiopsis aegyptia* DSM 44442, respectively (Table 2 and Figure 3). None of these isolates showed antibacterial or antifungal activity (Table 1). On the other hand, only one isolate (AE13) isolated from marine soft coral, *Dendronephthya hemprichi* (collected from Hurghada, Egypt) and identified as a member of the genus *Pseudonocardia*, which showed 99.39 % identity to *Pseudonocardia carboxydivorans* Y8 without antibacterial or antifungal activity (Tables 1, 2 and Figure 3).



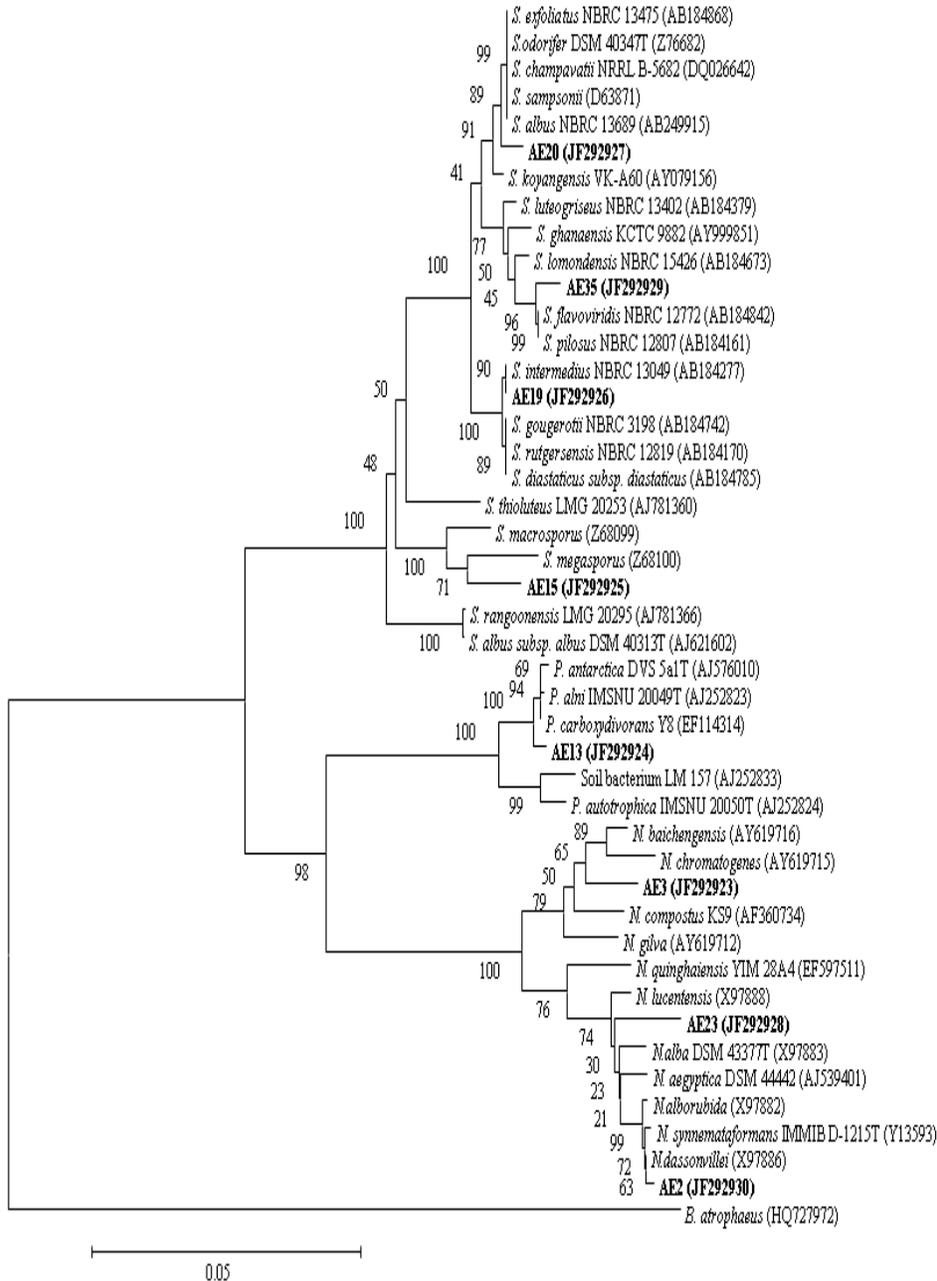
**Fig. 1:** Agarose gel electrophoresis of full-length sequences of 16S rDNA amplification products (Approximately 1500 bp) from cultured endophytic actinobacteria isolates associated with marine soft corals (a) and sponges (b), M; DNA molecular weight marker X (0.07-12.2kbp).



