Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

Short Communication

Molecular systematics of the genus *Holothuria* in the Mediterranean and Northeastern Atlantic and a molecular clock for the diversification of the Holothuriidae (Echinodermata: Holothuroidea)

Giomar Helena Borrero-Pérez [a,*], Jesús Gómez-Zurita [b], Mercedes González-Wangüemert [a], Concepción Marcos [a], Angel Pérez-Ruzafa [a]

[a] Departamento de Ecología e Hidrología, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain
[b] Institut de Biologia Evolutiva, CSIC-UPF, Pg. Marítim de la Barceloneta 37, 08003 Barcelona, Spain

ARTICLE INFO

ABSTRACT

This work investigates the systematics of the genus *Holothuria* in the Mediterranean Sea and Northeastern Atlantic in the light of a wider molecular phylogenetic hypothesis of Holothuriidae, and it also provides a time-scale for the family diversification using mitochondrial markers and the molecular clock hypothesis. The subgenera *Holothuria* and *Roweothuria* are retrieved as paraphyletic. At least four separate lineages, with quite different time frameworks were identified. There are at least three species with an apparent long evolutionary history, *H. forskali, H. sanctori* and *H. impatiens* and six species belonging to *Holothuria, Roweothuria* and *Vaneyothuria*, which have diverged relatively recently.

1. Introduction

The sea cucumber family Holothuriidae is the most diverse in the Holothuroidea class, and includes predominantly tropical, shallow water animals; only a few species inhabit warm temperate environments. The family includes five extant genera representing 187 described species: *Actinopyga* Bronn, 1860 (17 species), *Bohadschia* Jaeger, 1833 (11), *Holothuria* Linnaeus, 1767 (150), *Labidodemas* Selenka, 1867 (8), and *Pearsonothuria* Levin et al., 1984 (1) (Samyn et al., 2005, 2006; O'Loughlin et al., 2007). The genus *Holothuria* is currently recognized to include 18 subgenera and is the only holothuriid present in the Mediterranean Sea (MS) and Northeastern Atlantic (NEA), where thirteen species from seven of these subgenera occur. Two of these subgenera, *Holothuria* and *Roweothuria* Thandar, 1988, including six out of the thirteen species, are restricted to the eastern Atlantic, MS and Red Sea, with exception of *H. (H.) dakarensis* Panning, 1939, which was also recorded from the Gulf of Mexico (Pawson and Shirley, 1977). The subgenus *Pannin-*

gothuria Rowe, 1969 was considered a monotypic subgenus exclusive of this area, however, a second species from Australia has been recently described (O'Loughlin et al., 2007). The other four subgenera, *Platyperona* Rowe, 1969, *Vaneyothuria* Deichmann, 1958, *Thymiosycia* Pearson, 1914, and *Semperothuria* Deichmann, 1958, are widely distributed, including one or two species in the area of interest. Some of these species are restricted to the eastern Atlantic; however other ones are common in the western Atlantic (recorded in the Eastern Atlantic only in the Cape Verde Islands (Pérez-Ruzafa et al., 1999)) or reported as circum-tropical.

Sea cucumber fishing is increasing globally mainly for export, and there is overexploitation due to the demand from Asian markets (Toral-Granda et al., 2008). New economically important species include the Mediterranean *H. mammata, H. tubulosa, H. polii* and *H. arenicola* (Aydin, 2008; Abdel Razek et al., 2007). Ecological and commercial importance of sea cucumbers has recently spurred systematics work in this group, mainly carried out by the Aspidochirote Working Group, engaged in the taxonomic revision of the Aspidochirotida worldwide (http://guammarinelab.com/peetcukes/index.html). Nevertheless, modern treatises about the higher level systematics of Holothuriidae are scarce. The relationships of the five genera were documented from morphological (Samyn et al., 2005) and molecular (Kerr et al., 2005; Kamarul Rahim

* Corresponding author. Fax: +34 868 88 3963.
E-mail addresses: gborrero@um.es (G.H. Borrero-Pérez), j.gomez-zurita@ibe.upf-csic.es (J. Gómez-Zurita), mergonza@um.es (M. González-Wangüemert), cmarcos@um.es (C. Marcos), angelpr@um.es (A. Pérez-Ruzafa).

et al., 2006) studies. However, the relationships among subgenera of *Holothuria* were only hypothesized by Rowe (1969), although some new information was presented by Samyn et al. (2005) using morphological data, with molecular information still being scarce.

Sea cucumbers are soft animals which leave a poor fossil record and often fail to provide useful taxonomic information at low hierarchical levels. These circumstances preclude a solid reconstruction of the time-scale for the evolution of these animals, so that the timing of the diversification of holothuriids using DNA sequence data and the molecular clock hypothesis constitutes an appealing, almost unavoidable strategy. Despite the few fossils recorded for the family, their age (the earliest one is from the middle Triassic (approximately 237 and 245 mya; Reich, 2004) and the seemingly ancestral Tethyan distribution of the group suggest the antiquity of several groups within the Holothuriidae (Kerr et al., 2005).

In this study, we examine the systematics of MS and NEA species of *Holothuria* in the light of a wider phylogenetic hypothesis of the Holothuriidae and provide a time-scale for the family diversification presenting a molecular clock calibration.

2. Material and methods

We sampled nine species out of thirteen distributed in the MS and NEA, including all currently recognized subgenera ocurring in this area, except *Semperothuria*. We also used all sequence data of Holothuriidae available in GenBank, analyzing a total of 25

Table 1
Species of Holothuriidae included in this study, with references, GenBank accession number and sample location. Voucher numbers are also given for individuals sequenced in this study and deposited at the University of Murcia (Ho).

