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Molecular Phylogeny of Egyptian Sea Cucumbers As Predicted From 16s Mitochondrial rRNA Gene Sequences

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Abstarct: A molecular phylogenetic analysis of some Holothuroidea was undertaken in order to clarify the systematics and taxonomy of this class in the red sea. DNA sequence for portions of 16S mitochondrial genes was obtained from 4 species of sea cucumbers. The resulting molecular phylogeny using maximum likelihood methods of the Holothuroidea conflicting existing taxonomy, which is based largely on the morphology of calcareous parts. In particular, evolutionary relationships among the brooding species were clarified. The current results confirmed that the species *Holothuria fuscogilva*, *Actinopyga mauritiana*, *Holothuria nobilis* and Sea cucumber leaves , were identical to *Cucumaria frondosa*, *Pentacta pygmaea*, and *Cucumaria pseudocurata Psolus fabricii* , respectively with Query coverage 100%, Zero E- value and Max identical 100%. It was suggested that an in-depth phylogenetic analysis of sea cucumber families will require examination of additional, more slowly evolving, regions of the genome(s).

Key words: Sea cucumber · 16s Mitochondrial rDNA · Sequence · Red Sea

INTRODUCTION

Sea cucumber belongs to phylum Echinodermata. This soft-bodied marine-dwelling echinoderm from class Holothuroidea is unique due to the existence of evolved skeleton (i.e. ossicles or spicules) and ancient-looking respiratory system called respiratory tree possessed by few species. However, the systematic of sea cucumbers based on morphology particularly in Egypt is still unclear, thus, it requires molecular methods as alternatives to address the problem [1].

According to Kerr *et al*. [2], the molecular phylogeny of sea cucumbers inferred from 16S mitochondrial rRNA gene sequences summarizes that genus *Actinopyga* and *Bohadschia* are each monophyletic and *Pearsonothuria* is a sister species to *Bohadschia*. The phylogeny of *Holothuria* due to unexpected grouping of *Holothuria excellens* out of *Holothuria* genus subsequently suggests that in few cases molecular phylogeny might not be in accord with the existing taxonomy of sea cucumbers as well as the evolutionary background speculated previously as well. However, from another aspect, such unclear taxonomic status from species level up to the highest level in linear classification leads to the suggestion and requirement for an alternative taxonomic scheme to replace the present classification [3].

There are few characteristics of mitochondrial DNA (mtDNA) that make this genetic component the most preferred model in molecular genetics ecology such as effective maternal inheritance, apparent haploid genome, non-recombination, continuous replication and the rate of substitution in mtDNA is within the range of 5 to 10 times greater than in 'single-copy' nuclear DNA [4, 5]. Phylogenetic inference from 16S mitochondrial rRNA gene shown by previous studies suggests the ability of such gene to support the relationship between morphology and genetics.

The previously unclear and problematic identification of sea cucumber species as well as the not up-to-date and incomplete documentation on the species presence and distribution in Egypt have led the way to this further study. This study aimed to obtain 16S mitochondrial ribosomal RNA gene sequences for selected sea cucumbers from several locations in Egypt and to apply the 16S mitochondrial ribosomal RNA gene sequences in taxonomic and phylogenetic analyses of sea cucumbers from Egypt to verify the outcomes.

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MATERIALS AND METHODS

Collection and Identification: Specimens were collected subtidally by scuba diving, dredge or by hand in the intertidal zone. The species were collected from two locations, the first is west coast of the Red Sea (Safaga to Hurghada area) and the second is Goluf of aqaba (Sharm El-Shakh area) Red Sea, Egypt as shown in Table 1.

Table 1: Species of sea cucumber identified at Sharm El-Shakh and Hurghada, Egypt

No	Species	Location
1	*Holothuria fuscogilva*	Sharm El-Shakh and Hurghada
2	*Actinopyga mauritiana*	Sharm El-Shakh and Hurghada
3	*Holothuria nobilis*	Sharm El-Shakh and Hurghada
4	Sea cucumber leaves	Hurghada

Upon collection, specimens were cleaned in seawater, immediately frozen on dry ice, and consequently stored at -70°C. Identifications were based on inventive descriptions and, in some cases, examination of the type specimens. Informative characters at this level are mainly ossicle structure or form. Ossicles were isolated from mid-dorsal skin by dissolution in fresh bleach, rinsed with water, and permanently mounted on glass slides [3].

DNA Extraction: Typically 200 mg of oral tentacle was ground in liquid N2 in the presence of 600 µl of a proteinase solution (50 mM Tris–HCl, pH 7.5, 50 mM EDTA, pH 8.0, 0.4% SDS, 0.5 mg/ml Proteinase K). The ground samples were immediately placed at 65°C for a minimum of 2 h until tissue was completely digested. The digested samples were repeatedly extracted with an equal volume of phenol:chloroform:isoamyl alcohol, 25:24:1, until the interface was clear. The resultant aqueous phase was adjusted to 0.5 M NaCl and to 1% cetyltrimethylammonium bromide (CTAB) and incubated for a further 20 min at 65°C. After two final phenol/chloroform extractions, total DNA was precipitated by adding an equal volume of isopropanol followed by sedimentation at 13,000 rpm for 20 min in a microcentrifuge. The DNA pellet was rinsed with 500 µl of 70% ethanol, air-dried, and resuspended in 200 µl of TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA).

