Bacterial Communities Associated with the Sea Squirts of the Genus *Ecteinascidia* from the Red Sea and Florida

Mohamed Ahmed Elbaz
B.S., Mansoura University, 1998
Diploma, Alexandria University, 2001
M.Sc., Alexandria University, 2003

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CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Mohamed Ahmed Elbaz

has been approved

Dr. Margo G. Haygood, Thesis Research Advisor
Professor

Dr. Michiko M. Nakano
Associate Professor

Dr. Jorge Crosa
Professor
Department of Molecular Microbiology and Immunology

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Bacterial Communities Associated with the Sea Squirts of the Genus *Ecteinascidia* from the Red Sea and Florida

Mohamed Ahmed Elbaz, MSc.

Master of Science
Division of Environmental and Biomolecular Systems within
The Department of Science & Engineering
and the Oregon Health & Science University
School of Medicine

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Thesis Advisor: Margo G. Haygood

Marine natural products attract the attention of scientists from different fields due to their proven clinical importance. Ecteinascidin 743 or Et 743 is one of the compounds known from the colonial ascidian *Ecteinascidia turbinata* in the Caribbean and Mediterranean (Berrill, 1932) and from *Ecteinascidia thurstoni* in Thailand (Chavanich *et al.*, 2009) and in the Red Sea Egypt (Gab-Alla, 2008). Et 743 is a cyclic peptide with a potent anti-cancer activity and it is currently in Phase II clinical trials (Rinehart *et al.*, 1990; Jimeno *et al.*, 1999; Ryan *et al.*, 2002; Laverdiere *et al.*, 2003). The chemical structure of Et 743 is similar to other bacterial secondary metabolites. This suggests that these chemical agents are produced from bacteria that are in symbiotic association with these marine invertebrates. There are recent evidences for the presence of persistently associated bacteria in *E. turbinata* from both the Caribbean (Salomon, 2001; Pérez-Matos *et al.*, 2007) and the Mediterranean Sea (Moss and Green, 2003). A putative bacterium

"*Candidatus* Endoecteinascidia frumentensis" has been reported to be associated with *E. turbinata* and it was proposed to be the source of Et 743 (Moss and Green, 2003; Pérez-Matos *et al.*, 2007).

This study highlights a general strategy for the identification of different *Ecteinascidia spp.* using different gene markers. The results showed how conserved are the 18S rRNA gene and *cox*I gene along these tunicate species, and showed the need of a new nuclear markers in order to further resolve the phylogeny of this keystone group in chordate evolution.

While this study does not provide direct evidence of *Candidatus* E. frumentensis to be the source of Et 743, our results yield useful information on bacteria that are persistently and specifically associated with filter feeders in different tissue samples and may help to elucidate the biological and ecological role of these endosymbionts. The identification of the persistent bacteria associated with different *Ecteinascidia spp.* collected from different sites in Mediterranean Sea and Atlantic Ocean is novel data. It has never been known that *E. frumentensis* to exist outside *E. turbinata.* Our results showed *E. frumentensis* is present as a persistent bacterium in *E. thurstoni* and *Ecteinascidia*. We are hoping that our information can be utilized for the high-throughput culturing, metagenomics, phylogenetic studies and identifying the candidate biosynthetic genes responsible for the Et 743.

CHAPTER 1
INTRODUCTION

For almost a century, the ascidians have been considered as model organisms for the evolutionary developmental studies that aim to understand the origins of the tunicates. They were revealed as an important key group in the phylogenetic relationships and taxonomic status of all the chordates. Over 2500 species have been identified and studied; yet the tunicates evolutionary history is still ambiguous. *Ecteinascidia turbinata* Herdman (1880) is one of the important ascidians that attract scientists' attention due to showing biological, evolutionary phylogenetic and economic importance.

Biologically, *E. turbinata* is described as a colonial ascidian from the family Perophoridae that belongs to the largest one of 3 classes of the Tunicate (Berrill, 1932; Van Name, 1945). Its colony-forming individuals or zooids, 30 mm in length, are connected through a series of root-like stolons at the base of the colony. Stolons provide blood to the zooids, attach them to each other, help budding new zooids during asexual reproduction, and act as the storage organ during colony regression (Moss and Green, 2003). Each zooid has its own tunic which is opened to the water column via a siphon. The tunic has sparse pigments which give the colonies a transparent appearance, and make the internal anatomy visible (Fig. 1.1). In most of the populations, the tunics of the zooids have a yellow, orange or pink cast (Lyerla *et al.*, 1975; Voss, 1980). The common name sea squirt arises from their habit of squirting a jet of water, when you stand on or near them when they are uncovered at low tide.

E. turbinata colonies have an interesting habitat and distribution. They have a patchy distribution and shows seasonal population. They grow at the warm shallow water of the Gulf of Mexico, the east coast of Florida, Bermuda and the Caribbean Sea (Van Name, 1945). In the spring and summer months, many of these sessile individuals were

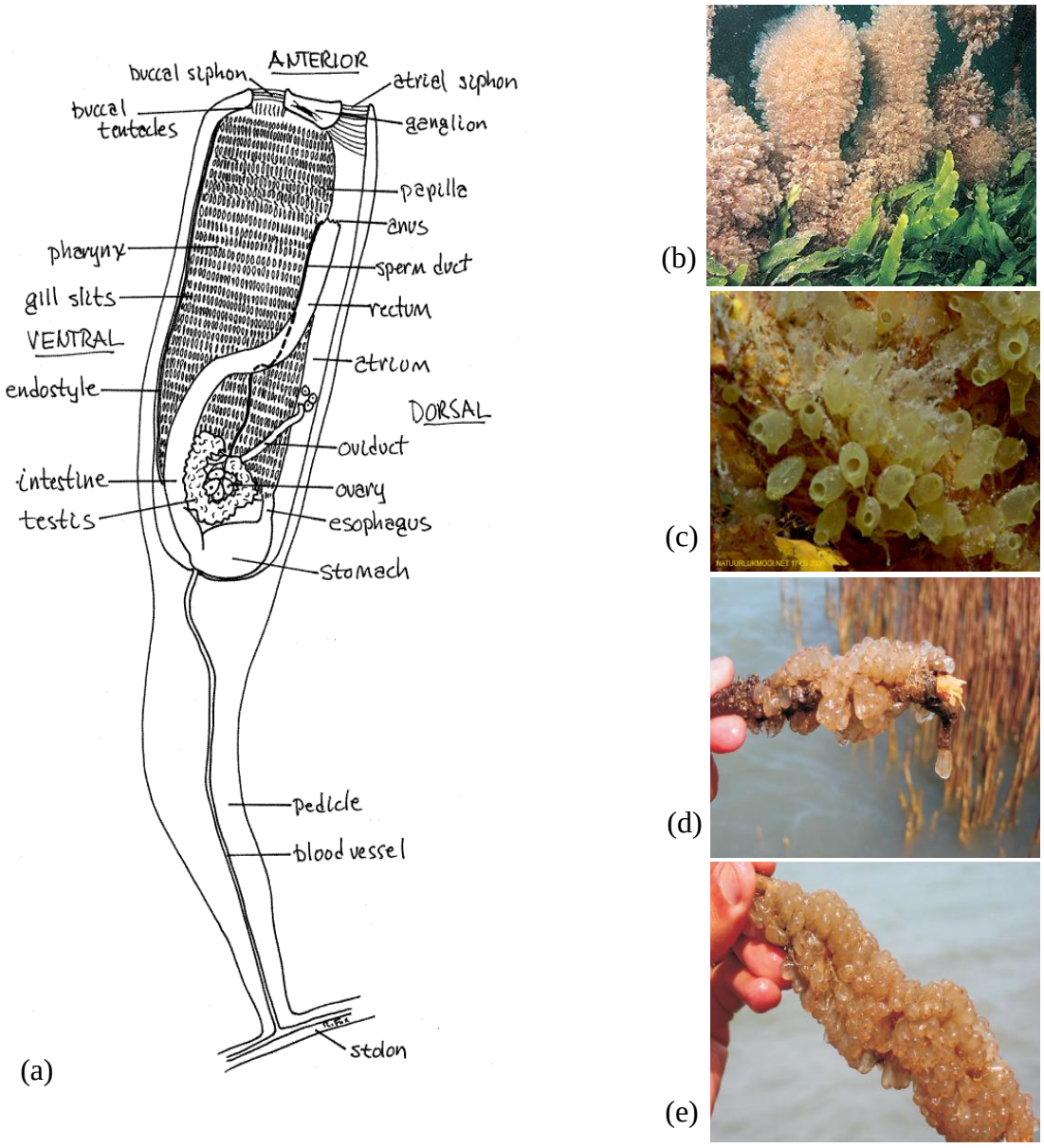

Fig. 1.1 (a) *Ecteinascidia* zooid anatomy as described in the Invertebrate Zoology textbook (Ruppert *et al*., 2004). (b) *Ecteinascidia turbinata* (photo from PharmaMar). (c) *Ecteinascidia thurstoni*, Herdman 1890 (Chavanich *et al.*, 2009). (d) Medium-sized colony, Wadi Ra'ada, Red Sea. (e) Large colony, Wadi Ra'ada, Red Sea (photos from Gab-Alla, 2008).

monitored to be as orange to reddish orange clusters (175 zooids/m^2) in the Mediterranean (Carballo *et al.*, 2000), and Chesapeake Bay (Calder *et al.*, 1966) and on reefs off the Carolinas (Pearse and Williams, 1951). They were found attached to rocks, floating docks, and sea grass remains (Young and Bingham, 1987; Ruppert and Fox, 1988) or growing on the submerged lengths of the mangroves. Colonies of the mangrove tunicate grow on prop roots of the red mangrove, *Rhizophora mangle*, in subtropical and tropical areas in both hemispheres (Vázquez and Young, 1996).

Adults of *E. turbinata* are hermaphrodites. They reproduce sexually or asexually. Sexual reproduction occurs when water temperature becomes warm in the spring and summer seasons (25–32°C). A tadpole larva matures in the peribranchial cavity for 7–9 days after fertilization and is then released into the water column (Carballo, 2000). The swimming larvae have a sharp, bright yellowish orange color. They are 4.5 mm in length (Young and Bingham, 1987). They swim to find a suitable habitat to settle down before food reserves are exhausted. After succeeding to attach themselves to the substrate, they metamorphose into juvenile tunicates (Young, 1986). It takes few weeks till the larva grows to adult size and starts to reproduce forming a full mature population. By the end of the autumn, when the water temperature drops down to 17–18°C, these populations disappear completely and the colons are then reduced to the mere stolon until the following spring (Bingham and Young, 1991; Carballo *et al.*, 1997).

Most recent phylogenetic analyses argue the classical origin of tunicates to be one of the chordates. Tunicates are morphologically divided into three major clades: (1) Phlebobranchia + Thaliacea + Aplousobranchia, (2) Appendicularia, and (3) Stolidobranchia according to their life style and metamorphosis (Nishino and Satoh, 2001). From the molecular point of view, tunicates show more complex evolutionary relationships among these morphologically distinct groups. The analysis of the 18S rRNA sequences was previously reported to show several odd clades that do not match with the classical arrangement (Delsuc *et al.*, 2006, 2008; Dunn *et al.*, 2008; Putnam *et al.*, 2008; Tsagkogeorga *et al.*, 2009). This makes the tunicates a more interesting group for further comparative genomic studies.

The economic importance of this marine mangrove tunicate was highlighted when an extract of the *E. tunicata* was found to have potent cytotoxic properties. In 1990, Rinehart's group identified and characterized many of these ecteinascidin compounds. The biological role of these compounds within the animal and how these secondary metabolites produced are still unknown. Some of the literature supports a role in chemical defense, anti-fouling, anti-infective or anti-predation agents (Pennings *et al.*, 1994; Waddell and Pawlik, 2000). As little as is known about the production of these secondary metabolites by the marine invertebrates, there is evidence that supports the hypothesis that some of these compounds have a bacterial origin. The bacterial symbionts associated with the marine invertebrates may be the real source of these compounds and produce them either by their own or in association with their hosts (Bewley *et al.*, 1996; Haygood *et al.*, 1999; Davidson *et al.*, 2001). Many examples from sponges that show bacterial symbionts complementation and activity were addressed (Vacelet and Donaday, 1977; Santavy *et al.*, 1990; Schmidt and Obraztsova, 2000; Webster *et al.*, 2001), molluscs (Distel *et al.*, 1991; McFall-Ngai, 1999; Nyholm *et al.*, 2000), bryozoans (Woollacott, 1981; Haygood and Davidson, 1997) and echinoderms (Holland and Nealson, 1978; Kelly and McKenzie, 1995). Larvae were also found to be chemically defended against predators (Young and Bingham, 1987; Lindquist *et al.*, 1992). This also supports the hypothesis of bacterial vertical transmission from the adults to their larvae (Usher *et al.*, 2005).

Ecteinascidin-743 (also known as Et 743 or Trabectedin) is one of the important characterized ecteinascidins. This bioactive tetrahydroisoquinoline alkaloid showed a high potency as an anti-tumor drug. It was approved for the treatment of breast, prostate, colon, renal, ovarian, pediatric soft tissue sarcomas and melanoma cell lines (Zewail-Foote and Harley, 2001; Rinehart *et al.*, 1990). Trabectedin, marketed under the brand name Yondelis, is currently in phase I clinical trials in the European Union, Russia and South Korea (Cuevas and Francesch, 2009) and phase II clinical trials in the United States (Amant *et al.*, 2009; Schöffski *et al.*, 2008). The biological mechanism of action is not known yet. Et 743 is believed to bind to the minor groove of DNA by alkylation of the N2 of guanine. This binding induces a bend towards the major groove and

mechanistically disrupts the microtubule network which inhibits cell proliferation (Pommier *et al.*, 1996; Moore *et al.*, 1997). Another theory suggested that Et 743 may be involved in the production of superoxide near the DNA strand via an unusual auto-redox reaction which results in DNA backbone cleavage and p53-independent apoptosis of cancer cells (Seaman and Hurley, 1998).