Species	Reference	GenBank Accession number		Voucher/location
		rrnL	cox1	
Holothuria (*Holothuria*) *mammata*	1/This study	EU191949	GQ214743	Ho1293/Canary Islands, Spain (9)
Holothuria mammata	1/This study	EU191957	GQ214744	Ho1804/Azores Islands, Portugal
Holothuria mammata	This study	GQ21 4729	GQ214745	Ho1824/Cabo de Palos, Spain
Holothuria mammata	1/This study	FJ231190	GQ214746	Ho1855/Algarve, Portugal
Holothuria mammata	This study	GQ21 4730	GQ214747	Ho1873/Gerona, Spain
Holothuria (*Holothuria*) *tubulosa*	1/This study	FJ231192	GQ214748	Ho1828/Cabo de Palos, Spain
Holothuria tubulosa	This study	GQ21 4731	GQ214749	Ho1870/Aguilas, Spain
Holothuria tubulosa	1/This study	EU191974	GQ214750	Ho1869/Aguilas, Spain
Holothuria tubulosa	This study	GQ214732	GQ214751	Ho1884/Gerona, Spain
Holothuria (*Holothuria*) *dakarensis*	1/This study	EU191979	GQ214752	Ho321/Cape Verde Islands (9)
Holothuria dakarensis	1	EU191980	no	Ho312/Cape Verde Islands (9)
Holothuria (*Roweothuria*) *arguinensis*	This study	GQ214734	GQ214754	Ho1191/Canary Islands, Spain (9)
Holothuria arguinensis	This study	GQ214735	GQ214755	Ho1841/Algarve, Portugal
Holothuria arguinensis	This study	GQ214736	GQ214756	Ho1741/Canary Islands, Spain
Holothuria arguinensis	This study	GQ214737	GQ214757	Ho1223/Canary Islands, Spain (9)
Holothuria (*Roweothuria*) *polii*	This study	EU191981	GQ214759	Ho1835/Cabo de Palos, Spain
Holothuria polii	This study	GQ214738	GQ214758	Ho1838/Mar Menor, Spain
Holothuria (*Vaneyothuria*) *lentiginosa lentiginosa*	This study	GQ214733	GQ214753	Ho1021/Alboran Island, Spain
Holothuria (*Thymiosycia*) *impatiens*	This study	GQ214739	GQ214760	Ho273/Cabo de Palos, Spain
Holothuria (*Panningothuria*) *forskali*	This study	GQ214740	GQ214761	Ho1856/Algarve, Portugal
Holothuria forskali	1 /This study	EU191983	GQ214762	Ho1857/Algarve, Portugal
Holothuria (*Panningothuria*) *austrinabassa*	2	EU220797	EU220818	West Australia
Holothuria (*Platyperona*) *sanctori*	This study	GQ214741	GQ214763	Ho1451/Canary Islands, Spain (9)
Holothuria sanctori	This study	GQ214742	GQ214764	Ho1833/Cabo de Palos, Spain
Holothuria (*Platyperona*) *excellens*	2	EU220796	EU220817	Palau Island
Holothuria (*Halodeima*) *atra*	2	EU220799	EU220820	Hawaii island
Holothuria (*Halodeima*) *edulis*	2	EU220811	EU220830	Okinawa, Japan
Holothuria (*Halodeima*) *floridana*	2	EU220803	EU220822	Florida
Holothuria (*Halodeima*) *grisea*	2	EU220800	No	Florida
Holothuria (*Halodeima*) *kefersteini*	2	EU220801	No	Panama
Holothuria (*Halodeima*) *mexicana*	2	EU220802	EU220821	Belize
Holothuria (*Halodeima*) *nigralutea*	2	EU220805	EU220824	West Australia
Holothuria (*Halodeima*) *signata*	2	EU220812	EU220831	Rangiroa, French Polynesia
Holothuria (*Microthele*) *fuscogilva*	3	No	AY700769	Fiji Islands
Holothuria (*Microthele*) *nobilis*	3	No	AY1 76775	La Reunion
Holothuria (*Microthele*) *whitmaei*	3	No	AY1 76777	Fiji Islands
Holothuria (*Microthele*) *whitmaei*	4	AY509147	No	Ilot Maitre, New Caledonia.
Holothuria (*Metriatyla*) *scabra*	4	AY509130	No	Ilot Maitre, New Caledonia.
Holothuria (*Metriatyla*) *scabra var. versicolor*	4	AY509145	No	Ilot Maitre, New Caledonia.
Holothuria (*Mertensiothuria*) *leucospilota*	5	AY338419	No	Guam, Mariana Islands
Holothuria (*Mertensiothuria*) *leucospilota*	6	FJ223871	No	Tiom an Island, Malaysia
Actinopyga mauritania	5	AY33841 4	No	Guam, Mariana Islands
Actinopyga miliaris	3	No	AY700773	Linnet Reef, Great Barrier Reef
Actino pygaobesa	2	EU220794	EU220815	Hawaii island
Bohadschia marmorata	7	AY574877	AY574883	Pohnpei Island, Micronesia
Bohadschia argus	7	AY574870	AY574878	Pohnpei Island, Micronesia
Labidodemas semperianum	5	AY338420	No	Guam, Mariana Islands
Pearsonothuria graeffei	5	AY338421	No	Guam, Mariana Islands
Pearsonothuria graeffei	7	AY574868	No	Chuuk Islands, Micronesia
Isostichopus macroparentheses	5	AY33841 5	No	Guana Islands, British Virgin Islands
Isostichopus fuscus	8	No	AF486429	Coast of Jalisco, Mexico
Stichopus ocellatus	2	EU220793	EU220814	Papua New Guinea

Refs. (1) Borrero-Pérez et al. (2009); (2) O'Loughlin et al. (2007); (3) Uthicke et al. (2004); (4) Uthicke et al. (2005); (5) Kerr et al. (2005); (6) Kamarul Rahim et al. (2006); (7) Clouse et al. (2005); (8) Solís-Marín et al. (2004); (9) Individuals collected during Macaronesia 2000 project (Museo de la Naturaleza y el Hombre de Santa Cruz de Tenerife, Canary Islands, Spain).

species in 10 subgenera of *Holothuria* and 7 species belonging to the other four genera of Holothuriidae (Table 1). Three species of Stichopodiidae were used as outgroups.