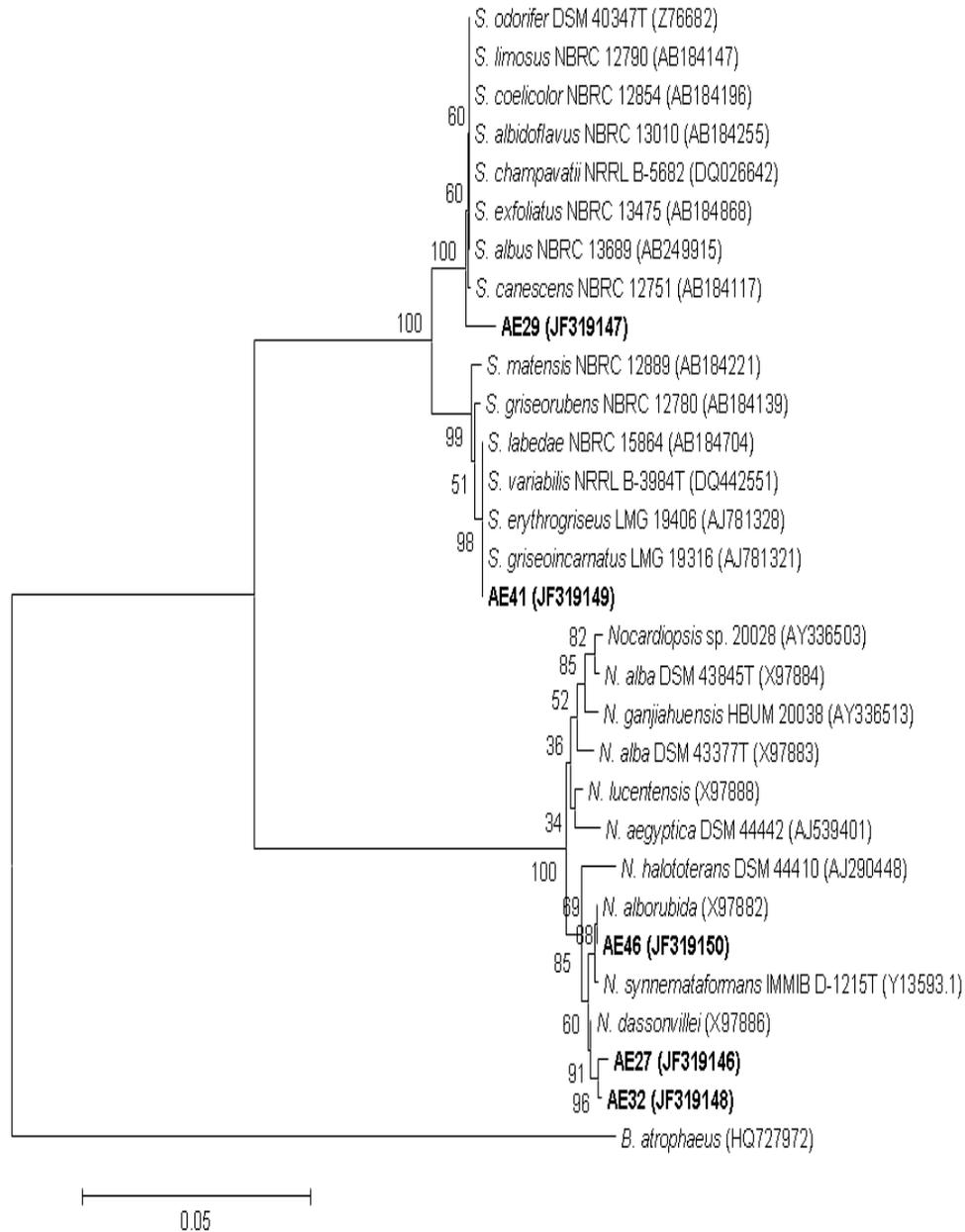
**Fig. 2:** Antimicrobial activity of endophytic marine actinobacteria isolates derived from marine soft corals and sponges.