The remarkable structure similarity between the Et 743 and saframycin antibiotics isolated from the bacteria *Pseudomonas fluorescens* (Meyers *et al.*, 1983), *Streptomyces lavendulae* (Arai *et al.*, 1977) and *Myxococcus xanthus* (Irschick *et al.*, 1988) arise the idea of Et 743 has a bacterial origin. Et 743 cyclic peptide structure is similar to the structure of drugs isolated from well-studied sponge-associated bacteria such as jorumycin, isolated from *Jorunna funebris* (Fontana *et al.*, 2000); renieramycins, isolated from the marine sponge *Reniera spp.* (Frincke and Faulkner, 1982) and cribrostatin, isolated from *Cribrochalina spp.* (Pettit *et al.*, 2000). Fig. 1.2 shows the chemical structures of Et 743 and other similar metabolites.

The major problem in the study of this anticancer agent Et 743 is that the large-scale production from their natural source is difficult and costly. Every one gram of this cancer-fighting agent requires collecting and processing of about one metric ton of *E. turbinata* (Proksch *et al.*, 2003). Pharmaceutical industries produce trabectedin through semisynthesis in order to eliminate the need to waste the original natural source (Cuevas and Francesch, 2009).

The study of the microbial community associated with *E. turbinata* from both the Caribbean (Salomon, 2001; Pérez-Matos *et al.*, 2007) and the Mediterranean (Moss and Green, 2003) showed a putative bacterium, *Candidatus* Endoecteinascidia frumentensis, as a persistently associated bacterium, and it was proposed to be the source of Et 743 (Moss and Green, 2003; Pérez-Matos *et al.*, 2007). The success of identifying the bacterial biosynthetic source of Et 743 in this ascidian will help in the cultivation trials as well as identification of the biosynthetic genes, cloning and expression steps.

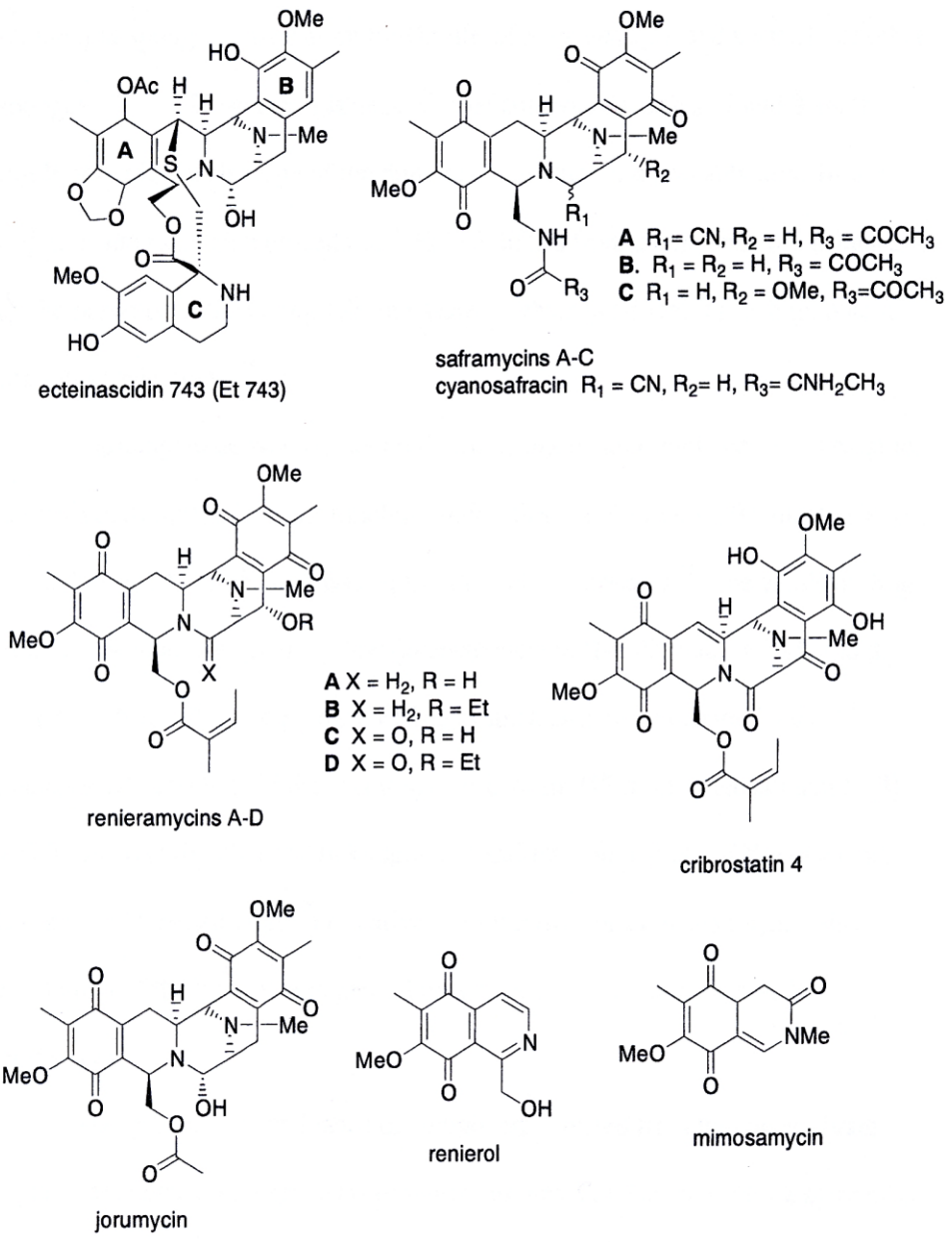

Fig. 1.2 Ecteinscidin-743 and structurally related compounds.

CHAPTER 2
AIM OF RESEARCH

The purpose of the current study is the investigation of the microbial community associated with *E. turbinata* samples collected from different stations in Florida, USA and *Ecteiniscidia spp.* samples collected from Suez Canal and the Egyptian Red Sea. By comparing the different samples, if similar symbiont sequence(s) is found from different places and habitats and found in two closely related species, which produce the Et 743 compound as well, it strengthens the evidence that the organism is a source of the compound and be a targeted for further studies. The conformation of the symbionts species will support the trials for culturing this bacterium in the laboratory using the standard techniques, and identifying the Et 743 biosynthetic genes in the candidate symbiont.

CHAPTER 3
MATERIALS AND METHODS

3.1 Sample Collection

Samples were collected and shipped to Haygood's lab either by the lab members during their visits to the different locations in Egypt and Florida or through collaboration with the Egyptian ecologist group in the marine science department, faculty of science, Suez Canal University, Egypt. Samples were sorted, preserved in RNA*later*, frozen in special coolers and sent back to Haygood's lab in OHSU (USA) until used. Samples of *Ecteinascidia spp.* were undertaken in 2005–2007 period by SCUBA diving or snorkeling from different sites along the Harbor branch oceanographic institution (HBOI) at Fort Pierce, the Red mangrove (*Rhizophora mangle*) at the keys, Florida USA; and the coasts of the Suez Canal and the Egyptian Red Sea waters. The specimens were identified as *Ecteinascidia spp.* based on morphology references (Berrill, 1932; Van Name, 1945). The ecology and distribution of this marine invertebrate in Egyptian water was investigated in another publication by our collaborators (Gab-Alla, 2008). An effort was made to collect the animal in different life stages (e.g., adults with zooid and stolen, larvae, and embryos).

Three different samples from the Egyptian collection were chosen to check the bacterial communities associated with *Ecteinascidia thurstoni*: (a) the metal pilings of jetties at Fayed (Ismalia) on the Bitter Lakes in the Suez Canal coasts, (b) El-Rawisia—north of *Avicennia marina* mangroves—at the Nabq Protected Marine Park; Gulf of Aqaba, and (c) Wadi El-Ra'ada which is more to the south of *Avicennia marina* mangroves at the Red Sea waters. Six different samples from the Florida collections were chosen to be processed. Three samples from Harbor Branch Oceanographic Institution (HBOI), which is located along the Indian River Lagoon in Fort Pierce, and three other samples from the

key West, Florida. Table 3.1 showed the name, habitats, characterization and time of collection of samples collected from the Egypt and Florida. Fig. 3.1 showed the locations of sampling on maps from Egypt and Florida.

Table 3.1

Samples of *Ecteinascidia* Species Collected from Different Sites, Habitats and Collection Times

Sample name	Collection site	Habitat	Time of collection	Type and characterization of sample
Egy-1	Fayed, Ismailia 30° 19´ ‖ 32° 19´	Metal pilings of jetties at Suez canal	May 2005	Adult Zooid*
Egy-2	Sharm El-Sheikh 27° 44´‖ 34° 15´	*A. marina* mangrove (North Red Sea)	June 2005	Adult Zooid
Egy-3	Fayed, Ismailia 30° 19´ ‖ 32° 19´	Metal pilings of jetties at Suez canal	June 2005	Stolon from adult
Egy-4	Wadi El-Ra'da 24° 19´ ‖ 35° 20´	*A. marina* mangrove (South Red Sea)	April 2006	Adult Zooid
SEV43B	HBOI***	Clam docks	Oct 2006	Adult Zooid
SEV43C	HBOI	Clam docks	Oct 2006	Adult Zooid
SEV45C	HBOI	Clam docks	Oct 2006	Juveniles with stolon
SEV43H	HBOI	Clam docks	Oct 2006	Whole animal**
SEV43E	Keys FL	*R. mangle* (Red mangrove)	Feb 2007	Adult Zooid
SEV43F	Keys FL	*R. mangle* (Red mangrove)	Feb 2007	Stolon from adult

* The gonads were carefully removed from adult zooids.
** The whole animal includes the zooids, stolons, gonads and the colony base.
***HBOI is Harbor Branch Oceanographic Institution

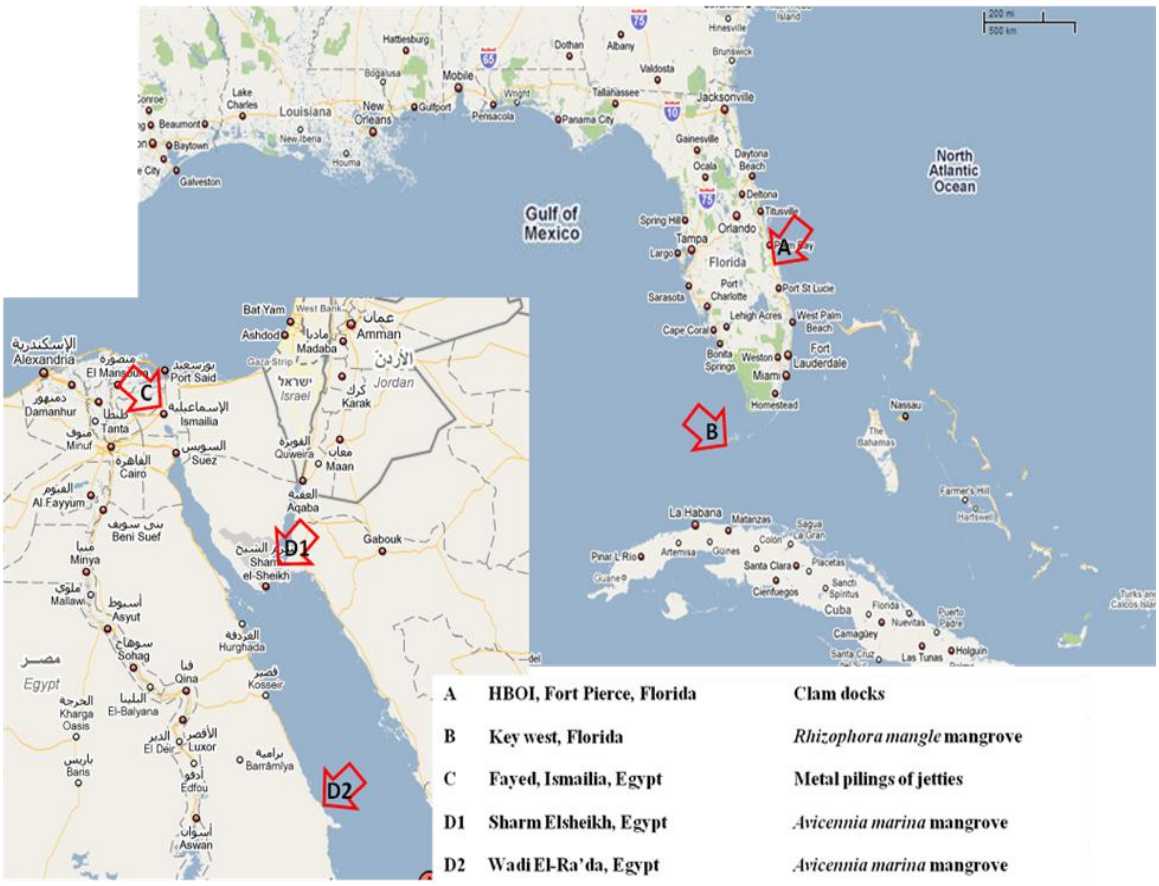

Fig. 3.1 The locations of sampling on maps from Egypt and Florida. Three different samples have been collected from Egypt and two different samples from Florida, USA.

