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EFFICIENCY OF GENETIC MARKERS FROM STUDIES OF SEA CUCUMBERS (HOLOTHUROIDEA: HOLOTHURIIDA)

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ABSTRACT: Sea cucumbers are a group of large marine benthic invertebrates, that play an important role in marine ecosystems. They are also in demand for commercial and pharmaceutical purposes. The most traded sea cucumbers are those in the order Holothuriida, making them a particularly interesting subject for study. Initially, morphological methods were used to study these sea cucumbers, and then genetic information has since been incorporated into the research. The aim of this study is to investigate the genetic differentiation and evaluate the effectiveness of genetic markers in contributing to the taxonomy of sea cucumbers within the order Holothuriida. Nucleotide sequence data was collected from the National Center for Biotechnology Information (NCBI), and the sequences were edited and aligned using BioEdit 7.2 and MEGA 11 software. The results indicate that the cytochrome c oxidase subunit I (COI) genetic marker is suitable for species identification but does not perform well in resolving phylogenetic relationships. In contrast, the 16S rRNA genetic marker can be used for both species identification and for examining relationships between different species within the genera. The 18S rRNA genetic marker is not suitable for species identification but can be used to study evolutionary relationships in order or family levels. Therefore, the choice of genetic marker depends on the specific objectives of the research.

Keywords: Sea cucumber, Holothuriida, genetic markers, COI, 16S rRNA, 18S rRNA

INTRODUCTION

Sea cucumbers within the class Holothuroidea are a group of invertebrates belonging to the phylum Echinodermata. They are large benthic animals with a cylindrical shape and play a crucial role in marine ecosystems. Sea cucumbers can be found in various marine benthic environments, from intertidal zones to deep-sea regions, including rocky shores, sandy beaches, and seagrass beds. They exhibit significant diversity, with more than 20 species found in some areas (Kerr *et al.* 1993), and are in high demand for commercial and pharmaceutical purposes. In the commercial sector, sea cucumbers are harvested for domestic sale and export, with the most traded species belongs to the families Holothuriidae and Stichopodidae (Purcell *et al.* 2012). This makes them particularly interesting for study.

Initial research on sea cucumbers utilized taxonomic methods based on morphology. However, genetic information has since been incorporated to support or refine traditional taxonomy, especially in cases of morphological complexity (Madduppa *et al.* 2017). Genetic studies utilize specific DNA markers to enhance understanding of evolutionary relationships, genetic diversity, and population structure within sea cucumbers.

The use of genetic markers in the study osea cucumbers involves both nuclear DNA and mitochondrial DNA markers. Nuclear DNA is inherited equally from both parents and exhibits a high degree of genetic diversity, making it challenging to identify specific markers. In contrast, mitochondrial DNA is inherited solely from the mother and typically contains about 15,000 to 16,000 base pairs, making it easier to identify markers (Wallace and Chalkia, 2013; Bronstein *et al*., 2018). Therefore, selecting appropriate genetic markers in conjunction with traditional taxonomic methods signifies a major advancement in sea cucumber research. This integrated approach provides effective data that contributes to SDG 4 (Quality Education), aiding in future management and utilization. The objective of this study is to investigate genetic distance and evaluate the effectiveness of genetic markers as tools in the taxonomy of sea cucumbers within the order Holothuriida.

MATERIALS AND METHODS

Nucleotide sequences at the cytochrome c oxidase subunit I (COI), 16S ribosomal RNA (16S rRNA) and 18S ribosomal RNA (18S rRNA) regions for a total of 22 taxa of sea cucumbers including 19 taxa from the family Holothuriidae, specifically from four genera *Actinopyga, Bohadschia, Holothuria*, and *Pearsonothuria*, and one taxon from the family Mesothuriidae, specifically from the genus *Zygothuria* (Table 1), were used in this study. The sequences are obtained from the NCBI (National Center for Biotechnology Information) database at https://www.ncbi.nlm.nih.gov/. Subsequently, the sequences were edited and aligned using BioEdit 7.2 (Tippmann, 2004) and MEGA 11 (Tamura *et al*., 2021) software. For the analysis of genetic distances, both within and between genera genetic distances were calculated using the Kimura-2 parameter model (Nishimaki and Sato 2019) with 1000 bootstraps. A phylogenetic tree was constructed using the maximum likelihood method, which can create highly accurate phylogenetic trees and help estimates substitutions/ sites, it is a complete and reliable method (Felsenstein, 1981), implemented in MEGA 11 with the TrNef model (Tamura and Nei 1993). We used following sequences as outgroup taxa: *Opheodesomagrisea* (Genbank accession number PP735437) and *Synapta maculata* (Genbank accession number OR145351) for the phylogenetic analysis based on the COI, *Cucumaria frondosa* (Genbank accession number KF479389) and *Colochirus robustus* (Genbank accession number KX856781) for that based on the 16S rRNA, and *Cucumaria frondosa* (Genbank accession number AM422388) and *Colochirus robustus* (Genbank accession number KX856832) for than based on the 18S rRNA regions.