Extraction of total genomic DNA was carried out following standard procedures by Sambrook et al. (1989) or DNAzol reagent (GIBCO BRL, Carlsbad, CA) from longitudinal muscle preserved in absolute ethanol. Partial fragments of mitochondrial genes, the subunit 1 of the cytochrome c oxidase (cox1) and the large ribosomal subunit (rrnL), were amplified from different dilutions of the original DNA using the primers CO1eF 5′ATAATGA TAGGAGGRTTTGG3′ and CO1eR 5′GCTCGTGTRTCTACRTCCAT3′ for the former, and 16SA 5′CGCCTGTTTATCAAAAACAT3′ and 16SB 5′CTCCGGTTTGAACTCAGATCA3′ for the latter (Palumbi, 1996; Arndt et al., 1996). PCRs were carried out following the same conditions as Clouse et al. (2005). Annealing temperature varied between 39 °C and 47 °C depending on the tissue quality and the gene. PCR products were purified using Exo/SAP-IT (USB Co., Cleveland, OH) or through dilution in water, and sequenced using the BigDye 3.1 (Applied Biosystems) technology.

Sequences were aligned using the L-INS-i method implemented in MAFFT 6. Pairwise uncorrected distances and distances using GTR+I+G correction were calculated among Holothuriidae sequences using PAUP*4.0b10. Data congruence between both genes was tested and confirmed using the partition homogeneity test ($p = 0.89$) as implemented in PAUP* and they were consequently combined in all analyses. The best substitution model was searched using the Akaike Information Criterion implemented in Modeltest 3.7. Phylogenetic relationships were inferred using Bayesian inference (BI), maximum likelihood (ML) and parsimony. BI analysis was run in MrBayes version 3.04 using unlinked GTR+I+G evolutionary models for each gene partition. The data set was run twice, using for each analysis four Markov chains for five million generations to estimate posterior probabilities. Trees were sampled every 500 generations and the first 750,000 generations were discarded. ML analysis was performed in PhyML using a single GTR+I+G model where model parameters were fixed according to the Modeltest results; support was assessed in this case by 500 bootstrap pseudoreplicates. Parsimony was implemented in PAUP* with a heuristic search using 500 replicates of random sequence addition and TBR branch swapping, with node support assessment by 1000 bootstrap pseudoreplicates. In the latter analysis, gaps were treated either as a fifth character state, as missing data or, alternatively, stretches of gaps were recoded as discrete indel events using FastGap 1.1. No method is considered as superior and differences in node resolution are pertinently discussed, although the BI method was selected to represent the phylogeny of this group. The Shimodaira–Hasegawa (SH) test as implemented in PAUP* was used to test for the monophyly of different taxa using 1000 RELL replicates in every case.

To test if sequences accumulate substitutions in a clock-like manner, a likelihood ratio test (LRT) was conducted between the optimal topology and the topology recovered enforcing a molecular clock in PAUP*. The diversification of Holothuriidae was timed implementing a relaxed clock in r8s 1.71 to the ML tree topology including all taxa and sequence data available for analysis. Branch lengths were optimized in PAUP* using ML and a single GTR+I+G model where model parameters were fixed according to the Modeltest results; PAUP* ML was preferred over other alternatives (e.g., PHYML), because it allows topology constraints as required for branch length linear regression analysis. The latter was needed because some species had missing data either for rrnL or cox1 (see Table 1). Thus, we corrected and proportionally increased the affected branch lengths using the method of Ribera et al. (2008), where the branch lengths are corrected on the original tree using a regression equation relating any given length due to one gene to the expected contribution of the other. This tree topology, with corrected branch lengths, was linearized using the penalized likelihood method in r8s, via a previous cross-validation procedure using sequential increases of the smoothing parameter value (exponentially from 1 to 5). Absolute ages were established based on the oldest known Holothuriidae fossil (245 mya; Reich, 2004). A 95% confidence interval for these estimates was added by analyzing 100 bootstrapped datasets of the original data matrix obtained with seqboot in PHYLIP v. 3.66 and their optimal ML topologies analyzed as before with r8s.

3. Results

The mtDNA alignments of rrnL and cox1 had 461 (including 43 positions with gaps) and 532 characters, respectively. Pairwise sequence divergence (*p*-distance) among *Holothuria, Vaneyothuria, Roweothuria* and *Halodeima* subgenera ranged from 0.046 to 0.137. Higher values (from 0.163 to 0.219) were found among *Thymiosycia, Microthele* Brandt, 1835, *Platyperona* and *Panningothuria* (Table 2; see also electronic Appendix 1 for corrected distances).

ML and BI analyses generated trees with −ln score of 10401.01 and 10385.74 (Fig. 1), respectively. Parsimony analysis produced multiple equally parsimonious trees and the tree lengths were 2336 (CI = 0.36, RI = 0.63), 2424 (CI = 0.37, RI = 0.63) and 2586 (CI = 0.36; RI = 0.65) steps, treating gaps as missing data, as an additional character state, or with gap length-recoding, respectively. All tree reconstruction methods produced similar topologies, with parsimony resulting in poorly resolved trees, particularly at basal branching events (Fig. 1; and see electronic Appendix 2 for results obtained with each method).