Amplification Primers: Amplification of specific 16S mitochondrial ribosomal DNA fragments was accomplished by designing conserved primers based on published sea urchin and sea star sequence data [6, 7]. Two universal primers were used for isolation of partial 16S mitochondrial ribosomal RNA (rRNA) region (approximately 450 bp-500 bp): 16s-L (forward) 5'GACGAGAAGACCCTGTGGAGC3' and 16s-R (reverse) 5'AC TTAGATAGAAACTGACCTG3'

Thermal Cycle Amplification: One hundred nanograms of template DNA and 25 pmol of each primer were placed in a Taq DNApolymerase reaction mixture according to the manufacturers' specifications (Biorn, Germany). The amplification profile was as follows: An initial cycle of 95°C for 60 seconds(s); 50°C, 1 mins; 72°C, 2 mins; followed by 30 cycles of 95°C, 30 s; 50°C, 30 s; 72°C, 60 s; followed by a final cycle with a 10-min extension time.

Product Purification: Amplified products were separated by electrophoresis through 1% agarose gels in a 40 mM Tris–acetate, 2 mM EDTA buffer (1X TAE) containing 0.1 µg/ml ethidium bromide. Product bands, visualized with UV transilluminator and photographed using UVP gel documentation system, were excised with a razor blade and purified using QIAquick Gel extraction kit, Qiagen, Germany.

Sequencing: The dideoxyribonucleoside chain termination procedure, originally developed by Sanger *et al*. [8] was employed for sequencing the double-stranded 16S mitochondrial ribosomal DNA by automated DNA sequencing method performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (3100 Genetic Analyzer).

Sequence Alignment and Analysis: All sequences were manually entered and aligned using the Blast programs from National Center for Biotechnology Information (NCBI), USA http://www.ncbi.nlm.nih.gov/BLAST), to detect sequence homologies, initially the 16S mitochondrial ribosomal DNA sequences were aligned using ClustalW (MegaAlign, LaserGene, DNASTAR, Inc., Madison, WI). Phylogenetic trees were constructed with the neighbor-joining method [9] in MEGA [10], by the maximum likelihood method, DNAML [11, 12], by parsimony using PAUP [13], and by the Splits tree program [14] using a LogDet transformation [15, 16].

RESULTS

The amplified 16S mitochondrial DNA products from all four selected sea cucumbers showed approximately 480-500 base pairs (bp) of expected length as shown in Fig. 1.

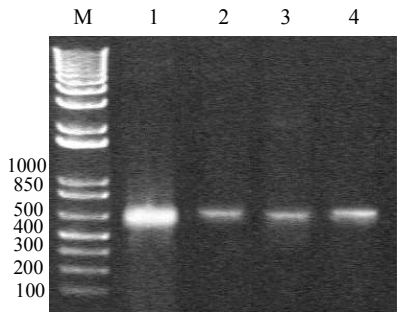

Fig. 1: PCR of 16S rDNA fragment of the four Sea cucumber species
(1) *Holothuria fuscogilva*, (2) *Actinopyga mauritiana*, (3) *Holothuria nobilis* (4) Sea cucumber leaves

All the four partial DNA sequences of 16S mtDNA gene were forwarded to phylogenetic analyses. The mean length of the partial DNA sequences is approximately 470 bp.

Basic Local Alignment Search Tool (BLAST) program provided in the GenBank database was used to search for the corresponding sequences to the partial DNA sequences obtained in this study based on the E value and Score (S). Corresponding sequence of the individually aligned BLAST results indicated that the first DNA sequences of 16S mtDNA gene of *Holothuria fuscogilva* are genetically close to sequences of *Cucumaria frondosa*, *Pentacta pygmaea* vouche, *Cucumaria pseudocurata* and *Psolus fabricii* with scores

Table 2: Homology results of Holothuria fuscogilva 16S rDNA using BLAST (NCBI) database

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U15598.1	*Cucumaria frondosa* mitochondrion 16S ribosomal RNA, partial sequence	852	852	100%	0	100%
AY182376.1	*Cucumaria miniata* mitochondrion, complete genome	806	806	100%	0	98%
DQ777097.1	Pentacta pygmaea voucher AMCC 113256 16S ribosomal RNA gene, partial sequence; mitochondrial	562	562	100%	5.00E-157	88%
U15597.1	*Psolus fabricii* mitochondrion 16S ribosomal RNA, partial sequence	433	433	100%	4.00E-118	84%
U32212.1	*Cucumaria pseudocurata* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	412	412	57%	6.00E-112	94%
U32213.1	*Pseudocnus astigmatus* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	394	394	57%	2.00E-106	93%
U32216.1	*Cucumaria lubrica* intertidal specimen cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	394	394	57%	2.00E-106	93%
U32214.1	*Cucumaria lubrica* subtidal specimen cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	370	370	58%	4.00E-99	91%

Table 3: Homology results *Actinopyga mauritiana* 16S rDNA using BLAST (NCBI) database

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ777097.1	*Pentacta pygmaea* voucher AMCC 113256 16S ribosomal RNA gene, partial sequence; mitochondrial	972	972	100%	0	100%
AY182376.1	*Cucumaria miniata* mitochondrion, complete genome	638	638	100%	1.00E-179	88%
FJ794474.1	*Isostichopus* sp. IF-2009 large subunit ribosomal RNA gene, partial sequence; mitochondrial	364	364	100%	2.00E-97	79%
AY338415.1	*Isostichopus macroparentheses* 16S ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	329	329	100%	7.00E-87	78%
AY338418.1	*Holothuria excellens* 16S ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	322	322	100%	1.00E-84	78%
EU220801.1	*Holothuria kefersteini* voucher UF 3359 16S ribosomal RNA gene, partial sequence; mitochondrial	318	318	99%	1.00E-83	78%
EU220796.1	*Holothuria excellens* voucher UF E1595 16S ribosomal RNA gene, partial sequence; mitochondrial	316	316	99%	5.00E-83	78%
FJ589208.1	*Actinopyga lecanora* large subunit ribosomal RNA gene, partial sequence; and cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	303	303	100%	4.00E-79	78%
EU822449.1	*Actinopyga spinea* isolate FAO01 16S ribosomal RNA gene, partial sequence; mitochondrial	298	298	99%	2.00E-77	78%
EU822448.1	*Actinopyga spinea* isolate FAO03 16S ribosomal RNA gene, partial sequence; mitochondrial	298	298	99%	2.00E-77	78%
EU822447.1	*Actinopyga spinea* isolate FAO02 16S ribosomal RNA gene, partial sequence; mitochondrial	298	298	99%	2.00E-77	78%
EU822442.1	*Holothuria hilla* isolate FAO17 16S ribosomal RNA gene, partial sequence; mitochondrial	294	294	99%	3.00E-76	77%