The 16S rDNA genes of five strains (AE27, AE32, AE29, AE41 and AE46) isolated from two different Sponges (*Haliclona* sp. and *Callyspongia* sp. collected from Sharm El-Sheikh and Hurghada, Egypt, Table 1) were fully sequenced and subjected to phylogenetic analysis (Figure 4). The sequences results indicate that the three strains (AE27, AE32 and AE46) from two sponge species clustered in genus *Nocardiopsis* and were only distantly related to their closest described relatives, which include 99.80 %, 99.73 % and 99.93 % identity to *Nocardiopsis dassonvillei* DSM 43111, *Nocardiopsis dassonvillei* DSM 43111 and *Nocardiopsis dassonvillei* DSM 40465T, respectively (Table 2 and Figure 4). No antimicrobial activity was detected for these strains (Table 1). The strains (AE29 and AE41) isolated from *Callyspongia* sp. (collected from Hurghada, Egypt) clustered with genus *Streptomyces* and showed 99.33 % and 99.93 % homology to *Streptomyces albidoflavus* strain HD-109 and *Streptomyces griseoincarnatus* LMG 19316, respectively (Table 2 and Figure 4). These isolates showed strong anticandida and anti-positive Gram bacteria activities (Table 1). The phylogenetic analysis (Table 2 and Figures 3, 4) revealed that four culturable actinobacteria strains (AE2, AE27, AE32 and AE46) were more distantly related to other previously published *Nocardiopsis dassonvillei*. Their highest 16S rDNA gene sequence similarities to published sequences obtained from NCBI/BLAST and RDP were 99.73 %, 99.73 %, 99.73 % and 99.73 %, respectively.

99.73 % (AE2 and AE32) homology to *Nocardiopsis dassonvillei* DSM 43111, 99.80 % (AE27) homology to *Nocardiopsis dassonvillei* DSM 43111 and 99.93 % (AE46) homology to *Nocardiopsis dassonvillei* DSM 40465. Prescreening for antimicrobial activity revealed that out of the 13 culturable actinobacteria isolates belonged to three known genera, only five (AE15, AE19, AE20, AE29 and AE41) belonged to genus *Streptomyces* have anticandida or anti-positive Gram bacteria activity.



**Fig. 3:** Neighbor-joining phylogenetic tree from the analysis of full-length sequences of 16S rDNA genes from cultured endophytic actinobacteria associated with marine soft corals. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 re-sampled data sets. The scale bar represents 0.05 substitutions per nucleotide position. *Bacillus atrophaeus* (HQ727972) used as outgroups.



**Fig. 4:** Neighbor-joining phylogenetic tree from the analysis of full-length sequences of 16S rDNA genes from cultured endophytic actinobacteria associated with marine sponges. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 re-sampled data sets. The scale bar represents 0.05 substitutions per nucleotide position. *Bacillus atrophaeus* (HQ727972) used as outgroups.