3.2 Genomic DNA Preparation

All DNA manipulations were performed as described by Sambrook *et al.* (1989), with minor modifications. About 300 g (wet weight) of the whole *E. turbinata* was used for the extraction of the genomic DNA and the rest of the sample was preserved for further chemical extraction experiments. Samples were centrifuged and the filtrates were collected in new clean autoclaved tubes. *E. turbinata* tissue from each station was rinsed twice with filter-sterile artificial sea water, frozen for 15–30 s in liquid N_2 and crushed with a sterile mortar and pestle. The whole homogenate was used for total genomic DNA preparation. Lysis buffer (100 mM Tris-HCl, 10 mM EDTA, 37 µM Triton X100, 100 µg RNase A ml^{-1}) was added to the pulverized tissue and allowed to thaw. The

homogenized tissue was immediately transferred to a sterile tube in which Lysozyme (200 µg ml^{-1} final concentration) was then added, and the samples were incubated for 30–60min at 37°C. Proteinase K (400 µg ml^{-1} final concentrations) was added and the suspension was incubated for further 15 min at 70°C. CTAB extraction buffer (1.4N NaCl, 20 mM EDTA, 100 mM Tris-HCl, 3% CTAB, and 1% β-mercaptoethanol) was added to the suspension and sample was further incubated for 30 min at 65°C. DNA was purified with phenol:chloroform extraction and ethanol precipitation. The DNA pellet was air-dried and stored in TE buffer (10 mM Tris-HCl, pH8.0; 1mM EDTA) at –20°C.

3.3 PCR and rRNA Gene Libraries Construction

Universal 16S rRNA bacterial PCR primers (Table 3.2) were used to amplify the total bacterial 16S rRNA gene from the extracted DNA using Gene Amp PCR System 9700 (Applied Biosystems) thermocycler [cycles as follows 94°C for 2 min, followed by 30 cycles of 94°C (30 s), 52°C (30 s) and 72^{o}C (1 min 30 s), and a final elongation step of 72°C for 10 min]. 16S rRNA gene was amplified from several DNA dilutions to obtain optimal results, with the suitable positive and negative controls for each PCR reaction. The PCR product (approximately 1400 bp) was confirmed by 1% agarose gel electrophoreses. For the 18S rRNA gene analysis; a different combination of the previously reported primers (Table 3.2) was used with optimized PCR reactions [cycles of 94°C for 2 min, followed by 30 cycles of 94°C (30 s), 60^{o}C (30 s) and 72°C (1 min 30 s), and a final elongation step of 72°C for 10 min] to amplify almost 1500 bp to represent the 18S rRNA gene. The PCR products were then purified from the agarose gel using QIAEX® II Gel extraction kit (Qiagen) and cloned in TopoTA cloning kit® for sequencing (Invitrogen).

Electrocompetent Top10® *Escherichia coli* cells were used as the host cells as recommended by the manufacturer.

Table 3.2

List of Primers Used in the Construction and Sequencing
of the 18S rRNA and 16S rRNA Libraries

Primer name	Sequence	Reference
Universal 27F (8–27)*	AGAGTTTGATCMTGGCTCAG	Lane, 1990
Universal 1492R (1492–1513)*	TACGGYTACCTTGTTACGACTT	Lane, 1990
18S rRNA-F1**	CTGGTTGATCCTGCCAG	Wada & Satoh, 1994
18S rRNA-F2	AACCTGGTTGATCCTGCCAGT	Yokobori *et al.*, 2006
18S rRNA-F3	GATCCTGCCAGTAGTBATAT	Yokobori *et al.*, 2006
18S rRNA-R1**	CACCTACGGRWACCTTG	Wada & Satoh, 1994
18S rRNA-R2	TCTGCAGGTTCACCTACGG	Yokobori *et al.*, 2006
18S rRNA-R3	TGATCCTTCTGCAGGTTCA	Yokobori *et al.*, 2006
LCO1490***	CATAACAGGAAGAGGTTTAAG	Folmer *et al.*, 1994
HCO2198***	TGTTGGTATAGAATAGGATC	Folmer *et al.*, 1994

* 16S rRNA primers positions are according to *Escherichia coli* (Weisburg *et al.*, 1991).
** The 18S-F1and 18S-R1 primers were as described by Wada and Satoh (1994) with a slight modification to 18S-R1.
***The numbers in the names of primers refers to the corresponding positions of the 5' nucleotide in the annotated mitochondrial genome of *Drosophile yakuba* (Clary and Wolstenholme 1985; GenBank accession: NC001322).

3.4 PCR and *cox*I Gene Llibraries Construction

Mitochondrial DNA (mtDNA) was extracted using the QIAamp DNA Micro Kit (Qiagen). The universal primers LCO1490 and HCO2198, described in Folmer *et al.* (1994), were used to amplify a segment of the *cox*I mitochondrial gene. Amplification was performed in a 25 μL total-reaction volume with: 1.25 μL of each primer (10 μM), 2.5 μL dNTP's (2 mM), 2.5 μL 10× buffer, 2 μL $MgCl_2$, 0.2 μL *Taq* polymerase 5 U, and 1 μL DNA. Using a Gene Amp PCR System 9700 (Applied Biosystems) thermocycler [cycles as follows: 95°C for 5 min, followed by 35 cycles of 95°C (1 min), 55°C (1 min) and 68°C (2 min), and a final elongation step of 72°C for 10 min]. PCR products were purified using the Qiagen PCR purification kit, and cloned in TopoTA

cloning kit® for sequencing (Invitrogen). Electrocompetent Top10® *E. coli* cells were used as the host cells as recommended by the manufacturer.

3.5 Sequencing and Phylogenetic Analysis

Some of the positive clones were randomly selected to check the insert. Plasmid DNA was isolated for DNA sequence analysis according to the manufacturer's instructions (Qiagen spin Mini-preps, USA) and PCR reactions with designating primers confirmed the right inserts. Sequences were primed in the forward direction and checked by reverse-strand sequencing where necessary. All the positive clones carrying the 16S rRNA partial gene were inoculated in 96 well plates, and prepared for sequencing in Washington University sequencing facility. The sequencing reaction was carried out with the BigDye TM terminator v3.1 using the same primers as in the amplification step. Sequences were obtained on an ABI Prism 3100 automated sequencer.

Sequencing data was edited using GENEIOUS program (Biomatters Ltd.). Sequences were trimmed and cleaned and carefully checked—using the online tools VECSCREEN from NCBI and CHIMERA CHECK from the ribosomal database project (RDPII) websites—to ensure that no sequence is contaminated or chimeric. Sequences imported to the ARB-Silva program SSURef 96 database (Pruesse *et al.*, 2007) were multiple-aligned with reference and global sequences.

The analysis was performed on the Phylogeny platform at www.phylogeny.fr (Dereeper *et al.*, 2008). Sequences were aligned with MUSCLE v3.7 (Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). After alignment, ambiguous regions (i.e., containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) (Castresana, 2000) using the following parameters (5 as the minimum length of a block after gap cleaning, positions with a gap in less than 50% of the sequences were selected in the final alignment if they were within an appropriate block, all segments with contiguous nonconserved positions bigger than 8 were rejected, 55% as the minimum number of sequences for a flank position). The phylogenetic tree was reconstructed using

the maximum likelihood method implemented in the PhyML program v3.0 aLRT (Guindon and Gascuel, 2003). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.039) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=0.595). Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates) (Anisimova and Gascuel, 2006). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Chevenet *et al*., 2006).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Conformation of the Sea Squirt *Ecteinascidia* Samples Identity

4.1.1 Using the 18S rRNA gene phylogenetic analysis

Near-complete nucleotide sequences of the 18S rRNA gene (~1600 bp) amplified from the different samples collected in Florida USA, the Egyptian Suez Canal and Red Sea coasts have been analyzed. Fig. 4.1 shows the maximum likelihood phylogenetic tree. The tree shows the aligned 18S rRNA gene sequences of four different samples from the Egyptian Red Sea (EU662243-46), six different samples from Florida Keys (EU662235-37) and HBOI (EU662234, EU662238-39), and larvae sequence (EU662247) with some other different ascidians for identification of the query samples. The recently published sequences of *Ecteinascidia herdmani* (FM244847) and *E. turbinata* (FM244848) (Tsagkogeorga *et al.*, 2009) were used as reference sequences. The tunicate *Crytococcus remirezgomezianus* was used as out group sequence.

All of the eleven examined samples appear in one clade with high sequence identity to different eukaryotic members of Chordata/Tunicata/Ascidiacea. The 97% identity with other ascidians like *Perophora sagamiensis, Chelyosoma siboja* and *Ciona intestinalis* (Miwata *et al.*, 2006) proves the identity of our samples as members of the *Ecteinascidia spp.* The analysis of the 18S rRNA gene sequences failed to show the evolutionary difference between the Egyptian and American samples. As much as the sequences of the Egyptian samples are identical to each other, they did not form a separate clade from Florida samples. The percent identity indices (Table 4.1) showed that the Egyptians samples are ≥97% identical to each other and ≤97% identical to the samples collected from different sites in Florida, USA.

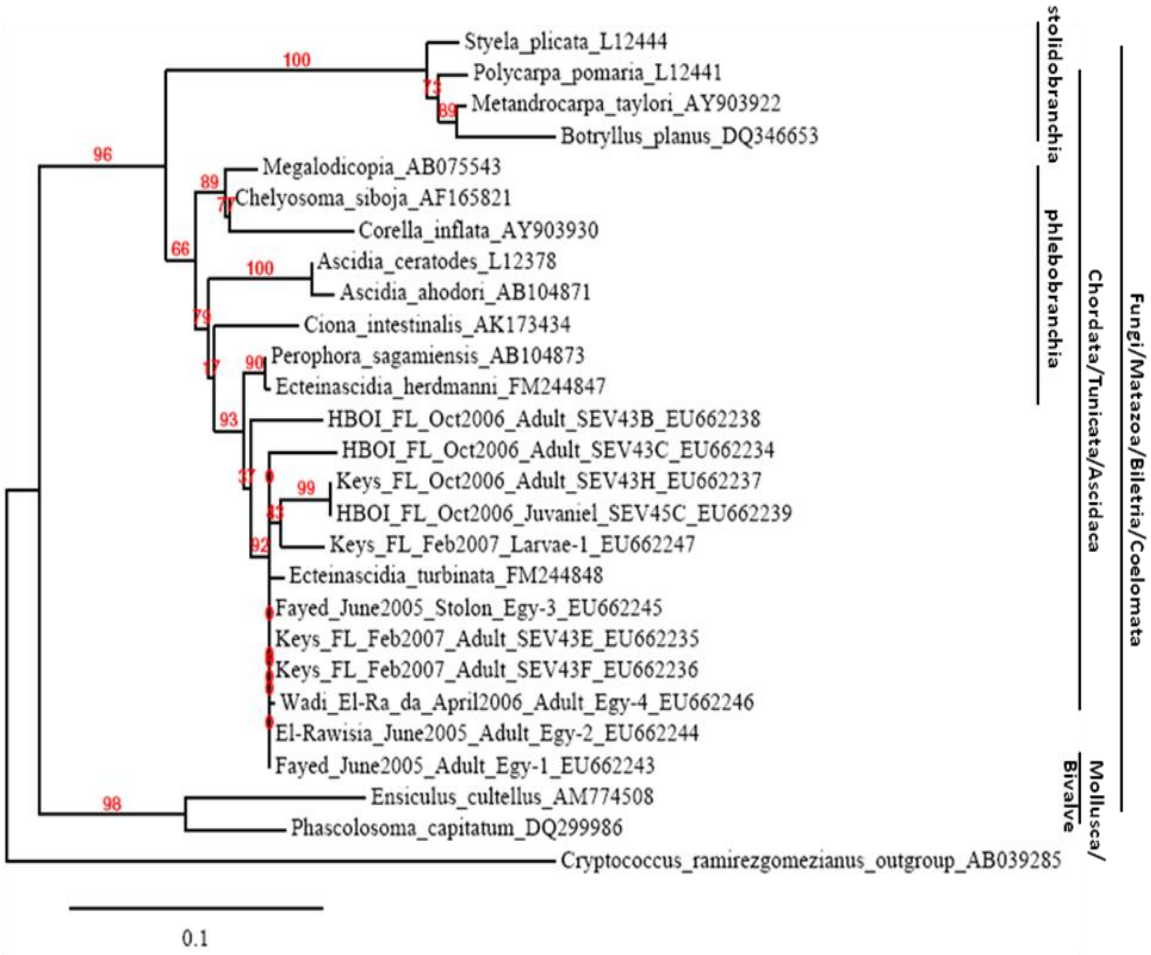

Fig. 4.1 Maximum likelihood Phylogenetic tree showed the aligned 18S rRNA gene sequences of eleven different samples of *Ecteinascidia spp. Crytococcus remirezgomezianus* is used as outgroup. The scale bar represents 0.1 substitutions per nucleotide position. Nucleotide accession numbers are shown alongside each representative organism.

The sequences of Egy (1:4) samples showed 99% identity to *E. turbinata* published sequence (FM244848). These samples were collected and shipped to our lab from Egypt. They were collected from the same studied locations as in our collaborator publication (Gab-Alla, 2008). In their publication they morphologically identified most of their samples as *E. thurstoni*, which is very closely related species to *E. turbinata*.