Fig. 2: Illustration and sequence alignment of *Holothuria fuscogilva* 16S mtDNA gene

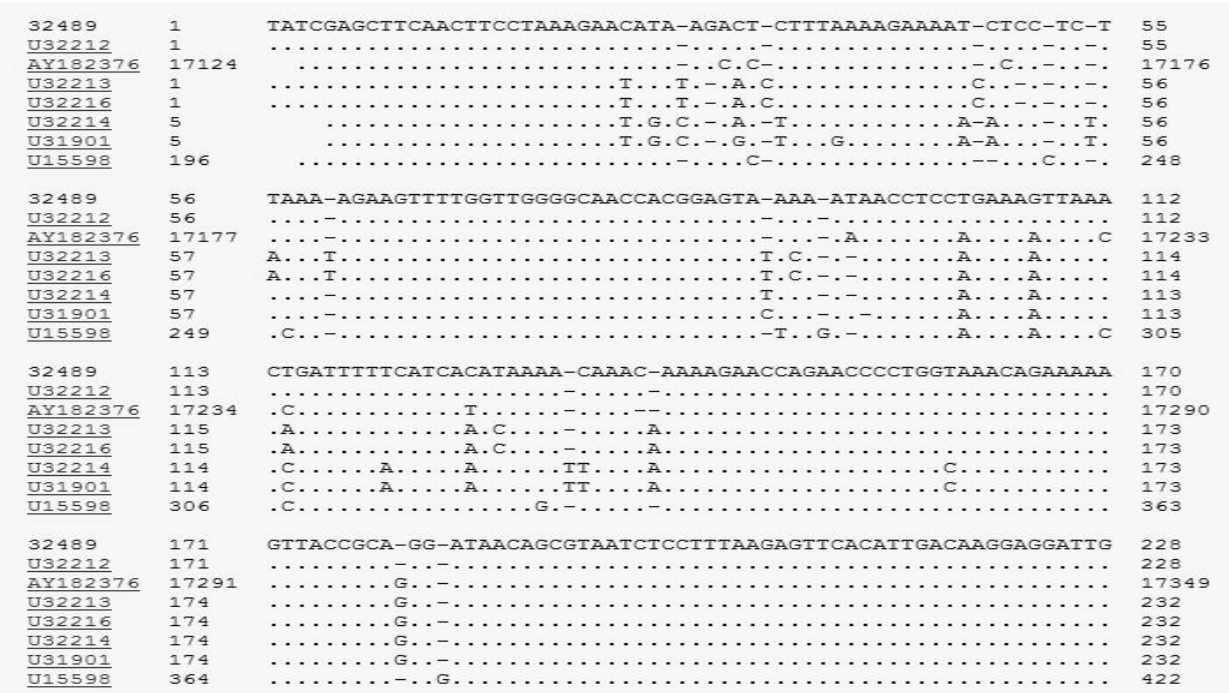

Fig. 3: Illustration and sequence alignment of *Actinopyga mauritiana* 16S mtDNA gene

852, 806, 562 and 433, respectively. Three of them corresponding to16S mtDNA gene of sea cucumbers and one -which was *Cucumaria pseudocurata* - corresponding to the 5' half of the Cytochrome Oxidase subunit 1 (COI) mtDNA gene as shown in Table 2 and Fig. 2.

The second sequences of 16S mtDNA gene of *Actinopyga mauritiana* are genetically close to sequences of *Pentacta pygmaea* vouche, *Cucumaria miniata, Isostichopus macroparentheses, Isostichopus sp, Holothuria excellens,* and *Actinopyga lecanora* with scores 972, 638, 364, 329, 322 and 303, respectively. Four of them corresponding to16S mtDNA gene of sea cucumbers, while *Actinopyga lecanora* was corresponding to the 5' half of the Cytochrome Oxidase subunit 1 (COI) mtDNA gene as shown in Table 3 and Fig. 3.

The third sequences of 16S mtDNA gene of *Holothuria nobilis* are genetically closed to sequences of *Cucumaria pseudocurata* with scores 832. It was corresponding to the 5' half of the Cytochrome Oxidase subunit 1 (COI) mtDNA gene as shown in Table 4 and Fig. 4.