Table 1. Sea cucumber species and GenBank data

Taxonomic rank	GenBank accession number		
	COI	**16S**	**18S**
Order Holothuriida			
Family Mesothuriidae			
Genus *Zygothuria*			
Zygothuria oxysclera (R. Perrier, 1899)	KX874393	KX856735	KX856825
Family Holothuriidae			
Genus *Actinopyga*			
Actinopyga lecanora (Jaeger, 1833)	FJ589208	EU822465	-
Actinopyga mauritiana (Quoy & Gaimard, 1834)	OP897946	KX856787	KX856851
Actinopyga obesa (Selenka, 1867)	OP897950	EU220794	-
Genus *Bohadschia*			
Bohadschia atra Massin, Rasolofonirina, Conand & Samyn, 1999	JN543447	JN543402	-
Bohadschia marmorata Jaeger, 1833	AY574881	JN543423	AY133478
Bohadschia vitiensis (Semper, 1868)	JN543500	JN543407	AY133477
Genus *Holothuria*			
Holothuria atra Jaeger, 1833	LC217309	EU220799	-
Holothuria cinerascens (Brandt, 1835)	JN207584	MK564650	-
Holothuria edulis Lesson, 1830	EU848269	MF616462	AY133471
Holothuria flavomaculata Semper, 1868	JN207630	KF479392	-
Holothuria leucospilota (Brandt, 1835)	MN276190	KY986423	AY133472
Holothuria (Theelothuria) notabilis Ludwig, 1875	KJ801945	KF479396	-
Holothuria (Thymiosycia) arenicola Semper, 1868	JN207610	JN207535	AY133475
Holothuria (Thymiosycia) impatiens (Forsskål, 1775)	PP735433	JN207526	AY133474

Taxonomic rank	**GenBank accession number**		
	COI	**16S**	**18S**
Holothuria (Mertensiothuria) hilla Lesson, 1830	JN207616	KX856783	KX856854
Holothuria (Lessonothuria) pardalis Selenka, 1867	OP898088	FJ223861	-
Holothuria (Stauropora) fuscocinerea Jaeger, 1833	JN207618	JN207560	-
Holothuria (Selenkothuria) erinacea Semper, 1868	JN207604	JN207508	-
Genus *Pearsonothuria*			
Pearsonothuria graeffei (Semper, 1868)	JN543503	KX856786	KX856853

RESULTS

The alignment of nucleotide sequences in the cytochrome c oxidase subunit I (COI) and 16S rRNA regions for 22 taxa revealed that 191 bases positions (in a total of 459 bp) and 174 positions (in a total of 361 bp) were variable for the COI and 16S rRNA genes, respectively. In the 18S rRNA region for 12 taxa, in a total of 1437 bp, 187 positions were variable. The genus *Holothuria* exhibited the highest variability due to its high species diversity, while non-changeable (n/c) variability was observed in the genera *Zygothuria* and *Pearsonothuria* because there was only one representative species in these genera. The details of the percentage variation are shown in Table 2.