E. turbinata and *E. thurstoni* morphology are much similar. The only visual difference between both species is that the body wall of *E. thurstoni* is surrounded by a layer of

Table 4.1

Percent Identity Indices for the 18S rRNA Gene Sampled from Different Sites in Florida, USA, and Egypt

Ecteinascidia sp. samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. EU662234 SEV43C	-	93	93	94	90	93	96	93	94	95	89	88	90	91
2. EU662235 SEV43E		-	93	92	93	95	99	93	96	96	93	92	93	94
3. EU662236 SEV43F			-	95	92	95	99	92	97	98	92	92	93	94
4. EU662237 SEV43H				-	95	92	99	95	95	95	94	93	93	93
5. EU662238 SEV43B					-	92	94	94	92	93	91	93	93	93
6. EU662239 SEV45C						-	96	92	95	95	94	91	92	93
7. EU662243 Egy-1							-	99	99	98	97	94	93	97
8. EU662244 Egy-2								-	97	96	94	92	93	94
9. EU662245 Egy-3									-	97	95	93	91	95
10. EU662246 Egy-4										-	97	90	91	93
11. EU662247 Larvae											-	91	92	95
12. AF165821 *helyosoma siboja*												-	96	95
13. AB104873 *Perophora sagamiensis*													-	96
14.AK173434 *Ciona intestinalis*														-

circular muscles which circle the whole body and are absent on the ventral border of the body wall. Also in their publication they mentioned that *E. thurstoni* pooled samples from all over the Egyptian Red Sea and Suez Canal waters were tested and confirmed to produce ET 743, ET 770, ET 729 and ET 759 compounds (Youssef, personal communication). This makes it difficult to distinguish between both species depending on the morphology or bioactivity.

This study revealed that 18S rRNA gene is highly conserved among *Ecteinascidia* species and the sequences cannot be used alone to classify species. As a gene marker, the 18S rRNA gene is not informative for relationships at lower taxonomic levels within this family. It was hard to show a difference among any of the samples.

4.1.2 Using Cytochrome *c* oxidase subunit I (*cox*I) gene phylogenetic analysis

The high degree of nucleotide variability of the mitochondrial gene cytochrome c oxidase subunit I (*cox*I) makes it one of the established tools for tracing evolution, population structure and phylogeography in marine invertebrates (Avise, 2000; Wilke and Davis, 2000; Duran *et al.*, 2004; Reuschel and Schubart, 2005). The sequence of the *cox*I gene from the same eleven DNA samples of *E. turbinata* was analyzed resulting in nucleotide sequences of 600–700 bp in length. Fig. 4.2 shows the maximum likelihood phylogenetic tree. The tree shows the aligned *cox*I gene sequences of four different samples from Suez Canal and the Egyptian Red Sea, six different samples from Florida Keys and HBOI, and larvae sequence with some other different ascidians for identification of the query samples. As reference sequences, *E. herdmani* (AY600967) (Turon and López-Legentil,

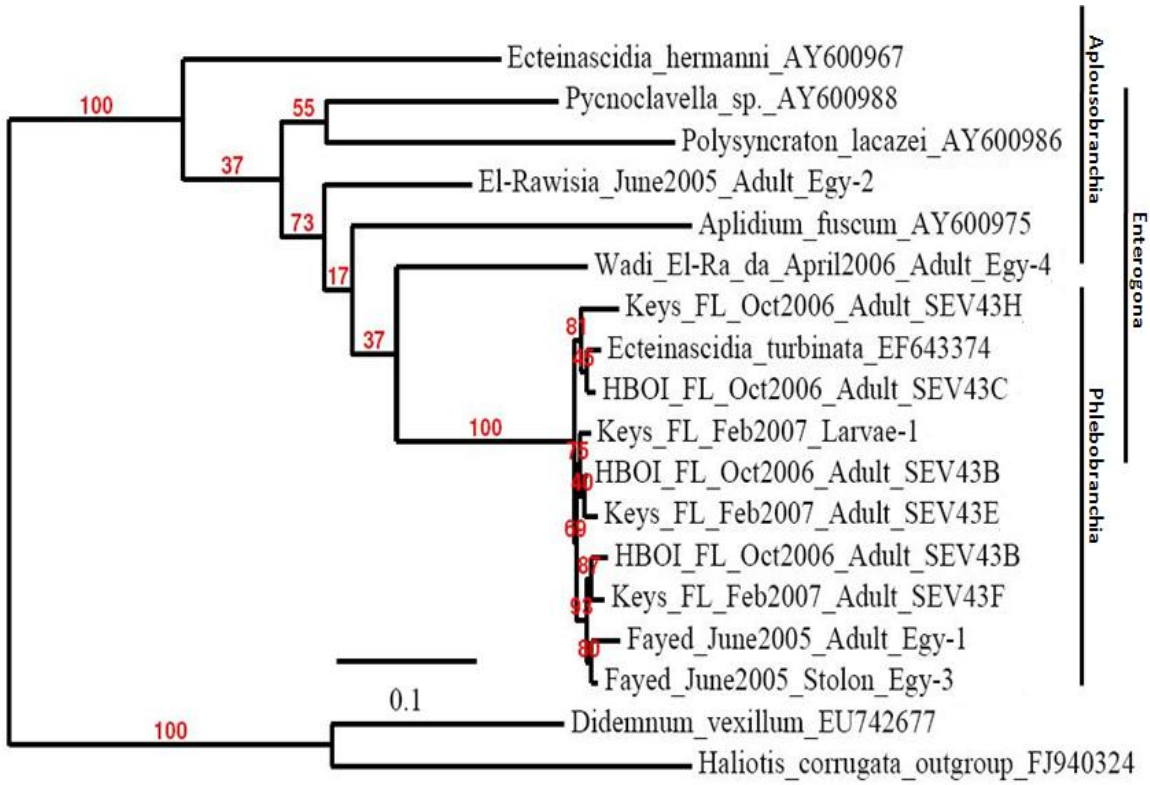

Fig. 4.2 Maximum likelihood Phylogenetic tree showed the aligned mitochondrial *cox*I gene sequences of eleven different samples of *Ecteinascidia spp*. *Haliotis corrugata* is used as outgroup. The scale bar represents 0.1 substitutions per nucleotide position. Nucleotide accession numbers are shown alongside each representative organism.

2004) and *E. turbinata* (EF643374) (Turon *et al.*, 2007) were used as they are the only available sequences for this genus on the databases. The mollusca *Haliotis corrugate* (FJ940324) was used as outgroup sequence. As expected all the six samples from the keys and HBOI Florida and the larvae sequences show 97–99% identity to the reference strain *E. turbinata* (EF643374). This reference sequence was the only haplotype sequence identified from 163 samples of *E. turbinata* collected over the Caribbean regions (Turon *et al.*, 2007). This result comes out with most of the previously reported data from Atlantic, Caribbean and Mediterranean regions (Salomon, 2001; Moss and Green, 2003; Pérez-Matos *et al.*, 2007).

Samples from Fayed (Egy-1 and Egy-3) fall in the same clade with 98% identity to *E. turbinata*. This makes sense as Fayed (Ismalia) is the nearest part of Suez Canal to the Mediterranean Sea and the highest possibility of getting the same ecology as the Mediterranean regions, plus Fayed area has busy ship traffic and marine activities. It is well-known that the standard methods of ascidian introduction include ship fouling or transportation in ballast water of ocean-going vessels. This enhances the idea that the settlement behavior of tunicate larvae in Fayed (Suez Canal) come to be the same as in the other different Mediterranean Sea studied regions.

It was not surprising to see a variation between the Egyptian samples. Samples from Sharm El-Sheikh (Egy-2) and Wadi El-Ra'da (Egy-4) collected from north and south Red Sea mangroves respectively were first morphologically identified by Gaba-Alla group as *E. thurstoni*. In our study, these sequences showed 92% identity to the *E. herdmani* and 73–75% identity to the *E. turbinata*. *E. herdmani* (Lahille, 1887) was first registered as one of the *Ecteinscidian* species in the "Marine species in Europe and a bibliography of guides to their identification" (Costello *et al*., 2001). The lack of the *E. thurstoni cox*I gene sequence in the databases was a barrier for testing these species relationship with different *Ecteinascidia spp*.

Morphological differences between samples collected from Red Sea (Egy-2,-4) and samples collected from Fayed (Suez Canal) Egy-1,-3) were also reported in Gab-Alla's

publication and explained due to the favorable environmental conditions that support *Ecteinascidia* colonies settlement and development (Gab-Alla, 2008). Egy-2 and Egy-4 came from more rural areas in the Red Sea mangroves (*R. mangle*), where the genetic drift or a selective sweep associated with the historical events are the minimum. This will suggest that these species may carry part of the native genotypes that had been disappeared through the fast evolutionary rate the other samples experienced in the Mediterranean Sea.

The failure to use the minor differences between these samples in the morphology and phylogenetic analysis make it difficult to identify the accurate *Ecteinascidia* species. The distinct differences in sequences among the samples suggest the difference in the mangrove populations and opens the suggestion of the presence of more than one species of *Ecteinascidia* in these samples that should be investigated further.

The alignment of both *Ecteinascidia* species showed great genetic variability (about 451 polymorphic sites out of a 616 bp sequence). This comes with the 23.6% nucleotide variation reported by Turon group, which is considered as low resolution to understand the evolutionary developmental history between the *Ecteinascidia* intra-species (Turon *et al.*, 2007).

4.2 Identification and Characterization of the Bacterial Communities in Red Sea Samples

Four different libraries, representing a partial 16S rRNA gene, were constructed from the samples collected from the four different stations in Egypt, Table 3.1. A total of 200 clones out of the four libraries were sequenced with forward and reverse universal primers, Table 3.2. Retrieved sequences from each clone were edited using GENEIOUS software and confirmed with individual BlastN process to the GenBank database. Sequences range from 800 to 1200 bp in length and with good quality was selected. 130 out of 200 sequences were selected for the further analysis. 105 different unique sequences were identified using the DOTUR free software (http://schloss.micro.umass.

edu/software/ dotur.html) (Schloss and Handelsman, 2005). Once the frequency data were assigned to the program, OTUs, rarefaction and collector curves were accurately calculated, Fig. 4.3 and Table 4.2. OTUs were identified from each station as sequences that showed ≥97% similarity to each other based on the ClustalW alignments (Thompson *et al.*, 1994). Each OTU represents a single bacterial species. Table 4.2 shows the number of the sequenced clones, operational taxonomic units (OTUs) and bacterial distribution for the four Egyptian stations. Classification and bacterial distributions in the samples were done online using the "Ribosomal Database Project" from Michigan state university web server http://rdp.cme.msu.edu. The hierarchical taxa is based on a naïve Bayesian rRNA classifier which assign the 16S rRNA gene sequences to the new phylogenetically consistent higher-order bacterial taxonomy proposed by J. M. Garrity (Wang *et al.*, 2007).

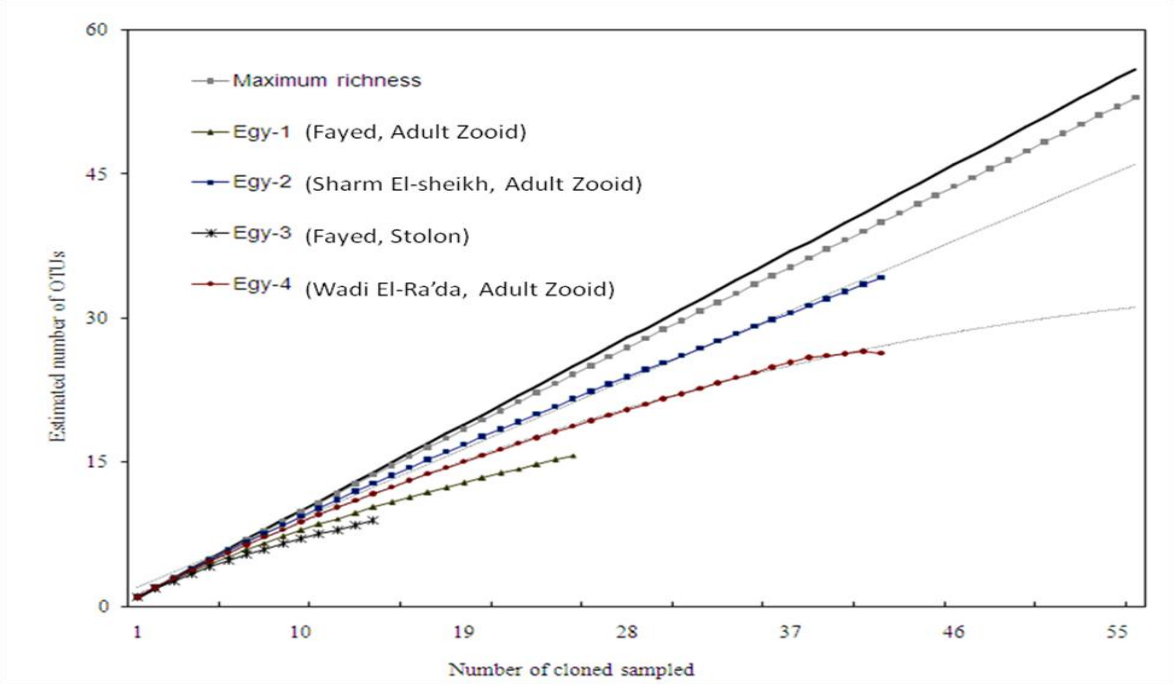

Fig. 4.3 Rarefaction curves of the 16SrRNA gene sequences constructed from the four different stations in Egypt. Clone data consisted of 800–1200bp DNA sequences assembled and aligned to the arb-silva SSURef 96 database. OTUs were defined as sequences at least 97% similar.