The Fourth sequences of 16S mtDNA gene of Sea cucumber leaves are closely related to sequences of *Psolus fabricii, Cucumaria miniata, Cucumaria frondosa*

Table 4: Homology results *Holothuria nobilis* 16S rDNA using BLAST (NCBI) database

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U32212.1	*Cucumaria pseudocurata* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	832	832	100%	0	100%
AY182376.1	*Cucumaria miniata* mitochondrion, complete genome	689	689	92%	0	96%
U32213.1	*Pseudocnus astigmatus* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	610	610	92%	2.00E-171	92%
U32216.1	*Cucumaria lubrica* intertidal specimen cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	610	610	92%	2.00E-171	92%
U32214.1	*Cucumaria lubrica subtidal* specimen cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	606	606	91%	2.00E-170	93%
U31901.1	*Cucumaria curata* cytochrome oxidase 1 (CO1) gene, mitochondrial gene encoding mitochondrial protein, partial cds	601	601	91%	1.00E-168	92%
U32211.1	*Cucumaria piperata* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	523	523	95%	2.00E-145	88%
U32220.1	*Psolus chitonoides* cytochrome oxidase 1 (CO1) gene, partial cds, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	357	357	92%	3.00E-95	82%
U32218.1	*Eupentacta quinquesemita* cytochrome oxidase 1 (CO1) gene, partial CDs, and large subunit rRNA gene, partial sequence, mitochondrial genes encoding mitochondrial products	333	333	93%	5.00E-88	81%

Table 5: Homology results of Sea cucumber leaves16S rDNA using BLAST (NCBI) database

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U15597.1	*Psolus fabricii* mitochondrion 16S ribosomal RNA, partial sequence	845	845	100%	0	100%
AY182376.1	*Cucumaria miniata* mitochondrion, complete genome	448	448	100%	2.00E-122	84%
U15598.1	*Cucumaria frondosa* mitochondrion 16S ribosomal RNA, partial sequence	433	433	100%	4.00E-118	84%
DQ777097.1	*Pentacta pygmaea* voucher AMCC 113256 16S ribosomal RNA gene, partial sequence; mitochondrial	425	425	100%	7.00E-116	83%
U15596.1	*Chiridota laevis* mitochondrion 16S ribosomal RNA, partial sequence	374	374	99%	3.00E-100	82%
EU822439.1	*Athyonidium chilensis* isolate FAO104 16S ribosomal RNA gene, partial sequence; mitochondrial	320	320	99%	4.00E-84	80%
EU822438.1	*Athyonidium chilensis* isolate FAO103 16S ribosomal RNA gene, partial sequence; mitochondrial	320	320	99%	4.00E-84	80%
EU220798.1	*Holothuria forskali* voucher UF E4480 16S ribosomal RNA gene, partial sequence; mitochondrial	246	246	99%	6.00E-62	77%
EU220797.1	*Holothuria* sp. NMV F110524 16S ribosomal RNA gene, partial sequence; mitochondrial	243	243	99%	8.00E-61	77%
EU220796.1	*Holothuria excellens* voucher UF E1595 16S ribosomal RNA gene, partial sequence; mitochondrial	241	241	99%	3.00E-60	77%
AY338418.1	*Holothuria excellens* 16S ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	241	241	99%	3.00E-60	77%

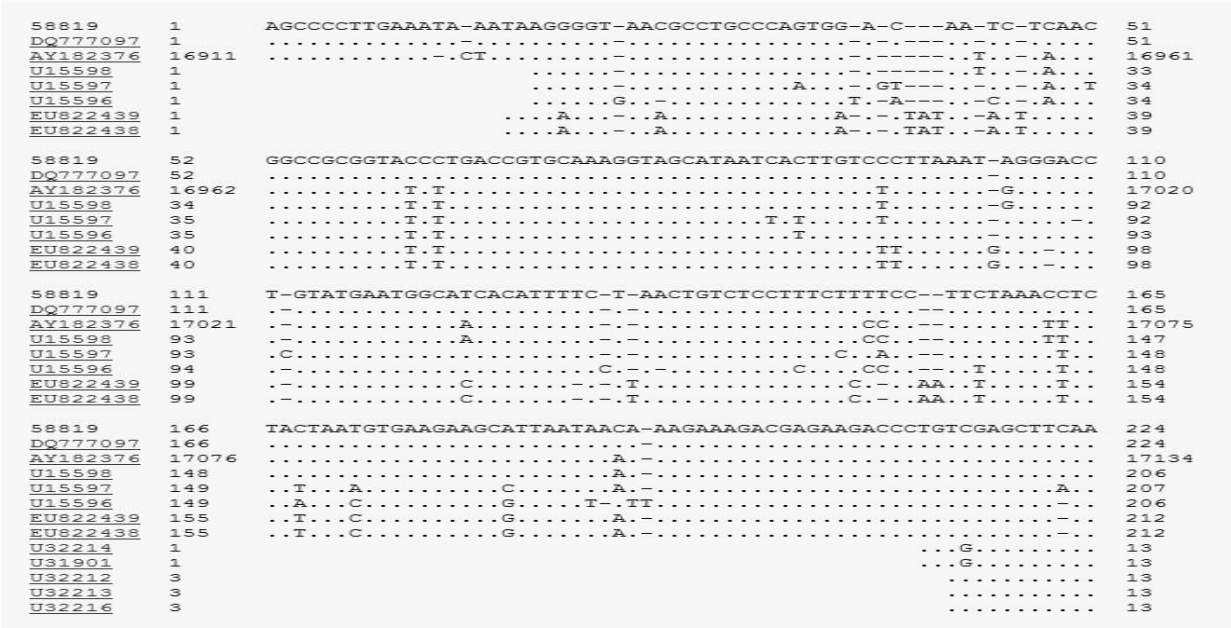

Fig. 4: Illustration and sequence alignment of *Holothuria nobilis* 16S mtDNA gene