Table 2. The percentage variation of nucleotide sequence positions in sea cucumbers within the order Holothuriida

Taxa	**species**	**percentage variation**		
		COI	**16S**	**18S**
Order Holothuriida	20	38%	45%	10%
Family Mesothuriidae	1	n/c	n/c	n/c
Genus *Zygothuria*	1	n/c	n/c	n/c
Family Holothuriidae	19	37%	40%	6%
Genus *Actinopyga*	3	22%	13%	n/c
Genus *Bohadschia*	3	18%	7%	1%
Genus *Holothuria*	12	34%	34%	4%
Genus *Pearsonothuria*	1	n/c	n/c	n/c

For the COI gene, genetic distances within each holothurian genus, ranged from 0.147 to 0.183 (Table 3). The highest value, 0.183, was observed in the genus *Holothuria*, while the lowest value, 0.147, was observed in the genus *Bohadschia*. For genetic distances between genera in family Holothuriidae, the values ranged from 0.219 to 0.316 (Table 3). The lowest value, 0.219, was between the genera *Bohadschia* and *Holothuria*. When examining the genetic distances between genera in different families (genera *Actinopyga*, *Bohadschia*, *Holothuria* and *Pearsonothuria* in the family Holothuriidae, and genus *Zygothuria* in the family Mesothuriidae (Table 3)), it is evident that there is more differentiation observed between genera in different families than between genera within the same family. This is consistent with the taxonomy that separates sea cucumbers in the order Holothuriida into two different families.

Table 3. Mean genetic distances within and between genera of sea cucumbers in the order Holothuriida retrieved from the COI gene data. Mean genetic distances within the same genus were in bold. "n/c" refers to no genetic distance observed.

Genera	*Actinopyga*	*Bohadschia*	*Holothuria*	*Pearsonothuria*	*Zygothuria*
Actinopyga	**0.181 ± 0.018**				
Bohadschia	0.236 ± 0.020	**0.147 ± 0.016**			
Holothuria	0.234 ± 0.018	0.219 ± 0.018	**0.183 ± 0.015**		
Pearsonothuria	0.227 ± 0.021	0.236 ± 0.024	0.234 ± 0.020	**n/c**	
Zygothuria	0.286 ± 0.026	0.287 ± 0.029	0.316 ± 0.028	0.294 ± 0.029	**n/c**

For the 16S rRNA gene, genetic distances within each genus ranged from 0.049 to 0.147 (Table 4). The highest value, 0.147, was observed in the genus *Holothuria*, while the lowest value, 0.049, was observed in the genus *Bohadschia*. For genetic distances between genera, the values ranged from 0.199 to 0.325. The lowest value, 0.156, was between the genera *Bohadschia* and *Pearsonothuria* (Table 4).

Table 4. Mean genetic distances within and between genera of sea cucumbers in the order Holothuriida retrieved from the 16S rRNA gene data. Mean genetic distances within the same genus were in bold. "n/c" refers to no genetic distance observed.

Genera	*Actinopyga*	*Bohadschia*	*Holothuria*	*Pearsonothuria*	*Zygothuria*
Actinopyga	**0.101 ± 0.015**				
Bohadschia	0.220 ± 0.026	**0.049 ± 0.010**			
Holothuria	0.236 ± 0.024	0.199 ± 0.020	**0.147 ± 0.014**		
Pearsonothuria	0.230 ± 0.028	0.156 ± 0.022	0.213± 0.023	**n/c**	
Zygothuria	0.325 ± 0.035	0.293 ± 0.034	0.309 ± 0.030	0.275 ± 0.033	**n/c**

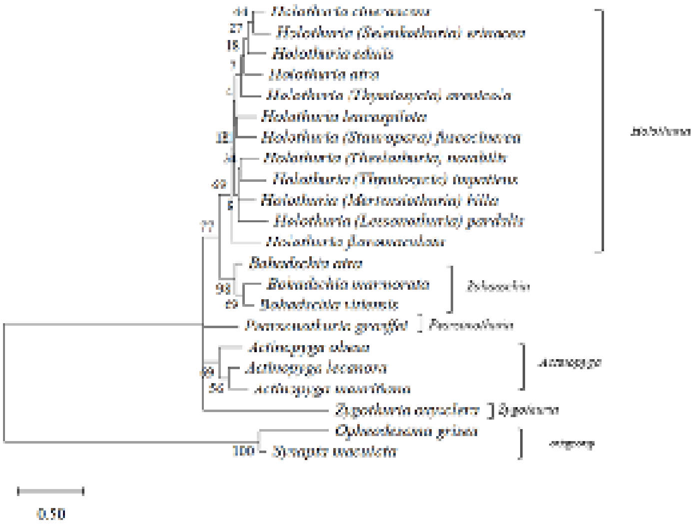
For the 18S rRNA gene, the genetic distances within genera were 0.01264 for the genus *Bohadschia* and 0.01784 for the genus *Holothuria* (Table 5). In addition, genetic distances between genera ranged from 0.00630 to 0.06684. The lowest value, 0.00630, was observed between the genera *Actinopyga* and *Pearsonothuria* (Table 5).