The family Holothuriidae appeared monophyletic (node A) in all analyses, except parsimony with gap recoding, and supported in the other two parsimony analyses. *Panningothuria* is the most basal subgenus in the Holothuriidae tree (node E) as reinforced by BI analyses. Node A thus defined a paraphyletic *Holothuria* (SH test, −ln L = 10433.83; p = 0.000) by non-ambiguously including *Labidodemas semperianum* Selenka, 1867 as sister to *H. leucospilota* (node J; subtending node C is also strongly supported in BI), as well as a poorly supported clade (node D) with the subgenus *Platyperona* and the genera *Actinopyga, Bohadschia* and *Pearsonothuria*. Nevertheless, an alternative monophyletic relationship between the subgenera *Platyperona* and *Panningothuria* is not rejected by the SH test (−ln L = 10347.26; p = 0.906).

Some relationships received high support by most if not all methods, including the monophyly of *Panningothuria* (node E), *Bohadschia* with *Pearsonothuria* (node G), *Metriatyla* Rowe, 1969 (node L), and all species in the subgenera *Roweothuria, Vaneyothuria* and *Holothuria* (node P and Q). The monophyly of *Platyperona* (node H), *Microthele* (node I), *Bohadschia* + *Pearsonothuria* + *Actinopyga* (node F) and the close relationships of *H. (H.) mammata* with *H. (R.) arguinensis* (node R) were obtained in BI and ML analyses, but strongly supported only by BI. *Halodeima* appeared paraphyletic (nodes M, N), however the SH test did not reject a monophyletic relationship (−ln L = 10437.25; p = 0.935). This subgenus is closely related to the subgenera *Roweothuria, Vaneyothuria, Holothuria* (node O; BI analysis) and *Metriatyla* (node K; BI analysis). Neither the subgenus *Roweothuria* nor *Holothuria* were retrieved as monophyletic, but while SH test clearly rejected the monophyly of *Roweothuria* (−ln L = 10437.93; p = 0.000), for *Holothuria s.str.* it was non-significant (−ln L = 10356.35; p = 0.582). While support was generally low or restricted to specific analyses for older nodes, most of the nodes from the top of the tree, which represent younger relationships, received high support regardless of the phylogenetic method used.

The MS and NEA Holothuriidae appeared in four separate lineages: (i) species in the subgenera *Holothuria, Roweothuria* and

Table 2

Pairwise uncorrected *p*-distances among Holothuriidae for combined rrnL and cox1 sequence data (below diagonal) and for each gene separately (above diagonal: cox1/rrnL). Missing sequence information for some specimens precludes distance estimation (−). Intra-subgeneric distances are shown in bold face.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. H. (Holothuria) mammata		0.080/0.049	0.113/0.043	0.070/0.040	0.080/0.030	0.132/0.085	0.152/0.112	−/0.153	0.170/0.154	−/0.165	0.184/0.180	0.184/0.194	0.176/0.218	0.209/0.201	0.227/0.204	−/0.164	0.209/0.233	0.178/0.214	−/0.213	0.183/0.272	0.195/0.289
2. H. (Holothuria) tubulosa	**0.066**		0.118/0.034	0.084/0.029	0.088/0.033	0.140/0.068	0.157/0.109	−/0.160	0.173/0.150	−/0.160	0.189/0.174	0.187/0.208	0.197/0.219	0.213/0.188	0.214/0.190	−/0.146	0.207/0.237	0.181/0.204	−/0.220	0.199/0.259	0.193/0.286
3. H. (Holothuria) dakarensis	**0.083**	**0.082**		0.100/0.012	0.120/0.024	0.138/0.061	0.145/0.107	−/0.158	0.185/0.142	−/0.144	0.191/0.175	0.178/0.197	0.203/0.212	0.190/0.193	0.203/0.204	−/0.153	0.214/0.234	0.175/0.208	−/0.217	0.195/0.270	0.173/0.290
4. H. (Vaneyothuria) lent. lent.	0.059	0.062	0.061		0.066/0.018	0.136/0.060	0.152/0.107	−/0.154	0.177/0.140	−/0.143	0.192/0.177	0.180/0.193	0.193/0.205	0.198/0.192	0.209/0.195	−/0.148	0.214/0.230	0.180/0.198	−/0.213	0.197/0.270	0.195/0.288
5. H. (Roweothuria) arguinensis	**0.059**	0.065	0.078	0.046		0.135/0.063	0.150/0.104	−/0.156	0.181/0.143	−/0.155	0.191/0.180	0.177/0.200	0.180/0.200	0.200/0.185	0.213/0.193	−/0.152	0.205/0.232	0.187/0.207	−/0.211	0.193/0.264	0.193/0.288
6. H. (Roweothuria) polii	0.113	0.110	0.106	0.102	**0.104**		0.152/0.082	−/0.140	0.181/0.131	−/0.150	0.184/0.156	0.194/0.180	0.189/0.187	0.217/0.169	0.205/0.187	−/0.132	0.206/0.217	0.190/0.191	−/0.200	0.184/0.273	0.193/0.271
7. H. (Halodeima)[a]	0.137	0.137	0.131	0.135	0.131	0.125		−/0.141	0.194/0.142	−/0.149	0.172/0.167	0.187/0.189	0.197/0.197	0.212/0.180	0.200/0.186	−/0.143	0.199/0.223	0.177/0.206	−/0.210	0.195/0.268	0.202/0.276
8. H. (Metriatyla)[a]	–	–	–	–	–	–	–		−/0.149	−/0.170	−/0.180	−/0.183	−/0.204	−/0.188	−/0.197	−/0.154	−/0.210	−/0.208	−/0.201	−/0.248	−/0.245
9. H. (Thymiosycia) impatiens	0.166	0.166	0.170	0.165	0.169	0.163	0.175	–		−/0.158	0.191/0.164	0.179/0.180	0.207/0.195	0.196/0.177	0.209/0.200	−/0.148	0.197/0.204	0.214/0.198	−/0.196	0.195/0.258	0.186/0.244
10. H. (Mertensiothuria) leucospilota	–	–	–	–	–	–	–	–	–		−/0.184	−/0.191	−/0.205	−/0.186	−/0.208	−/0.122	−/0.197	−/0.190	−/0.195	−/0.260	−/0.288
11. H. (Microthele)[a]	0.181	0.186	0.190	0.185	0.188	0.176	0.169	–	0.182	–		0.194/0.165	0.192/0.190	0210/0.162	0.205/0.174	−/0.150	0.200/0.218	0.191/0.196	−/0.218	0.193/0.265	0.206/0.242
12. H. (Platyperona) sanctori	0.190	0.198	0.188	0.188	0.190	0.190	0.189	–	0.181	–	0.181		0.173/0.160	0.169/0.161	0.195/0.190	−/0.157	0.214/0.204	0.198/0.192	−/0.196	0.195/0.258	0.186/0.244
13. H. (Platyperona) excellens	0.197	0.209	0.209	0.200	0.190	0.190	0.198	–	0.203	–	0.196	**0.169**		0.219/0.175	0.205/0.171	−/0.195	0.208/0.219	0.201/0.187	−/0.195	0.203/0.260	0.217/0.262
14. H. (Panningothuria) forskali	0.207	0.203	0.194	0.198	0.195	0.199	0.198	–	0.190	–	0.194	0.167	0.202		0.139/0.116	−/0.190	0.224/0.219	0.211/0.184	−/0.217	0.200/0.248	0.189/0.260
15. H. (Panningothuria) austrinabassa	0.219	0.206	0.208	0.207	0.208	0.201	0.196	–	0.205	–	0.193	0.195	0.195	**0.132**		−/0.195	0.233/0.231	0.220/0.199	−/0.213	0.209/0.260	0.203/0.252
16. Labidodemas semperianum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		−/0.212	−/0.179	−/0.195	−/0.267	−/0.252
17. Actinopyga[a]	0.224	0.229	0.234	0.227	0.224	0.217	0.218	–	0.195	–	0.213	0.212	0.210	0.224	0.240	–		0.218/0.208	−/0.214	0.214/0.265	0.213/0.250
18. Bohadschia[a]	0.197	0.194	0.191	0.190	0.198	0.192	0.191	–	0.209	–	0.196	0.196	0.195	0.200	0.213	–	0.218		−/0.175	0.216/0.270	0.193/0.267
19. Pearsonothuria graeffei	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		−/0.286	−/0.288
20. Isostichopus[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		0.167/0.174
21. Stichopus ocellatus	0.236	0.234	0.226	0.238	0.236	0.230	0.233	–	0.227	–	0.226	0.212	0.238	0.220	0.226	–	0.232	0.227	–	–	