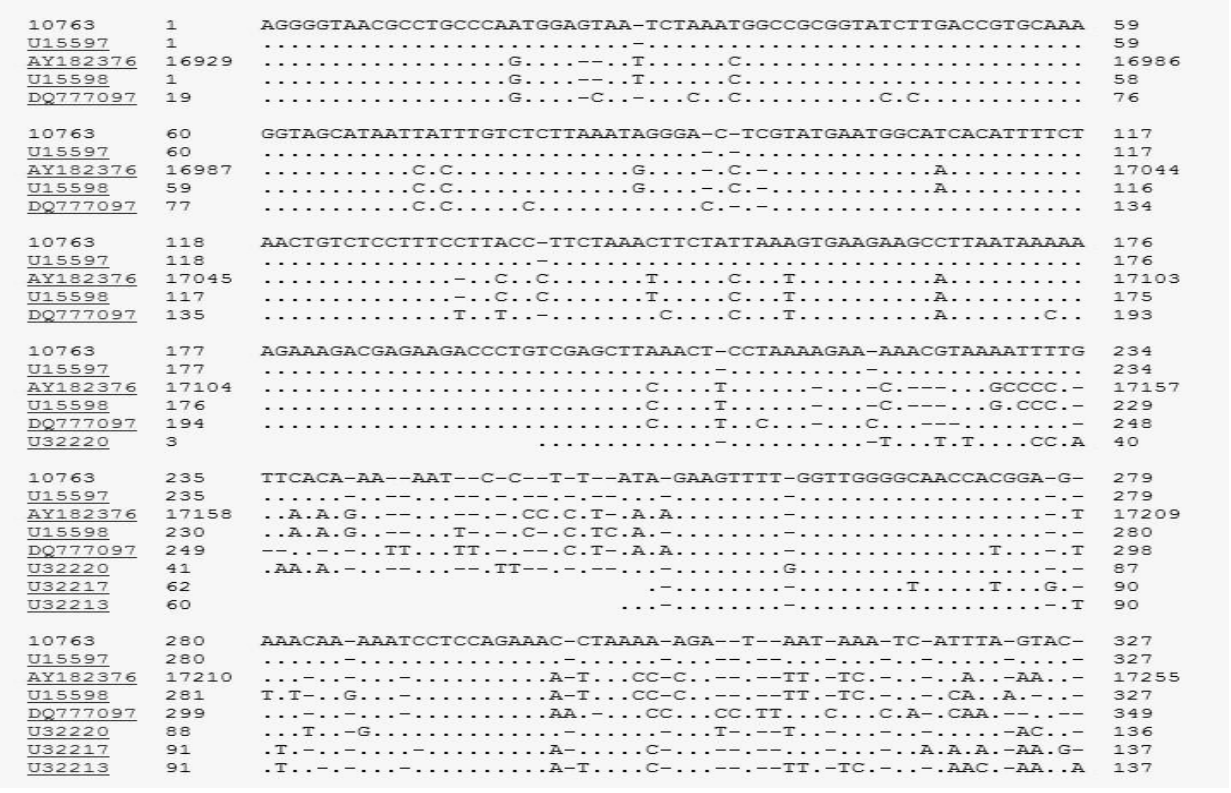

Fig. 5: Illustration and sequence alignment of Sea cucumber leaves =*Psolus fabricii* 16S mtDNA gene