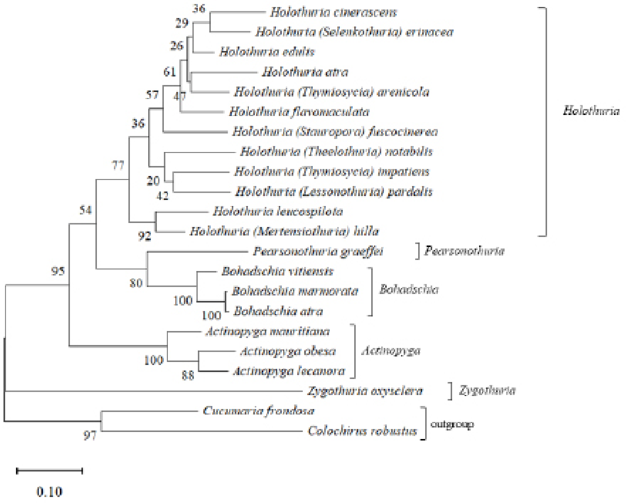
Table 5. Mean genetic distances within and between genera of sea cucumbers in order Holothuriida retrieved from the 18S rRNA gene data. Mean genetic distances within the same genus were in bold. "n/c" refers to no genetic distance observed.

Genera	*Actinopyga*	*Bohadschia*	*Holothuria*	*Pearsonothuria*	*Zygothuria*
Actinopyga	n/c				
Bohadschia	0.0070 ± 0.0018	0.0126 ± 0.0031			
Holothuria	0.0118 ± 0.019	0.0162 ± 0.0022	0.0178 ± 0.0022		
Pearsonothuria	0.0063 ± 0.0065	0.0108 ± 0.0024	0.0153 ± 0.024	n/c	
Zygothuria	0.0610 ± 0.067	0.0612 ± 0.0064	0.0668 ± 0.0066	0.0601 ± 0.0065	n/c

When analyzing phylogenetic trees using the maximum likelihood method, it is evident that sea cucumbers are grouped into different clades according to genera, except for the phylogenetic tree based on the 18S rRNA gene. In the phylogenetic tree based on the COI, sea cucumbers in the genera *Bohadschia* and *Holothuria* are closely related. In contrast, sea cucumbers in the genera

Actinopyga and Pearsonothuria are separated into another group and are closely related to the genus *Zygothuria*, which belongs to a different family. In the phylogenetic tree based on the 16S rRNA data, sea cucumbers from all genera within the family Holothuriidae are closely related and are clearly separated from the family Mesothuriidae and the outgroup. This is consistent with the morphological classification, which separates sea cucumbers in the order Holothuriida based on their peltate-tentacle morphology. In the family Mesothuriidae, ossicles form tables with large laced discs perforated with large holes. In the family Holothuriidae, ossicles form tables, buttons, rods, and rosettes (sometimes only rods are present). Within the genus *Holothuria*, the ossicles form table, while in the genera *Atinopyga, Bohadschia*, and *Pearsonothuria*, the ossicles are found as rosettes (Samyn and Vandenspiegel, 2016; Purcell *et al*., 2023). Regarding the phylogenetic tree based on the 18S rRNA data, sea cucumbers in the families Mesothuriidae and Holothuriidae are clearly separated. However, the genus *Bohadschia*, did not form a single clade.