Table 4.2

Number of Sequenced Clones, OTUs, and
Bacterial Distribution Percentages per Egyptian Stations

	Egy-1 Adult	Egy-2 Adult	Egy-3 Stolon	Egy-4 Adult
Number of sequenced clones	27	45	14	44
OTUs (# clones)	(10)	(42)	(14)	(39)
Actinobacteria	45.5	8.7	20.0	–
Alphaproteobacteria	–	26.1	–	30.6
Gammaproteobacteria	18.2	19.6	13.3	18.4
Epsilonproteobacteria	–	4.3	–	–
Proteobacteria (unclassified)	–	–	–	4.1
Cyanobacteria	–	2.2	6.7	22.4
Flavobacteria	–	2.2	–	6.1
Halobacteria	–	2.2	–	2.0
Planctomycetacia	–	23.9	–	–
Sphingobacteria	–	4.3	–	–
Verrucomicrobiae	–	2.2	–	–
Bacilli	9.1	–	–	–
Enterobacteriaceae (unclassified)	9.1	–	–	–
Acidobacteria	–	–	–	2.0
Other	18.2	6.5	40.0	14.3

From the rarefaction curves (Fig. 4.3), it is clear that samples from Sharm El-Sheikh (Egy-2) and Wadi El-Ra'da (Egy-4) showed the highest species richness of 42 and 39 OTUs respectively, while the other two samples from Fayed, Ismailia (Egy-1,-3) showed the lowest species richness. Some of the factors that affect the species richness are sampling, area latitudes, productivity and habitat diversity. Gaba-Alla (2008) showed that both the El-Rawisia (Egy-2) and Wadi El-Ra'da (Egy-4) were the most productive sites along the Red Sea coast as regards collection of this tunicate. They reported that the roots of the *Avicennia marina* mangrove support colonization of *Ecteinascidia spp.* and provide the best habitat for this tunicate.

The species richness estimates are dependent on the size of the analyzed clone libraries. To overcome sample size bias in species richness estimates in these complex microbial

communities, a new approach can be followed. This approach calculates the theoretical clone library sizes required to encounter the estimated species richness at various clone library sizes, using the curve fitting to determine the theoretical clone library size required to encounter the "true" species richness, and subsequently determined the corresponding sample size-unbiased species richness value (Youssef and Elshahed, 2008). Table 4.2 and Fig. 4.4 show the classification and bacterial distribution in the Egyptian sample. About 70% of the isolates were identified as different environmental species. These include types of *planctobacteria, Flavobacteria, actinobacteria, chloroflexi* and *cyanobacteria* species. The Adult stolon sample, collected from Fayed (Egy-3), was so difficult to analyze due to the insufficient unique sequences (OTUs) retrieved from this clone library. It needs to be repeated and further investigated, especially it is the only stolon sample we have. The bacterial distribution analysis of Egy-3 showed high percentage (~20%) of *actinobacteria*, 6% of *cyanobacteria* and other unknown species, which are typically the bacterial communities usually associated with a colonies-supporter or stolon and not involved in feeding as Zooids where you can get wide microbial profiles. The adult zooids sample (Egy-1) is related to Egy-3 sample as being sampled from the same area (Suez Canal). Egy-1 showed also a high percentage (~40%) of *actinobacteria* species. The presence of high percentage of *actinomycetes* in Fayed (Suez Canal) is normal due to the shipping and marine activities take place in this important part of the Mediterranean Sea (El-Shatouy *et al.*, 2004). The organic wastes and the remnants of navigation will be a great starter for *actinomycetes, bacilli, enterobacteria*, other bacterial communities in Fayed water. The distribution of the gamma-proteobacteria in the four stations is approximately the same (~18–19%) to what previously reported from the samples collected from the Mediterranean Sea (Moss and Green, 2003).

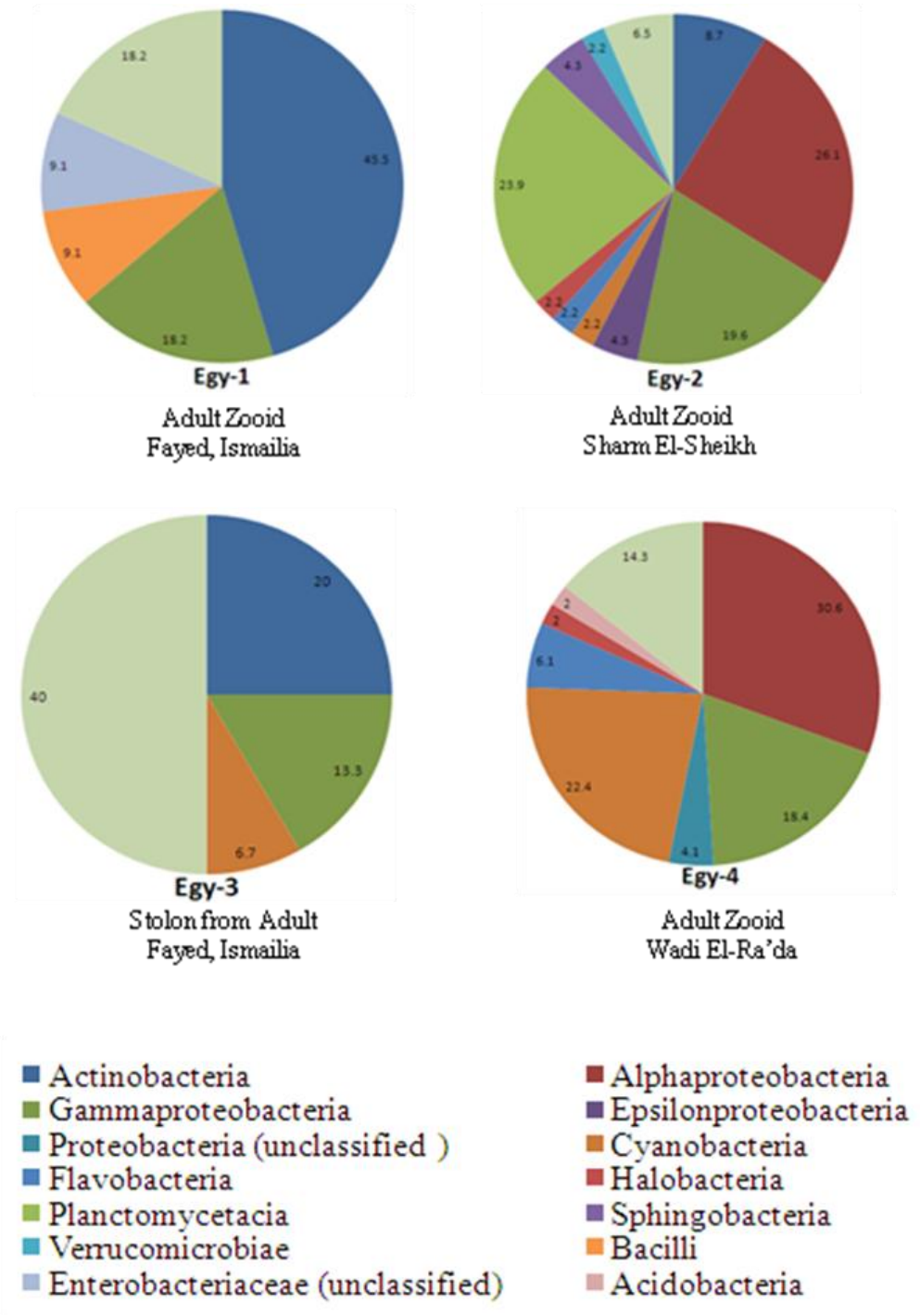

Fig. 4.4 The bacterial distribution and classification in the four Egyptian samples.

4.3 Identification and Characterization of the Bacterial Communities in Florida Samples

Six different libraries were constructed from the samples collected from the keys and Harbor Branch Oceanographic Institution (HBOI), Table 3.1. A total of 437 clones out of the six libraries were sequenced with forward and reverse universal primers, Table 3.2. Retrieved sequences from each clone were edited using GENEIOUS software and confirmed with individual BlastN process to the GenBank database. 303 sequences (900–1100 bp) were selected for the further analysis. One hundred and twenty different unique sequences were identified using the DOTUR free software (Schloss and Handelsman, 2005). Once the frequency data were assigned to the program, OTUs, rarefaction and collector curves were accurately calculated, Fig. 4.5 and Table 4.3. OTUs were identified from each station as sequences that showed ≥97% similarity to each other based on the ClustalW alignments (Thompson *et al.*, 1994). Table 4.3 shows the number of the sequenced clones, operational taxonomic units (OTUs) and bacterial distribution for the six stations in Florida. Classification and bacterial distributions in the samples were done online using the 'Ribosomal Database Project' from Michigan state university web server. The hierarchical taxa is based on a naïve Bayesian rRNA classifier which assign the 16S rRNA gene sequences to the new phylogenetically consistent higher-order bacterial taxonomy proposed by J. M. Garrity (Wang *et al.*, 2007).

Fig. 4.5 shows the rarefaction curves of the different samples collected from Florida station. None of these samples estimate reached the plateau as spices richness indicator. Sampling curves generally rise very quickly at first and then level off towards an asymptote as fewer new species are found per unit of individuals collected. This indicates that these samples need further sequencing and investigation. From the data we have in our hand, we can get a general idea of the bacterial distribution on these samples and linked to the others factors of sampling, ecology and colony productivity which may help in the future sample collection and in understanding the habitat diversity. As shown in Fig. 4.5a, the usage of the whole animal including the stolon, zooids and gonads does not help in extracting the right data. SEV43H rarefaction does not provide an accurate

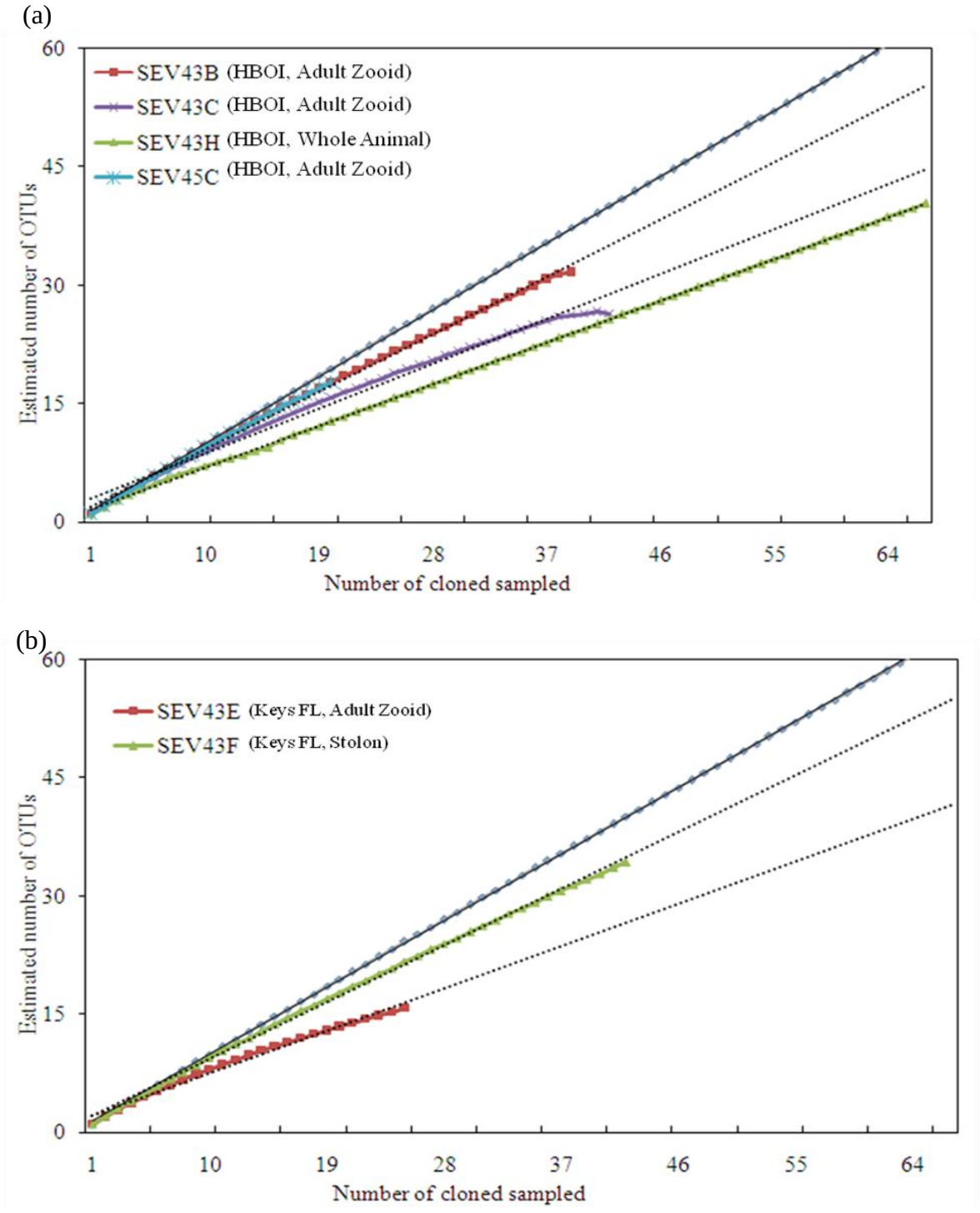

Fig. 4.5 Rarefaction curves of the 16SrRNA gene sequences constructed from the four different samples of HBOI (a), and two different samples of the keys (b). Clone data consisted of 900–1100bp DNA sequences assembled and aligned to the arb-silva (SSURef. 96 database). OTUs were defined as sequences at least 97% similar.