[a] Distances calculated from several species of the subgenus.
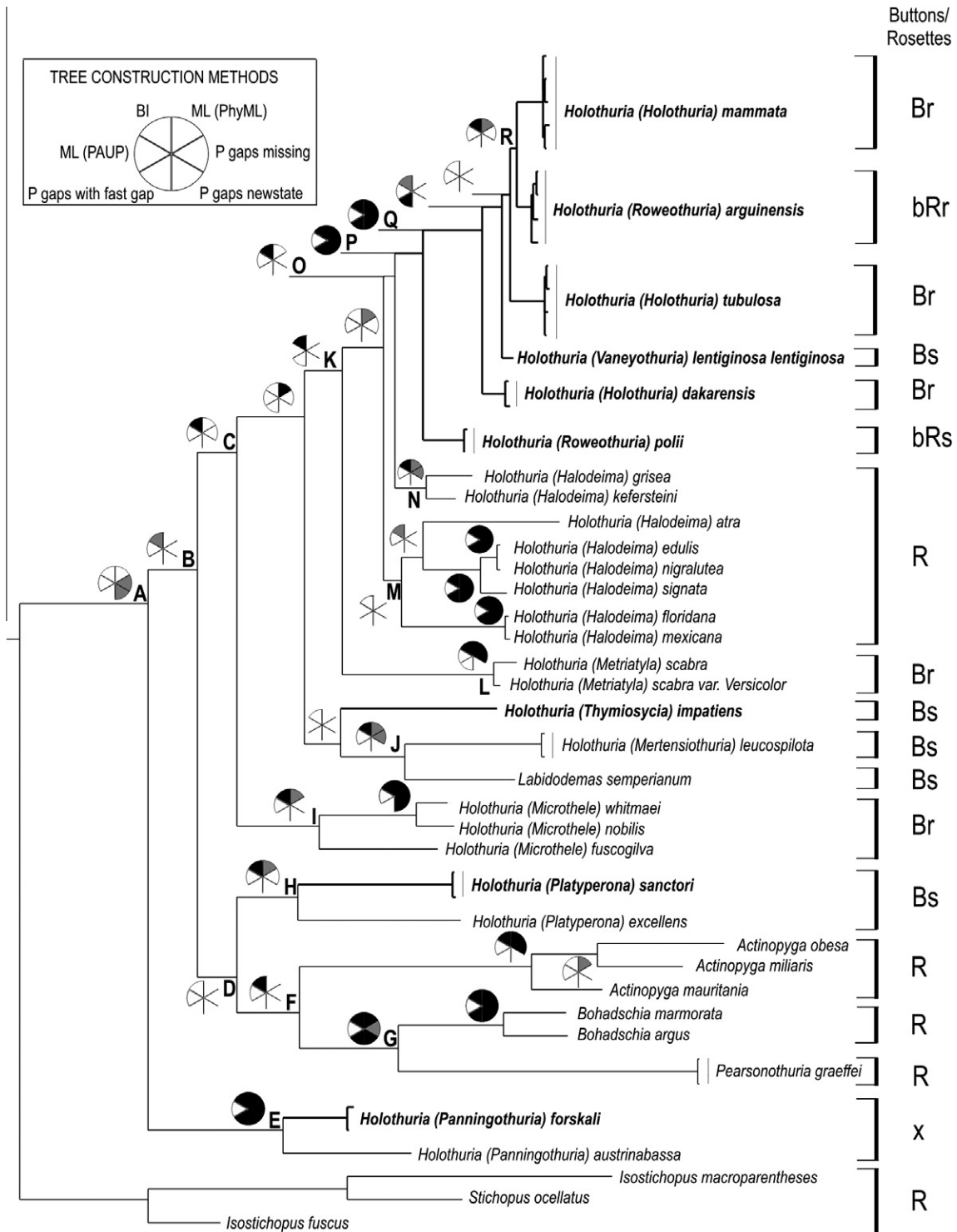
Fig. 1. Bayesian phylogram for the family Holothuriidae. Posterior probabilities (PP) and bootstrap percentages (BP) of several phylogenetic methods implemented are represented with pie charts where black indicates PP ⩾ 0.95 and BP ⩾ 70%, gray PP ⩾ 0.7 and BP ⩾ 50%, and white indicates PP < 0.7 and BP < 50 or recovered node in ML(PAUP); open sectors indicate nodes not recovered. Clades mentioned in the text are identified using letters, and thicker branches are used for lineages from the Mediterranean Sea and the Northeastern Atlantic. *Buttons-Rosettes column*: B: Buttons, R: Rosettes, bR: Buttons-like rosettes r: rugose, s: smooth, X: No buttons, no rosettes.