It has been shown from molecular diversity analysis of 16S rDNA sequences that actinobacteria occur abundantly in marine sponges and corals in several studies (Hentschel *et al.*, 2001; Imhoff and Stöhr, 2003; Montalvo *et al.*, 2005; Nithyanand *et al.*, 2011 and Webster *et al.*, 2001). It was found that 30% of the clone sequences obtained from *R. odorabile* (Webster *et al.*, 2001), more than 70% of the clones from specimens of *H. panacea* (Imhoff and Stöhr, 2003) were related to actinobacteria and 36% of the bacteria in the clone library of coral; *A. digitifera* were found to be novel after full length sequencing of the 16S rRNA gene wherein several clones were found to be novel at the Genus and species level. On the other hand, the use of the 16S rDNA gene as a phylogenetic marker enables the determination of the precise phylogenetic position of sponge and coral bacterial populations independent of their culturability. Previous studies demonstrated great differences between

the genetically verified diversity and the cultural spectrum of bacteria from sponges (Webster *et al.*, 2001 and Imhoff and Stöhr, 2003). However the molecular genetic analysis of the bacteria diversity indicated that phylum actinobacteria were the dominant group in the total bacterial assemblage (Nithyanand *et al.*, 2011 and Webster *et al.*, 2001). Moreover, Jiang *et al.*, (2008) found the culturable actinobacteria, isolated from the marine sponge *Iotrochota* sp. in the South China Sea belong to three actinobacteria genera, and one isolate may be a new species. *Streptomyces* appears to be the dominant genus among symbionts and adherents present in *Iotrochota* sp. in the South China Sea. Therefore, cultivation-based and genetic approaches are complimentary and should be combined to reveal the actinobacterial association with marine sponges and corals. It is necessary to carry out detailed molecular analyses e.g., using actinobacteria specific primers to reveal the true diversity even if actinobacteria are present as minor components of the total community. Therefore, it is important to recognize the advantages and limitations of both cultivation based and genetic approaches in revealing the actinobacterial diversity within marine sponges and corals.

In conclusion, differences in distribution and diversity of culturable actinobacteria were found in different marine sponges and soft corals which further supported the hypothesis that marine sponges and soft corals are a rich and novel source for actinobacteria and, potentially, natural products. The culturable actinobacteria, isolated from different marine sponges and soft corals and regions in the Red Sea, Egypt belong to three actinobacteria genera. *Streptomyces* and *Nocardiopsis* appear to be the dominant genera present in different marine sponges and soft corals (collected from Red Sea, Egypt). Only one strain which isolated from soft coral, *Dendronephthya hemprichi* (collected from Hurghada, Egypt) and identified as a member of the genus *Pseudonocardia*. Prescreening for antimicrobial activity revealed that only five culturable actinobacteria (AE15, AE19, AE20, AE29 and AE41) have anticandida or anti-positive Gram bacteria activity as well as all these strain belonged to the genus *Streptomyces*.