Table 4.3

Number of Sequenced Clones, OTUs,
and Bacterial Distribution Percentages per Station in Florida

	HBOI (OCT2006)				The Keys (FEB2007)	
	SEV43 B Adult-1	SEV43C Adult-2	SEV43H whole animal*	SEV45C Juveniles	SEV43 E Adult	SEV43F Stolon
Number of sequenced clones	39	41	67	47	52	57
OTU (# clones)	(18)	(19)	(35)	(15)	(18)	(15)
Actinobacteria	3.6	7.9	–	7.7	5.8	9.1
Alphaproteobacteria	–	–	10.5	11.5	3.8	9.1
Gammaproteobacteria	57.1	2.6	31.6	38.5	1.9	13.6
Epsilonproteobacteria	–	–	–	–	1.9	–
Deltaproteobacteria	–	–	–	11.5	–	–
Proteobacteria (unclassified)	1.8	–	–	–	–	–
Cyanobacteria	–	–	–	7.7	–	–
Flavobacteria	1.8	–	–	–	–	–
Planctomycetacia	–	–	–	–	7.7	4.5
Bacilli	–	3.9	–	–	–	–
Fusobacteria	–	–	–	3.8	23.1	40.9
Nitrospira	–	–	5.3	–	–	–
Bacteroidetes (unclassified)	–	–	–	–	3.8	4.5
Bacteria (unclassified)	–	–	31.6	7.7	–	9.1
Others	28.6	63.2	15.8	11.5	30.8	9.0

*The whole animal includes the zooids, stolons, gonads and the colony base.

estimate of asymptotic richness in this sample. The usage of the RDP II database web server to calculate and analyze the microbial distribution was so helpful (Maidak *et al.*, 1996). Fig. 4.6 and Table 4.3 show that the abundance of the gammaproteobacteria was so dominant in all of the samples examined with high percentages to the number of clones sequenced. The presence of the *actinomycites* in most of the samples was expected as both collection sites (the keys and HBOI) has recognizable features of the Atlantic Sea with the dense organic waste and high biological activity. Samples from

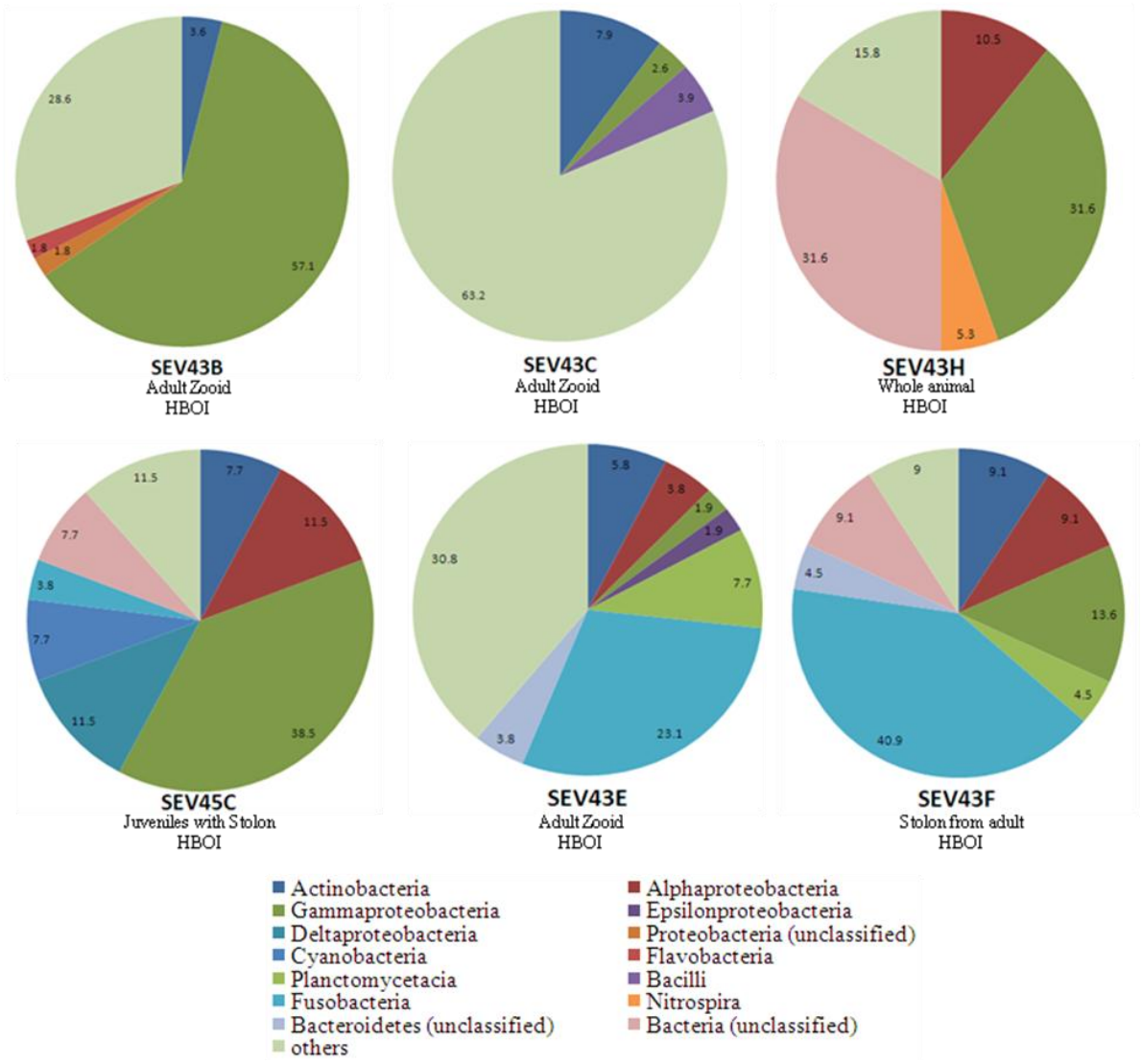

Fig. 4.6 The bacterial distribution and classification in the six Florida samples.

be different. In the Egyptian samples the ratio was 14–20%, while in Florida samples the range was wider from 14–57%. Many factors contribute in the formation of these distribution ratios, for example type of samples, preservation, DNA isolation, sequencing, species richness, dominancy, etc. This makes the results are more subject to different Florida (SEV43E and SEV43F) showed 3.8–4.5% of the population as *Bacteroides*. Moss and Green (2003) previously reported the same species in their samples from

Mediterranean Sea (Formentera, Spain). The presence of some bacteria as sulfur-reducing bacteria or nitrite-oxidizing bacteria (e.g., *Nitrospira*) is very important feature for marine habitats. The significance of these bacterial distribution differences is not biologically clear as it is related to the ecology, season of sampling and the method of preservation, but it reflects the environmental differences.

4.4 Searching *Candidatus* Endoecteinascidia frumentensis Sequence from the Gamma-Proteobacteria Pool

In order to get a more precise picture of the microbial communities in the different samples tested from different sites in the Mediterranean Sea and Atlantic Ocean, 16S clone libraries were constructed, screened, and positive clones were sequenced (see Material and Methods). Gammaproteobacteria distributions in each sample were found to interpretations and requires further investigation binding all the factors of the area ecology, host productivity, DNA isolation methods and microbial identification together.

Relating the gammaproteobacteria from each station to the reference sequences was a good method to identify the presence and/or the absence of the *Candidates* Endoecteinascidia *frumentensis*. *Endoecteinascidia frumentensis* (AY054370) was reported to be the most abundant bacteria associated with the *E. turbinata* in both adult and larvae tissues (Salomon, 2001; Moss and Green, 2003). The observation of related bacterial strains with similar levels of dominance in different samples from different sites suggests that these microbes are consistent and have important features to the host. By screening all the 16S clone libraries to find the much identical isolates to the *E. frumentensis*, 46 isolates showed ≥ 97% identical sequences.

From the Egyptian samples, Egy-4 sample collected from the mangroves, south of the Red Sea (Wadi El-Ra'da site) was the most well sequenced samples, and its bacterial distribution can be trusted as shown on the rarefaction curve (Fig. 4.3). 15–18% of the sequenced clones showed 98–99% identical sequence to the *E. frumentensis*. The other Egyptian samples showed the presence of related sequences (≤ 97% identical), but with

lower abundance level (< 3%). This can be related to the poor species richness in these samples as shown by the rarefaction curves and OTUs calculations (Fig. 4.3 and Table 4.2) and recommend more sample collection, metagenomics DNA isolation and sequencing to get the real estimate of species richness.

From the Florida samples, SEV43B and SEV43H samples collected from HBOI showed a similar percentage of abundance of identical sequences. 50% of the gamma-proteobacteria identified in SEV43B sample showed 98–99% identical sequence to the *E. frumentensis* (AY054370). In SEV43H sample, 15% of the gamma-proteobacteria showed similar identity. Other samples showed fewer ratios of abundance and identity to the reference sequence. To overcome this problem, two other references were included. DQ482575 and DQ494516 were identified as persistently associated bacteria from *E. turbinata* samples collected from different sites in the Caribbean Sea. These uncultured gamma-preteobacteria [clone 1–3h (DQ482575) and clone 2–24h (DQ494516)] showed >98% homology to *E. frumentensis* (Pérez-Matos *et al.*, 2007).

The recalculation of the neighbor-joining relations of all the gamma-proteobacteria from different samples and any of these references gave the same results of relation and identity. Therefore, to make it more visual, only the highly related sequences (≥ 98% identical sequences) were used to construct the maximum likelihood phylogenetic tree (Fig. 4.7). Fig. 4.7 show the phylogenetic relationship of these OTUs with other species from different families (e.g., *Enterobacteriacea, Ocenobacter, Pseudomonales, Thiotrichales* and *Legionellales*) based on the information available in the GenBank.

The majority of the clones fell within the same clade with the reference sequences (16 clones: 50% of the total clones studied). 9 clones isolated from the adult zooids of *E. turbinata* from the HBOI collection were 99% identical to E. *frumentensis* sequences. These clones represent 35% of the total gamma-proteobacteria isolated from this sample. From Florida the keys collection only two sequences showed > 98% identical sequence to the other two references. From Egyptian collections; Egy-4 samples collected from Wadi El-Ra'da showed the 99% identical sequences to the references, which is more than 35%

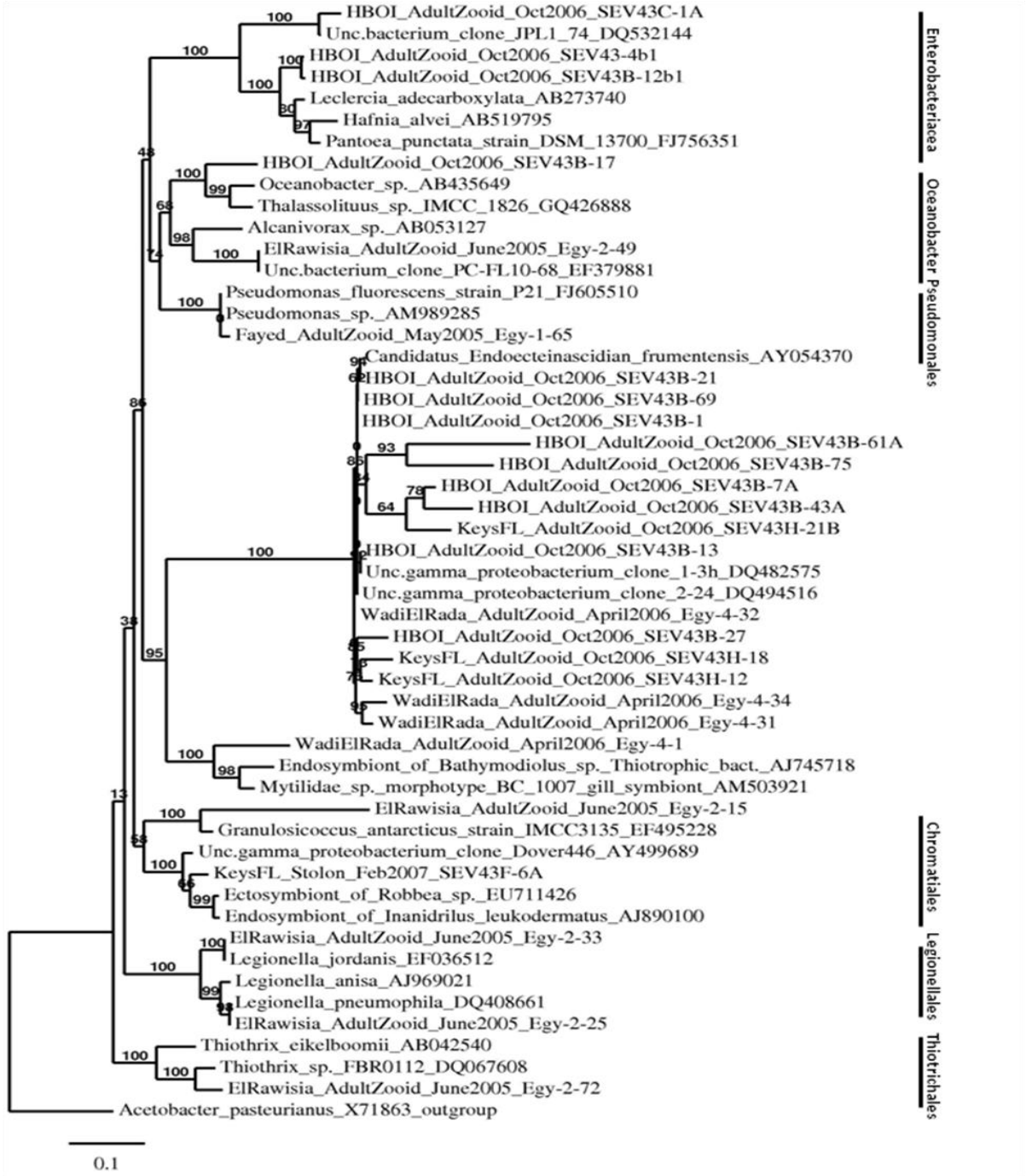

Fig. 4.7 Neighbor-joining analysis of partial 16SrDNA gene sequences of OTUs from the Egyptian and Florida samples. An alpha-proteobacterium, *Acetobacter pasteurians*, is used as the outgroup. The values (>50%) at the nodes show the bootstrap support, based on the neighbor-joining analysis. The scale bar represents 0.1 substitutions per nucleotide position. Nucleotide accession numbers are shown alongside each representative organism.

similarities with known endosymbionts of other species (e.g., AJ745718, EU711426, and AJ890100).