Vaneyothuria, with a deep divergence between *H.* (*R.*) *polii* and the other species; (ii) *H.* (*T.*) *impatiens*; (iii) *H.* (*P.*) *sanctori*; and (iv) *H.* (*P.*) *forskali* (Fig. 1).

Branch lengths of the individual genes were highly correlated ($r = 0.84$, $p = 0.000$) and branch lengths were thus corrected using the regression equation for cox1 versus rrnL distances. LRT of accumulation of substitutions in a clock-like fashion was non-signifi-

cant ($-\ln L$ without clock = 10348.00; $-\ln L$ with clock = 10396.42; 49 d.f.; $p = 0.42$). According to the fossil calibrated clock (Fig. 2), the basal lineages in Holothuriidae arose from the middle to upper Triassic (245–200 mya). These results place the divergence of *H.* (*Panningothuria*) *forskali* from *H.* (*P.*) *austrinabassa* species at 102 mya during the Cretaceous (confidence interval (ci): 142.8–73.2 mya), and the divergence of *H.* (*Platyperona*) *sanctori*
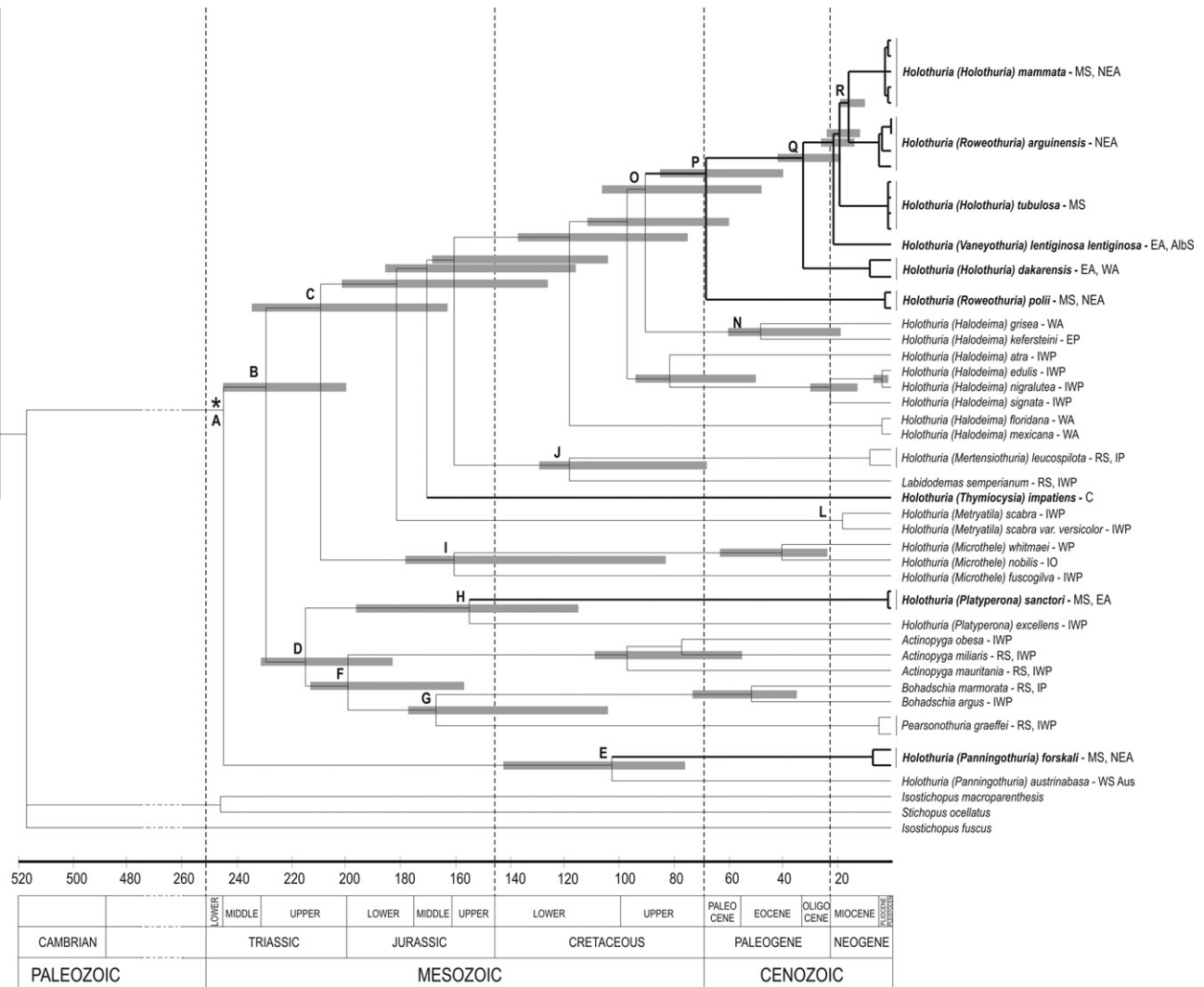
Fig. 2. Optimal ML tree topology constrained for a molecular clock. Gray bars represent 95% confidence age-intervals. The node used to calibrate the tree (earliest fossil of Holothuriidae – 245 mya) is indicated with an asterisk and lineages including species from the Mediterranean Sea and the Northeastern Atlantic are represented by thicker branches. MS: Mediterranean Sea, NEA: Northeastern Atlantic, AlbS: Alboran Sea, EA: Eastern Atlantic, WA: Western Atlantic, EP: Eastern Pacific, IP: Indo-Pacific, IWP: Indo-West Pacific, IO: Indic Ocean, WP: Western Pacific, RS: Red Sea, WS Aus: Western and Southern Australia, C: Circum-tropical.