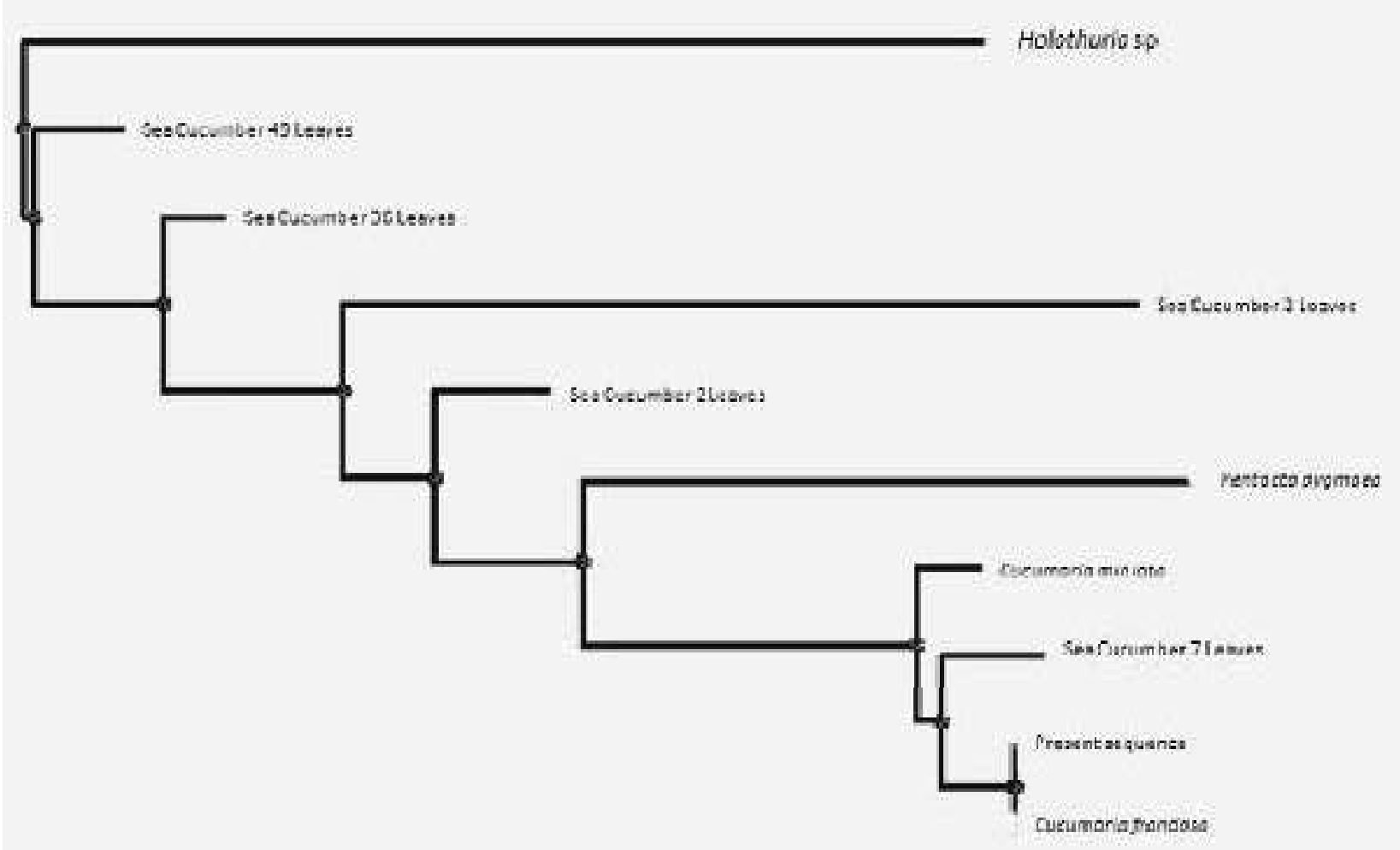

Fig. 6: Neighbor-joining Phylogenetic trees for the *Holothuria fuscogilva* based on the nucleotide sequence of the amplified 16S rDNA gene fragments using Tamura–Nei distance matrix

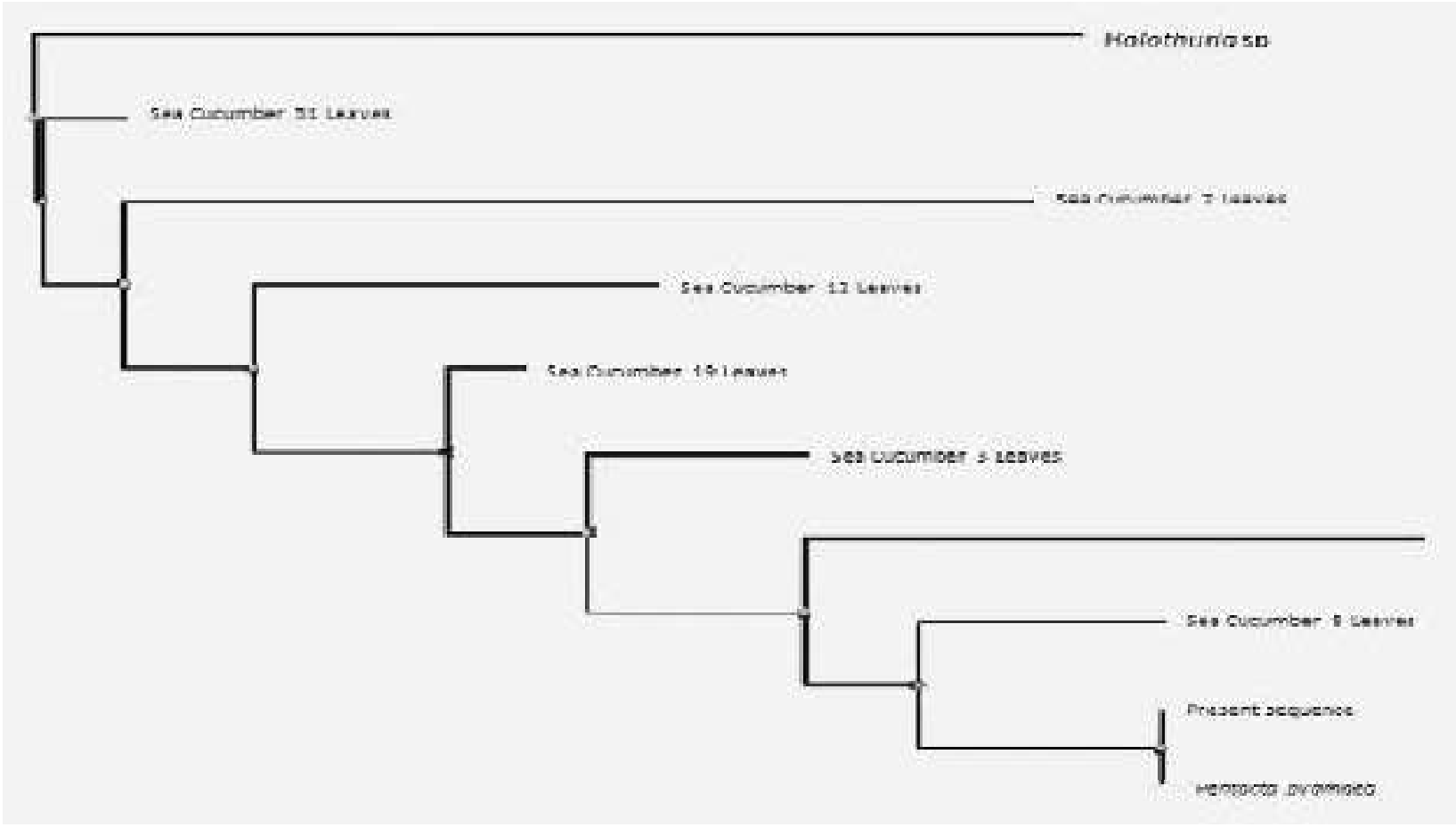

Fig. 7: Neighbor-joining Phylogenetic trees for the *Actinopyga mauritiana* based on the nucleotide sequence of the amplified 16S rDNA gene fragments using Tamura–Nei distance matrix