## REFERENCES

- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410.
- De Vos, L., K. Rützler, N. Boury-Esnault, C. Donadey, J. Vacelet, 1991. Atlas of sponge morphology. Smithsonian Institution Press, Washington, pp: 117.
- Faulkner, D.J., 2002. Marine natural products. Nat. Prod. Rep., 19: 1-48.
- Hentschel, U., M. Schmidt, M. Wagner, L. Fieseler, C. Gernert, J. Hacker, 2001. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiol. Ecol., 35: 305-312.
- Imamura, N., M. Nishijima, K. Adachi, H. Sano, 1993. Novel antimycin antibiotics, urauchimycins A and B, produced by marine actinomycete. J. Antibiot., 46: 241-246.
- Imhoff, J.F., R. Stöhr, 2003. Sponge-associated bacteria: general overview and special aspects of bacteria association with *Halichondria panacea*. In: Müller, W. E. G. (Ed.) Sponge (Porifera). Springer, Berlin, pp: 35-58.
- Jiang, S., X. Li, L. Zhang, W. Sun, S. Dai, L. Xie, Y. Liu, K.J. Lee, 2008. Culturable actinobacteria isolated from marine sponge *Iotrochota* sp. Mar. Biol., 153: 945-952.
- Labeda, D.P., M. Goodfellow, R. Brown, A.C. Ward, B. Lanoot, M. Vannanneyt, J. Swings, S.B. Kim, Z. Liu, J. Chun, T. Tamura, A. Oguchi, T. Kikuchi, H. Kikuchi, T. Nishii, K. Tsuji, Y. Yamaguchi, A. Tase, M. Takahashi, T. Sakane, K.I. Suzuki, K. Hatano, 2012. Phylogenetic study of the species within the family *Streptomycetaceae*. Antonie van Leeuwenhoek, 101(1): 73-104.
- Lane, D.L., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow M. (Eds.) Nucleic Acid Techniques in Bacterial Systematics, Wiley, New York, pp: 115-175.
- Lee, H.K., D.S. Lee, J. Lim, J. S. Kim, J.H. Jung, 1998. Topoisomerase I inhibitors from the *Streptomyces* sp. strain KM86-9B isolated from a marine sponge. Arch. Pharm. Res., 21: 729-733.
- Li, Z.Y., Y. Liu, 2006. Marine sponge *Craniella australiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active *Actinomycetes* screening, phylogenetic analysis. Letters in Appl. Microbiol., 43: 410-416.
- Montalvo, N.F., N.M. Mohamed, J.J. Enticknap, R.T. Hill, 2005. Novel actinobacteria from marine sponges. Antonie Van Leeuwenhoek, 87: 29-36.
- Nithyanand, P., T. Indhumathi, A.V. Ravi, S.K. Pandian, 2011. Culture independent characterization of bacteria associated with the mucus of the coral *Acropora digitifera* from the Gulf of Mannar. World J. Microbiol. Biotechnol., 27(6): 1399-1406.
- Reiswig, H., 1974. Water transport, respiration and energetic of three tropical marine sponges. J. Exp. Mar. Biol. Ecol., 14: 231-249.
- Rohwer, F., M. Breitbart, M.J. Jara, F. Azam, N. Knowlton, 2001. Diversity of the bacteria associated with Caribbean coral *Montastraea franksi*. Coral Reefs, 20: 85-91.

- Rohwer, F., V. Seguritan, F. Azam, N. Knowlton, 2002. Diversity and distribution of coral-associated bacteria. *Mar. Ecol. Prog. Ser.*, 243: 1-10.
- Saitou, N., M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
- Schmidt, E.W., A.Y. Obraztsova, S.K. Davidson, D.J. Faulkner, M.G. Haygood, 2000. Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel  $\delta$ -proteobacterium, "*Candidatus Entotheonella palauensis*". *Mar. Biol.*, 136: 969-977.
- Stierle, D.B., A.A. Stierle, 1992. Pseudomonic acid derivatives from a marine bacterium. *Experientia*, 44: 1165-1169.
- Taechowisan, T., J.F. Peberdy, S. Lumyong, 2003. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.*, 19(4): 381-385.
- Taechowisan, T., S. Lumyong, 2003. Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galangal* against phytopathogenic fungi. *Ann. Microbiol.*, 53: 291-298.
- Takahashi, Y., S. Omura, 2003. Isolation of new actinomycete strains for the screening of new bioactive compounds. *J. Gen. Appl. Microbiol.*, 49: 141-154.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using likelihood, distance, and parsimony methods. *Mol. Biol. Evol.*, 28(10): 2731-2739.
- Vera, T., S.C. Neulinger, T. Staufenberger, R. Schmaljohann, F.I. Johannes, 2006. Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. *FEMS Microbiol. Ecol.*, 59(1): 47-63.
- Webster, N.S., K.J. Wilson, L.L. Blackall, R.T. Hill, 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl. Environ. Microbiol.*, 67: 434-444.
- Webster, N.S., R.T. Hill, 2001. The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by  $\gamma$ -Proteobacterium. *Mar. Biol.*, 138: 843-851.
- Yang, Y., L. Xu, Q. Li, C. Jiang, 1995. A study on isolation methods of actinomycetes. *Chin. J. Microbiol.*, 22: 88-91.
- Yokota, A., 1997. Phylogenetic relationship of actinomycetes. *Atlas of actinomycetes*, Asakura Publishing Co. Ltd., Japan, pp: 194-197.