from *H.* (*P.*) *excellens* at 155.1 mya during the Jurassic to lower Cretaceous (ci: 197.6–115.1 mya). The separation of the lineage leading to *H.* (*Thymiosycia*) *impatiens* occurred at around the same period (170.3 mya; ci: 186.1–116.4 mya). The diversification of most species occurring in the MS and NEA region took place at the late Cretaceous and during the Paleogene (at 68.3 mya for *H.* (*R.*) *polii* [ci: 85.8–40.2 mya] and 32.2 mya for *H.* (*H.*) *dakarensis* [ci: 43.3–19.2 mya]), while the most recent diversification of extant lineages occurred during the early Miocene, at 21 mya for *H.* (*V.*) *lentiginosa lentiginosa*, 18.8 mya for *H.* (*H.*) *tubulosa* and 16.1 mya for *H.* (*R.*) *arguinensis* with regard to *H.* (*H.*) *mammata* (combined ci: 26.7–10.0 mya).

4. Discussion

Our mitochondrial phylogeny corroborates some aspects of the current classification of Holothuriidae (Kerr et al., 2005; Samyn et al., 2005; Kamarul Rahim et al., 2006): (1) paraphyly of the genus *Holothuria*, at least by inclusion of *Labidodemas*, (2) *Actinopyga* and *Bohadschia* deeply divergent from most *Holothuria*, and (3) an apparent sister relationship between *Pearsonothuria* and

Bohadschia. Although the genes investigated seem insufficient to resolve the relationships for basal nodes, the monophyly of *Actinopyga* + *Bohadschia* + *Pearsonothuria* and their basal position to *Labidodemas* and the majority of subgenera of *Holothuria* (exclusive of *Panningothuria* and *Platyperona*) is congruent with Samyn et al.'s (2005) (see below). These results apparently contradicts Kerr et al. (2005), where *Bohadschia* and *Pearsonothuria* were proposed as derived from *Holothuria*, while *Actinopyga* and *Bohadschia* were separated by 25 unambiguous changes on three branches. However, the latter authors only included two representatives of *Holothuria* (*H. excellens* and *H. leucospilota*) possibly biasing their results and interpretation.

The relationship among subgenera of *Holothuria* as inferred from the mtDNA data does not contradict that *Panningothuria* and *Platyperona* are among the oldest and highly divergent lineages in *Holothuria*, which in agreement with O'Loughlin et al., 2007, may warrant treating them as genera. Their basal position is consistent with their plesiomorphic calcareous rings, very similar to those observed in the fossils reported by Reich (2004). On the other hand, our results contradict several relationships hypothesized on the basis of morphology. Most notably, the subgenera *Holothuria* and *Roweothuria* do not appear as reciprocally

monophyletic groups (Rowe, 1969; Thandar, 1988). Both are paraphyletic with each other and by inclusion of *Vaneyothuria*: *H. (R.) arguinensis* is sister to *H. (H.) mammata*, the *H. (R.) arguinensis + Holothuria s. str.* clade includes *H. (V.) lentiginosa lentiginosa*, and *H. (R.) polii* is the sister to the entire group and very divergent genetically (Fig. 1 and Table 2).

Although we have examined only two out of the three currently valid *Roweothuria* species, the monophyly of this subgenus is clearly rejected in the mtDNA phylogeny (SH test −ln $L = 10437.93$; $p = 0.000$). This subgenus is characterized by flat spinose discs and smooth to rugose or spinose, rossete-like buttons (Thandar, 1988). Interestingly, *H. (R.) arguinensis,* the type species of *Roweothuria*, shares with *Holothuria s. str.* the rugose appearance of buttons, one of the most important diagnostic characteristics of the latter (Rowe, 1969), supporting their phylogenetic affinity, although *Holothuria s. str.* does not share the rossete-like buttons diagnostic of *Roweothuria* (Thandar, 1988). The monophyly of *Holothuria s.str.* is an issue difficult to solve with the data at hand, since, in spite of its close links with *Roweothuria* and *Vaneyothuria*, an SH test fails to reject it. Failure to settle this question is likely due in part to sampling limitations, as the analysis only includes three out of seven currently accepted species in this subgenus (Borrero-Pérez et al., 2009). However, not only the phylogenetic results showed that these three subgenera would be best treated as a single taxon, but also their representative sequences showed relatively high similarity being the same order as other *Holothuria* subgenera, such as *Panningothuria* (0.132), *Platyperona* (0.169) (Table 2) or *Halodeima* (up to 0.148).