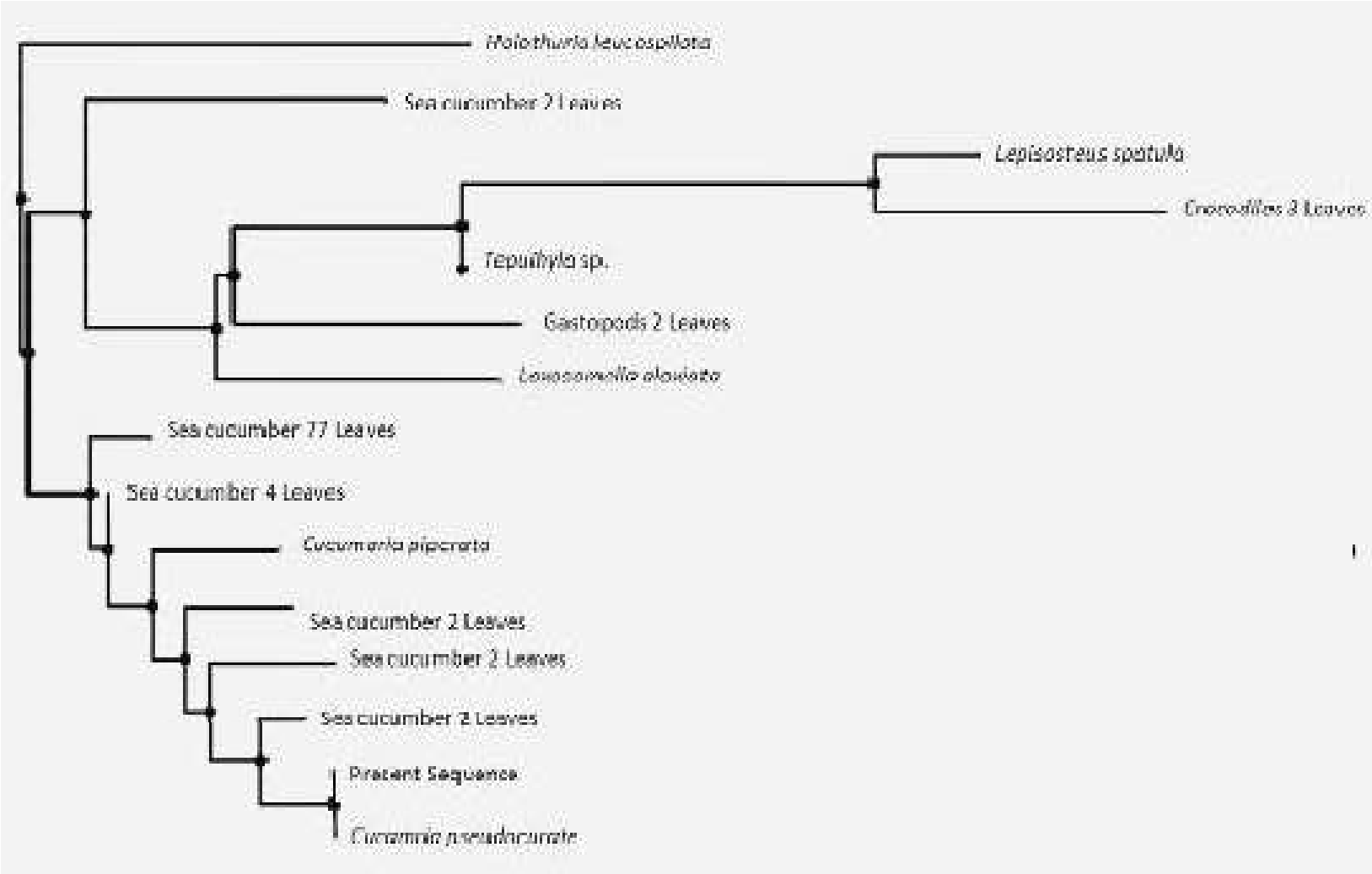

Fig. 8: Neighbor-joining Phylogenetic trees for the *Holothuria nobilis* based on the nucleotide sequence of the amplified 16S rDNA gene fragments using Tamura–Nei distance matrix