The rest of the clones showed as: 3 clones were closely related to *Enterobacteriacae*, 2 clones closely match *Oceanobacter*, one clone with *Pseudomonas spp.* ElRawasia-isolated 16S rRNA sequences (Egy-2-25, Egy-2-33) showed homology with the pathogenic *Legionella* species (Vinh *et al.*, 2007), while Egy-2-72 showed similarity to the sulfide oxidizing *Thiothrix spp.* (Cytryn *et al.*, 2006). A number of clones showed similarities with known endosymbionts of other species (e.g., AJ745718, EU711426, and AJ890100).

Using ClustalW as a multiple alignment tool, the specific primers for all the 16 identical clones with the reference sequences were computed (Fig. 4.8 and Table 4.4). The multiple alignment showed a hyper-variable regions that makes the usage of only one primer set as specific primers to *E. frumentensis* is not accurate. Different sets of primers were tested to confirm the region that can be amplified from most of the clones. Only the primer set (ET-7 FWD / ET-451 Rev) amplified the right amplicon size with the tested 27 clones. The other primers combination showed non-specific amplification even with optimized PCR conditions. Genomic DNA from *E. coli* and *Bacillus subtilis* were used as negative controls. Larvae and Flatworm DNA was also tested for the presence of this persistent associated bacterium. Fig. 4.9 show the agarose gel electrophoresis of the PCR amplification of the DNA isolated from the most promising sites to harbor the *E. frumentensis*-related bacteria. PCR signals were observed in the Egyptian samples (Egy-1, Egy-2 and Egy-4), Florida samples (SEV43B, SEV43H and SEV43F), the larvae-1 and Flat worm samples. The 16 clones showed by the phylogenetic analysis to be related to the reference sequences amplified the right amplicon size as expected (452bp). The interesting result was that some of the far-related isolates (e.g., SEV43B-4 and Egy-2-72) also amplified the right amplicon size, which can be explained as a high predominance of *E. frumentensis* in these samples, or unlikely amplification bias. Further trials are required for more accurate investigation. The weak signal from the stolon sample

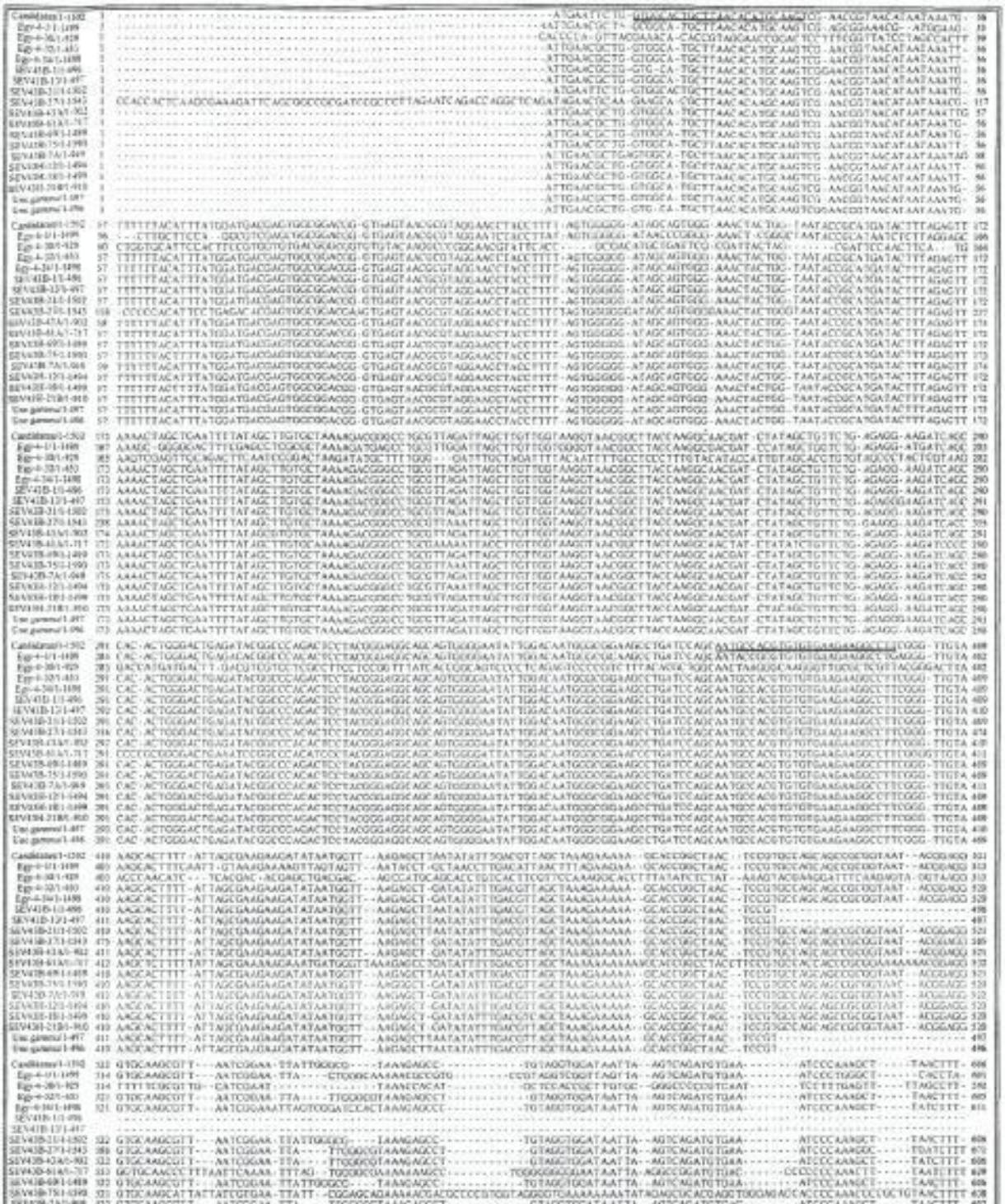

Fig. 4.8 ClustalW multiple alignments of 16 identical clones with the reference sequences *Candidatus* E. frumentensis and two uncultured gammaproteobacteria (2–24 and 1–3 h). ET-7 FWD / ET-542 Rev primer set is underlined.

Table 4.4

16S rRNA Gene Specific Primers Generated from the Consensus of the Multiple Alignments of the Highly Related Sequences and the Reference Sequences

Primers name	Primers sequence (5´–3´)
ET-7* Fwd	CTGGTGGCACTGCTTAACACA
ET-154 Fwd	GCATGATACTTTAGAGTTAAAAC
ET-728 Fwd	AACTGACACTGAGAGGCGAAA
ET-451 Rev	TTCACACACGTGGCATTGCT
ET-472 Rev	AATATATTTGACGTTAGCTAA
ET-1030 Rev	TGCATGGCTGTCGTCAGCTCGTGTCGTGA

*The numbers in the names of primers refers to the corresponding positions of the 5´ nucleotide in the annotated 16SrRNA gene *E. coli*.

(SEV43F-6) collected from the keys Florida may form the idea of bacterial localization in the zooids and partially on the stolons.

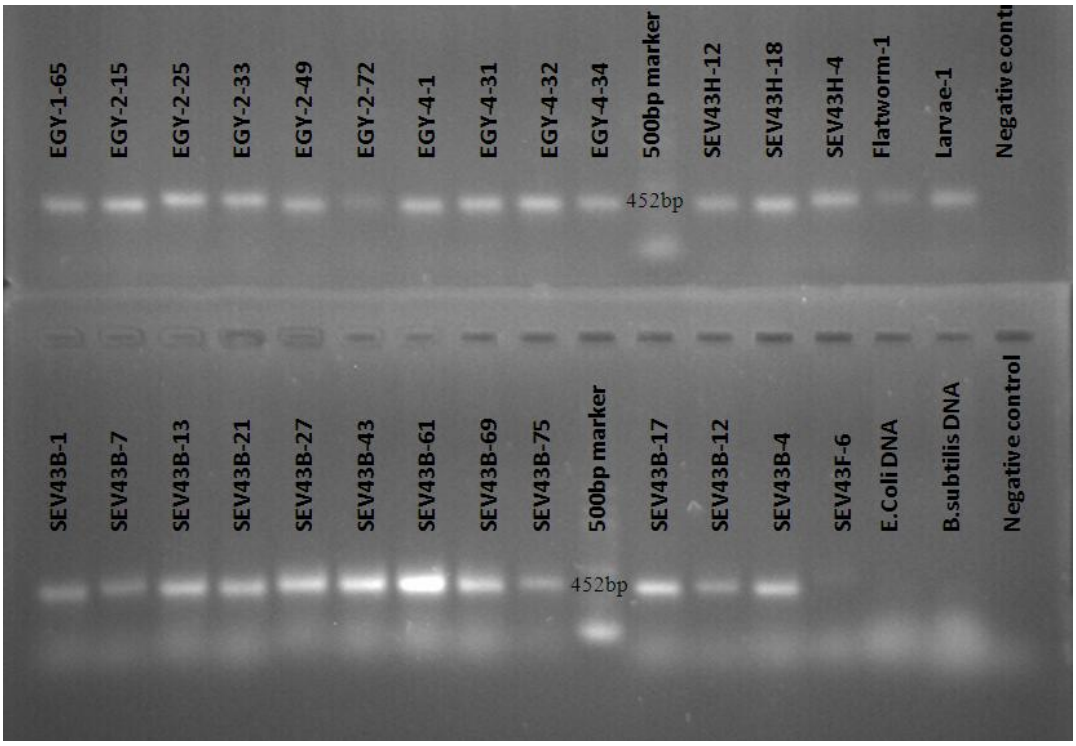

Fig. 4.9 PCR amplification of *E. frumentensis* bacterium using the primer set (ET-7 FWD / ET-451 Rev) with 27 tested clones. *E. coli*, *B. subtilis* DNA and no DNA samples were used as negative controls. 500bp marker was used.

4.5 *E. turbinata* Larvae and the Predator Flatworm May Have Microbial Communities

The most reliable method to study microbial associations and symbionts relationship with complex invertebrates is to focus on the non-feeding larvae. *E. turbinata* larvae do not have the feeding apparatus. It swims for a short time after release to the water column and usually does not filter feed before settle on a surface and initiating colonization. Many invertebrates associated with obligate symbionts are known to transmit bacteria vertically to their offspring; therefore any bacteria that are consistently and specifically associated with larvae are presumed to be true symbionts (Krueger *et al.*, 1996; Benayahu

and Schleyer, 1998; Hirose, 2000). Localization studies showed a vertical transmission process of the associated bacteria from adults to the larvae just before they leave the siphon. The exact mechanism of transfer of bacteria from adult to oocyte or embryo is not yet known (Moss and Green, 2003).

It was hard for our group to collect a reasonable amount and number of larvae to start this study as the collection process usually takes place manually in certain seasons of the year. Adult *E. turbinata* were shipped from Keys FL as wild colonies and maintained in static aquaria at 28^{o}C for up to 2 weeks. Larvae were either released from the colonies in the morning and individually collected or excised from adult individuals with a scalpel. The presence of sufficient larvae DNA samples from different location all over Red Sea and Florida sites will help to study the conservatively of the gene markers tested in most of the previous studies.

As much as the phylogenetic analysis of the larvae sequences showed 95% identical 18S rRNA gene (Fig. 4.1) and 94% identical *cox*I gene (Fig. 4.2) with the corresponding genes of the *E. turbinata,* the lack of enough DNA source suppressed this step for the time being. Thus I started looking to the microbial profiles on the adult zooids and stolen samples that were tested to produce Et 743 (unpublished data), hoping to identify the most abundant bacteria and investigating other dominant species that are not vertically transmitted to the offspring and may related to the Et 743 synthesis.

Fig. 4.10 show a gradient PCR amplification of the genomic DNA isolated from the Larvae-1 sample. The universal 27Fwd/ 1492 Rev primer set were used to identify 16S rRNA in the larvae-1. The success of the amplification of nearly the whole 16S rRNA gene was a good chance to study the microbial profile within this sample. The lack of enough DNA amount for further downstream processes suppress this step.