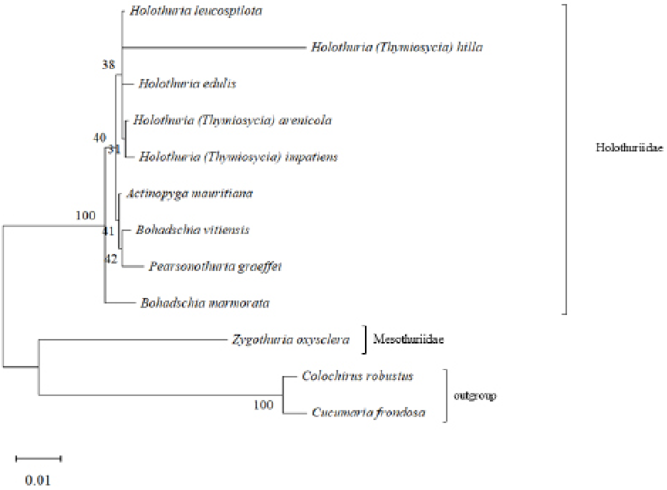


Figure 1. Molecular phylogenetic trees using maximum likelihood analysis of sea cucumbers in the order Holothuriida based on (a) the COI gene, (b) the 16S rRNA gene and (c) 18S rRNA gene.

DISCUSSION

Sea cucumbers in the order Holothuriida were classified into two families Holothuriidae and Mesothuriidae. The family Holothuriidae comprises five genera: *Actinopyga, Bohadschia, Holothuria, Labidodemas,* and *Pearsonothuria.* For the family Mesothuriidae two genera: *Mesothuria* and *Zygothuria* are recognized (Clark and Rowe 1971; Samyn and Vandenspiegel 2016; Mackenzie *et al*. 2021; Purcell *et al*., 2023; Prata and Christoffersen 2024). However, nucleotide sequence data deposited in GenBank for the genera *Labidodemas* and *Mesothuria* are insufficient due to limited information, and the genus *Zygothuria* has only one representative. Research on sea cucumbers has utilized DNA markers from both mitochondrial and nuclear DNA. In this study, the cytochrome c oxidase I subunit (COI) genetic marker showed 38% variability with genetic distances ranging from 0.147 to 0.316, consistent with the findings in Madduppa *et al*. (2017) which shows the genetic distance among the genera: *Actinopyga, Bohadschia* and *Holothuria* ranging from 0.2006 to 0.2057. However, this marker was less effective for phylogenetic relationships due to high genetic distances between species, making it suitable for identifying cryptic species (Panithanarak, 2021). This method is popular and successfully proven in other marine organisms (Jefri *et al*. 2015; Prehadi *et al.* 2015; Sembiring *et al*. 2015; Saleky *et al.* 2016). For the 16S rRNA, which showed 45% variability and genetic distances ranging from 0.049 to 0.325, the results aligned with the study by Eisapour *et al.* (2022). This marker could be used to distinguish species (Kamarudin *et al.* 2016) and phylogenetic relationships between genera with high bootstrap support 95%. For nuclear DNA, the 18S rRNA genetic marker was used, showing 10% variability with genetic distances ranging from 0.00630 to 0.06684. This marker was suitable for studying evolutionary relationships at the order or family, providing high bootstrap support 100% but the 18S gene is too conserved to resolve the phylogenetic relationships between genera (Lacey *et al.* 2005). In addition, it is necessary to select reliable reference sequences to ensure accurate representation of the genera within the order Holothuriida.

Recent advances in genetic studies have focused on population genetics using single nucleotide polymorphisms (SNPs) (Chieu *et al.* 2023) or genomic-level studies. For example, the study by Mongiardino *et al.* (2023) examined the genome-level genetics of sea cucumbers within the class Holothuroidea to resolve evolutionary relationships in the group Neoholothuriida. They used data from 1000 genes and 13 transcriptomes, analyzing the data using CAT-PMSF and ASTRAL-III methods. The CAT-PMSF method combines all data into a single analysis to build the phylogenetic tree while the ASTRAL-III method separates the data into smaller gene trees and then combines the results. Both methods provided good grouping at 80–100%, but the relationships between the orders Dendrochirotida and Synallactida differed. The CAT-PMSF analysis showed a closer relationship between Dendrochirotida and Synallactida than the ASTRAL-III analysis. Therefore, phylogenetic analysis based on specific gene positions could be more informative than the analysis using all available gene positions. Thus, choosing appropriate genetic markers suitable for the research objectives can enhance understanding and conservation of sea cucumbers. It is recommend studying genetic information in conjunction with morphology.

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