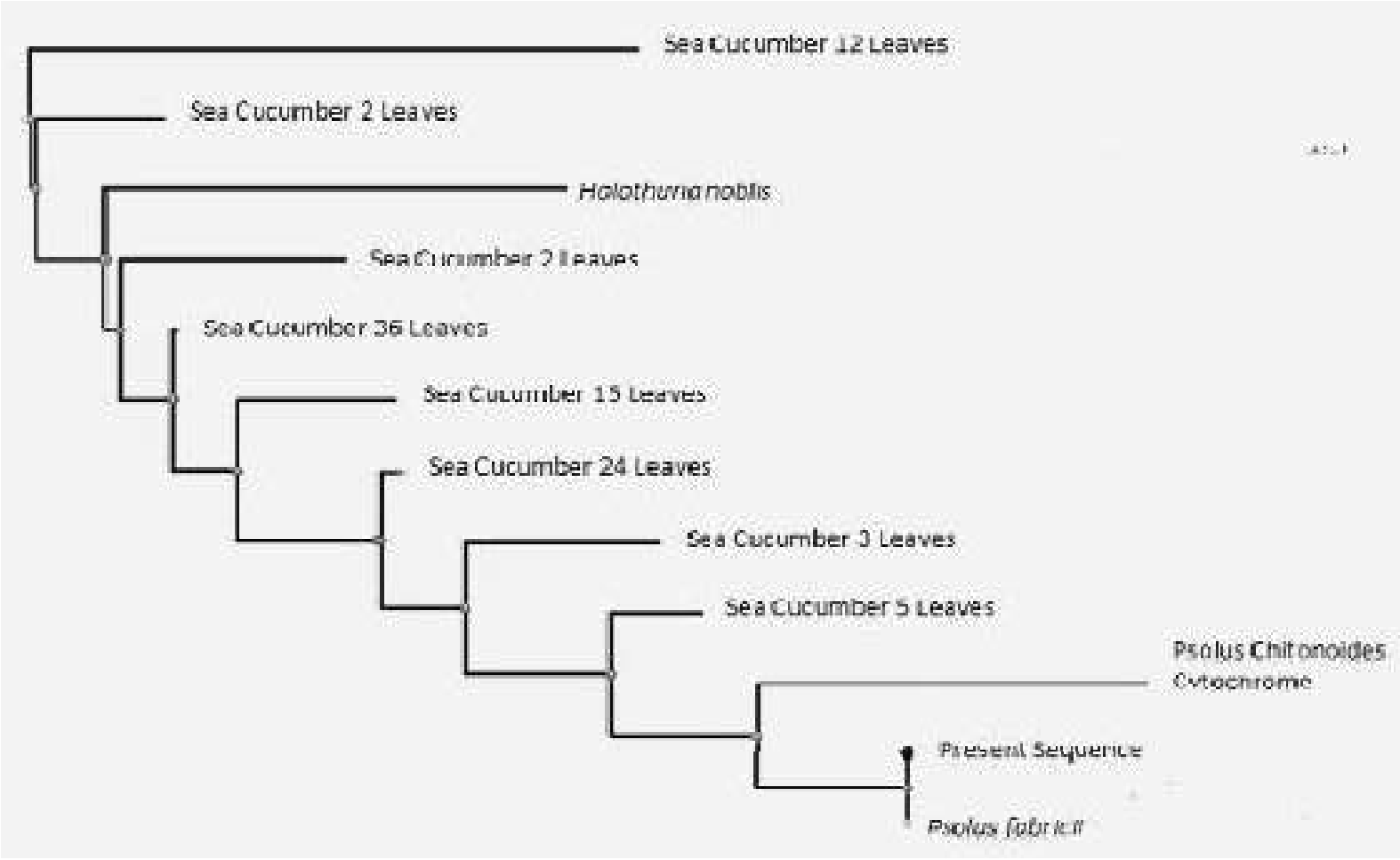

Fig. 9: Neighbor-joining Phylogenetic trees for the Sea cucumber leaves = *Psolus fabricii* based on the nucleotide sequence of the amplified 16S rDNA gene fragments using Tamura–Nei distance matrix

and *Pentacta pygmaea* vouche with scores 845, 448, 433 and 425, respectively. Three of them corresponding to16S mtDNA gene of sea cucumbers and *Cucumaria miniata* was corresponding to, complete genome (mitochondrion) as shown in Table 5 and Fig. 5.

Molecular Phylogeny and Systematics of the Cucumariidae: The four species was morphologically classified as *Holothuria fuscogilva, Actinopyga mauritiana, Holothuria nobilis and* Sea cucumber leaves that belonging to the same family Holothuriidae in the same order as the Aspidochirotida [17].

The Molecular genetic analysis of the collected samples clarified several controversial points concerning the identification and taxonomy of this group. The species *Holothuria fuscogilva, Actinopyga mauritiana, Holothuria nobilis and* Sea cucumber leaves *were* identical to *Cucumaria frondosa, Pentacta pygmaea, and Cucumaria pseudocurata Psolus fabricii, respectively* with Query coverage 100%, Zero E- value and Max identical 100% as shown in Tables 1-4.

For first sequence, the results of the 16S rDNA phylogenic trees are provided in Fig. 6, *Holothoria* sp. was utilized as an outgroup for our phylogentic tree, while *Cucumaria frondosa* is a sister clad for *Holothuria fuscogilva*. Moreover, in the second sequence *Holothoria* sp. were genetically distinct from the present sample (*mauritiana*) and *Pentacta pygmaea* was closely related to it (Fig. 7). In addition, *Holothuria leucospilota was* outgroup of sequence 3 (Fig. 8) and *Holothuria nobilis* is genetically close to *Cucumaria pseudocurata.* The sequanse 4 reveled that *Psolus fabricii* was quite closely related to our samples (Sea cucumber leaves) and most of other branches was Sea cucumber leaves (Fig. 9).

DISCUSSION

The curent results comprise the first phylogenetic test of the classification of the Holothuriidae. this phylogeny corroborated some aspects of the currently used classification; morphologically there is no database for identification and classification of the selected samples. The classical methods using for example spicules [18] appear to be not enough to the explicit taxonomy. However, the systematic of sea cucumbers based on morphology particularly in Egypt is still unclear thus, requiring molecular methods as alternatives to solve the problem [1,19]. It is clear from the previous and present results that there is a large gap between the morphological [20-23] and molecular phylogeny [3].

The present results showed that morphologically, three of the collected samples belonging to family Holothuriidae [24, 25], while it is typically with those of family Cucumariidae using the molecular phylogeny.

The Sea cucumber leaves collected from Hurghada was identical to *Psolus fabricii* which belonging to family Holothuroidea.

The present study has demonstrated the utility of molecular analysis in light of morphological evidence, particularly in systems such as the Holothuroidea where simple form. Phylogenetic analysis of the 16S genes has provided information at both the genus and family levels, which will hopefully serve as a foundation for further molecular analysis of this class of echinoderm.

It could be concluded that. The molecular phylogeny is more distinct than morphological approach. Using 16S rDNA is an easy way to identify any Sea Cucumber even it is a Sea cucumber leaves to the species level. Molecular phylogeny for Sea cucumber of the Red Sea needs further studies to establish a reliable database which can depend on it.

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