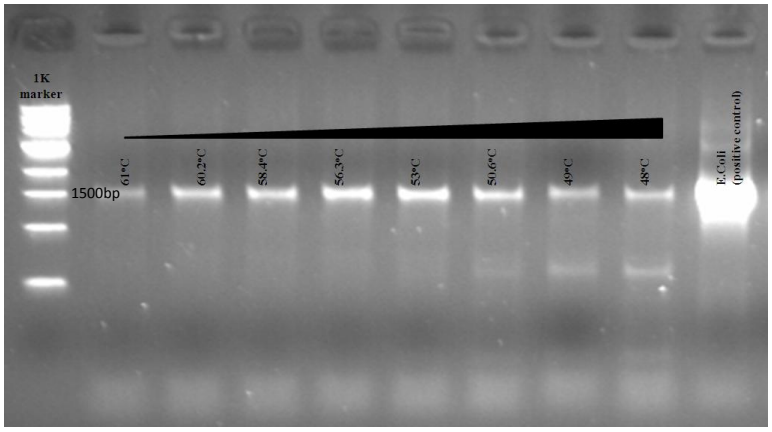

Fig. 4.10 Gradient PCR amplification of the genomic DNA isolated from the Larvae-1 sample. Floating larvae was collected from the shipment received from the keys Florida site. The universal 27Fwd/ 1492 Rev primer set were used to identify 16S rRNA in the larvae-1. *E. coli* DNA was used as positive control.

The tiger flatworm *Pseudoceros crozieri* is the only known and well studied predator for the orange tunicate *E. turbinata*. It inserts its pharynx into the zooid of the tunicate and secretes proteases for external digestion, then sucks the digested parts (Newman *et al.*, 2000). The flatworm was found to live on these ascidians in most tropical waters in Florida, the Caribbean and the Mediterranean Sea. The only economic importance of this flatworm is that it is able to provide substantial amounts of Et 743 (Proksch *et al.*, 2003). One hypothesis is that the flatworm is able to harbor the same Et 743-producing bacteria. This hypothesis is doubtful as the amount of Et 743 extracted from one flatworm was equally extracted from 1kg of tunicate. The other hypothesis is that one individual is able to consume an average of 19 zooids in 24 hours and accumulate the Et 743 compound in it body (Newman *et al.*, 2000). Both hypotheses need further investigation.

Fig. 4.11 show the trials to amplify the 16S rRNA gene from the genomic DNA sample extracted from flatworm sample. Universal primer sets (27Fwd / 1492Rev) and (27Fwd / 907Rev) did not show strong signals compared to the ones from the primer set (515Fwd / 1492Rev). This suggests the presence of bacteria in the flatworm DNA but cannot prove the presence of the *Candidatus* E. frumentensis.

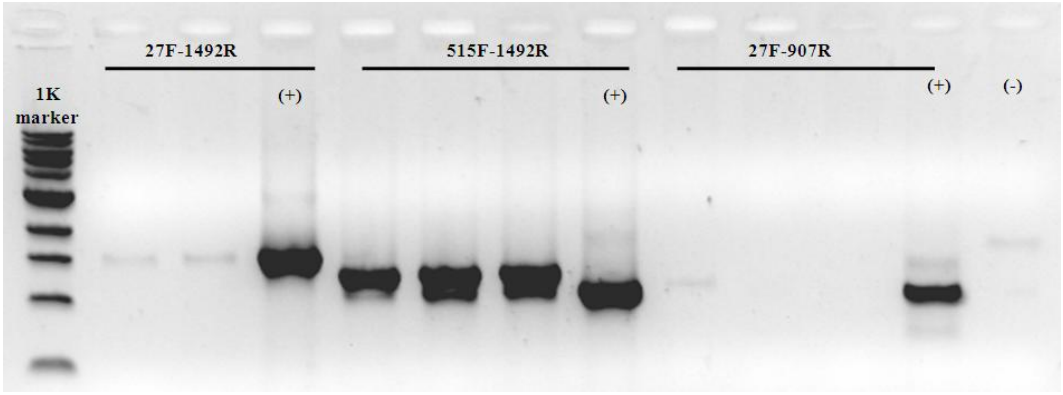

Fig. 4.11 PCR amplification of the 16S rRNA gene from flatworm genomic DNA sample using different universal primers. *E. coli* DNA was used as positive control.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

The study of the *E. turbinata* bacterial communities and trying to understand and identify the persistently associated bacteria that is proposed to be the source of Et 743 is still in its infancy. These types of studies must link all the environmental, biological, and genetic analysis together. The screening, sampling and processing of *Ecteinascidia spp.* must be done under the prospective well-studied protocols and procedures.

The differentiation between the different *Ecteniscidia spp.* depending on the morphology of the animal is not enough. They are highly related in their structures, behavior, pigmentation, biological activities and ecology. Thus the usage of suitable gene markers to differentiate between these closely related species is an established fact. The 18S rRNA gene sequences analysis was good enough to provide a clear view of the evolution of the major tunicate linage (Tsagkogeorga *et al.*, 2009), but in this study it appeared less informative for relationships at lower taxonomic levels. 18S rRNA gene was too conservative in all of the samples tested from Red Sea and Florida to show the minor genetic evolution between the *E. turbinata*, *E. thurstoni* and *E. herdmani*, in addition the lack of enough data about these species in the GenBank made it difficult to study the traditional phylogenetic relationships.

Turon's group in the University Du Barcelona, Spain spent more time to improve the phylogenetic frame work for the tunicate evolution by a wider taxonomic sampling than in the previous studies. They aimed to get more representation of major tunicate lineages and subdividing the previously odd long branches. They clarified that the 18S rRNA gene provided a clear view of the evolution of major groups, but appeared less supportive to several nodes in the reconstructed trees, even by using different reconstruction

approaches. In their recent research, they used more ribosomal markers, a non-parametric CAT mixture model and RNA-specific paired-site substitution models. These models allowed them to accurately account for site-specific heterogeneity of the evolutionary process and the occurrence of compensatory substitutions in the RNA stem regions by letting patterns of uniform substitution at different sites. Their results supported the division of the tunicates into three major clades, but failed to firmly resolve the positions of the species that have extremely high rates of changes in the secondary structure of their 18S rRNA, for example Aplousobranchia species (Tsagkogeorga *et al.*, 2009).

The analysis of the mtDNA (*cox*I gene) sequences showed the six samples from Florida (HBOI and the Keys) and the larvae sequences to be 99% identical to *E. turbinata*. Turon's group reported the lack of genetic variation in *cox*I sequences over the studied ascidian *E. turbinata* samples from the Mediterranean, Caribbean and Atlantic. They hypothesized that this gene may be unusually conserved in these species, or that there is a very slow evolutionary rate of *cox*I gene in *E. turbinata* which may be due to a favorable and widely spread mutation that spread and inherited in the mitochondrial DNA (mtDNA) or even due to the presence of a mitochondrial DNA mismatch-repair system (mtMSH gene) that keep this *cox*I gene highly conserved (Tsagkogeorga *et al.*, 2009). Samples from Red Sea and Fayed showed some variations. Samples from Fayed (Egy-1 and Egy-3) fall in the same clade with 98% identity to *E. turbinata*, while samples from Sharm El-Sheikh (Egy-2) and Wadi El-Ra'da (Egy-4) showed 92% identity to the *E. herdmani*. Samples Egy-2 and Egy-4 showed a remarkable nucleotide sequence variations (Fig. 4.2). They showed 86% identity to each other and 73–75% identity to the *E. turbinata*. This result suggested that there are two different species. The lack of the *E. thurstoni cox*I gene sequence in the databases was a barrier for computing these samples relationships. Thus our data suggested the presence of three putative species of *Ecteinascidia*; *E. turbinata* in Florida samples and Fayed (Suez Canal), and two other species in the other Egyptian samples, one of them may be *E. thurstoni* and/or the samples were a mixture of both species *E. herdmani* and *E. thurstoni*. The locations where these samples were collected in the Red Sea mangroves (*R. mangle*) were

considered as rural areas. This suggests that these samples may carry part of the native genotypes that had been disappeared through the fast evolutionary rate the other samples experienced in the Mediterranean regions.

Since it is hard to accurately differentiate between the closely related *Ecteinascidia* species depending on one aspect; either genetically or morphologically, it is important to develop more new phylogenetic markers for higher identification aspects, testing other genes (e.g., *hsp*70) is highly recommended for further study of tunicate evolutionary history. The study of the 28S rRNA gene sequences which is already available for some species is also a powerful tool of analysis if used in a combination with the 18S rRNA gene analysis.

The DNA isolation method, primers specificity and sequencing quality were critical factors in this study. Some of the samples processed did not show accurate or believable microbial distributions. The distribution of the gamma-proteobacteria in the most of the samples was much approximately the same (~18–19%) to what previously reported from the samples collected from the Mediterranean Sea (Moss and Green, 2003). From the rarefaction, OTUs and RDPII database analysis; three samples out of ten showed the highest species richness estimates. For future work, it is recommended to use more accurate "Species Richness eco-tool EstimateS", that implements a variety of analyses depending on a "species by sample" matrix analysis type (Colwell, 2009). Rarefaction is known to be unrealistic in its assumption of random spatial distribution of individuals, does not account for specific taxa and does not recognize species abundance, only species richness.

In this study I was not successful to find *Endoecteinascidia frumentensis*-like sequences in all the clone libraries studied. This may because the coverage was not extensive and they have been missed. Alternatively, these bacteria could be present in numbers below the detection levels of PCR amplification of mixed populations with a universal primer. *E. frumentensis*-like sequence was found in three samples: Egy-4 sample collected from the mangroves south of the Red Sea (Wadi El-Ra'da site), SEV43B and SEV43H

samples collected from HBOI site. 18% of the sequenced clones from each of these samples showed 99% identical sequence to the *Candidatus* E. frumentensis. Moss and Green (2003) identified the uncultured bacterium clones (2–24) and (1–3h) as *Candidatus* E. frumentensis. These two sequences were used as reference sequences and showed an identity of greater that 99% to most of the Egy-4, SEV43B and SEV43H clones.

The most interesting finding in this study is that it was not known for *E. frumentensis* to exist outside *E. turbinata*. The PCR amplification of the 16S rRNA gene using the specific primer set (ET-7 FWD / ET-451 Rev) from Egy-4 samples (Wadi El-Ra'da site) is novel results. Wadi El-Ra'da samples were first identified by our collaborators as *E. thurstoni*, and the *cox*I gene sequence analysis showed this sample to be 92% identical to the *E. herdmani*. This is consistent with the finding of Et 743 in *E. thurstoni* in Thailand (Chavenich *et al.*, 2009) and in the pooled samples from the Egyptian coasts (Youssef, personal communication). The high predominance of this endosymbiotic strain in three different tissues from different sampling sites cannot be unlikely explained by PCR bias. As yet we cannot assign *E. frumentensis* as the source of Et 743 compound as we are lacking these data, but we showed *E. frumentensis* sequence to be predominant in different *Ecteinascidia spp*., collected from different sampling sites in the Mediterranean, Caribbean and Atlantic.

The study of the non-feeding *Ecteinascidia* larvae is more reliable to come over the number of complications working with the filter feeding adults and the presence of large numbers of dietary-derived bacteria within the tunicate gut. As much as the biological roles of the Ecteinascidin compounds are unknown, the vertical transmission of these agents and their sources has been previously confirmed by localization studies (Moss and Green, 2003). The 16S rRNA gene specific primers (ET-7 Fwd / ET-542 Rev) designed during this study succeed to show the presence of the *E. frumentensis*-like sequence in both the Larvae and the predator Flatworm (Fig. 4.9). This can be a strong evidence for the presence of a significant population of bacterial symbionts in the ascidian larvae, and as the most putative candidate for the production of Et 743. The presence of *E. frumentensis*-like sequence on the flat worm sample is unknown, but it does not exclude

the possibility of *E. frumentensis* to be a potentially endosymbiotic bacteria to this animal.

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BIOGRAPHICAL SKETCH

Mohamed Ahmed Elbaz was born in Dumiatt, Egypt on April 1, 1976. He earned his Bachelor of Science in Biochemistry from Mansoura University, Egypt. In 2000–2003, he earned a Diploma in Bioscience and Technology (Biotechnology) and then a Master of Bioscience and Technology degree (Biotechnology) from the Institute of Graduate Studies and Research (IGSR)—Alexandria University, Egypt. In 2006–2009, he was awarded a Ford Foundation International Fellowship through which he joined the OHSU Department of Science & Engineering as a graduate student in the research groups of Drs. Michiko Nakano and Margo Haygood.

Professionally Mr. Elbaz was employed as a Lecturer at Mansoura University from 1998–99; as a Technical Chief Biochemistry at Delta Electricity Company from 1999–2000; as a Research Associate at Alexandria University from 2000–2003; as a Genetic Engineering Instructor and Program Manager at the Center for Biotechnology, Scientific Informatics Research Academy (SIRA) from 2004–2005; and as a Production Supervisor with Rhein-Minapharm (Biogenetics) Pharmaceutics from 2005–2006.

LIST OF PUBLICATIONS

1. Yukl, E.T., **Elbaz, M.A**., Nakano, M.M., and Moënne-Loccoz, P. (2008) Transcription factor NsrR from *Bacillus subtilis* senses nitric oxide with a 4Fe-4S cluster. *Biochemistry* 47(49), 13084–13092.

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