Our analyses are very valuable among other things because they provide the phylogenetic position of the type species of *Holothuria*, *H. (H.) tubulosa,* giving additional information about some of the proposals by Samyn et al. (2005). In spite of the low support of the basal nodes, the close relationship and the phylogenetic position of *Actinopyga + Bohadschia + Pearsonothuria* does not contradict an upgrade of their taxonomic rank, perhaps to family proposed by Samyn et al. (2005). However, it would be necessary to define the exact phylogenetic position of the subgenera *Panningothuria* and *Platyperona* and redefine their taxonomic rank, because considering Samyńs proposal, the names Holothuriidae and *Holothuria* would in this case apply exclusively to the clade including the type species *H. (H.) tubulosa* (Node C, Fig. 1), which would exclude *Panningothuria* and *Platyperona*. The same authors, Samyn et al., (2005), proposed a systematics classification based on their phylogeny, using the absence/presence of true buttons as a key character: thus, *H. (Halodeima) atra* would be a primitive lineage with rossetes and rods, but never true buttons (Samyn et al., 2005). However, our results, even though preliminary, offer a different picture. The subgenus *Halodeima* (node M and N) is not necessarily more primitive than other subgenera with true (yet variable in appearance, smooth or rugose) buttons (nodes I, J, L), and belongs in a clade (node K) which also includes other groups with true buttons and button-like rossetes (Fig. 1).

The implementation of a molecular clock to the mitochondrial phylogeny of the Holothuriidae suggests that the MS and NEA species had quite different time frameworks for their diversification. In the first place, there are at least three species with an apparent long evolutionary history, *H. (P.) forskali* and *H. (P.) sanctori* with no extant close relatives in the study area and *H. (T.) impatiens* with a single potential close relative, *H. (T.) arenicola*, the other only known species of *Thymiosycia* in the MS and NEA and unfortunately not available for analysis (Fig. 2). Second, there is a group of six species (node P, Fig. 2) which have diverged relatively recently and are closest relatives, with *H. (R.) polii* representing the most ancient and actually quite deep split (Fig. 2).

Panningothuria is an antitropical subgenus including *H. (P.) austrinabassa* and *H. (P.) forskali*, the southern- and northern-most

species of Holothuriidae, respectively (O'Loughlin et al., 2007). Our molecular clock suggests a Cretaceous separation of these species, which can be set within a largely vicariant framework based upon the disintegration of the super-continent Pangea and associated climatic and oceanographic changes, considered the oldest phase of antitropicality recognized by Crame (1993) for molluscs. *H. (Thymiosycia) impatiens* has a circum-tropical distribution, which compelled us to consider this species as a Tethyan relict. This hypothesis would be supported by an old origin, which is in line with our current molecular clock results (Fig. 2).

In spite of the large uncertainty of the time estimates, the origin and diversification of the lineage including the six most recent MS and NEA species of *Holothuria* (node P) seems contemporaneous with the northward drifting of major continental plates, specifically the divergence of *H. (R.) polii* approximately coincides with the collision of India with Asia (65 mya) (Kent and Muttoni, 2008) and the diversification of species in the node Q would have been originated during the Oligo-Miocene boundary when important geologic changes occurred in the Mediterranean area which ended up with the final closure of the Tethys Sea (10 mya) (Harzhauser et al., 2002). These dramatic changes created a variety of new habitats and induced alterations in sea currents that could be linked to the radiation of the genus, and possibly also to the extinction of other *Holothuria* lineages that could exist in this area. These events have promoted speciation in several marine organisms (Groenevelda et al., 2007, and references therein; Harzhauser et al., 2002).

According to our data, the estimated age of the most recent MS and NEA species (node P) predates the final Tethyan closure, and so it is possible that their ancestor could have reached the Mediterranean area from the Indo-West Pacific region through the Tethyan Seaway, or alternatively along the South African coast before the establishment of the Benguela current in the Miocene, which constitutes an effective barrier for dispersal of marine organisms (Siesser, 1980). Indeed, several species from *Roweothuria, Holothuria,* and *Vaneyothuria,* are from localities of the southwestern African coast. Another colonization route for the MS and NEA species (node P) could be from tropical America, where the diversity of the genus is higher, expanding eastward through the Tethys Seaway. The latter hypothesis finds additional support considering the oldest age of *H. (Halodeima) grisea* and *H. (Halodeima) kefersteini* (node O) from the western Atlantic and eastern Pacific, respectively. An Atlantic origin is also possible for node Q where, albeit without strong statistical support, the oldest species in this clade, *H. (H.) dakarensis* and *H. (V.) lentiginosa lentiginosa*, are exclusively from the Atlantic Ocean and distributed on both sides. Our results clearly point that the species currently present in the Mediterranean were originated well before the Messinian Salinity Crisis. Thus, it seems plausible that the Mediterranean species recolonized this sea from the Atlantic, although some species, particularly those endemic to the Mediterranean Sea, may have well survived in situ.

Our estimation of the separation of *H. (M.) fuscogilva* and (*H. (M.) nobilis + H. (M.) whitmaei*) in the subgenus *Microthele,* based on our molecular clock calibration (average substitution rate of 0.0042 (SD: 0.0011) per lineage per my), differs drastically from the scenario proposed by Uthicke et al. (2004), based on estimates of evolutionary rates for the cox1 inferred from the divergence after the closure of the Isthmus of Panama (0.010–0.025 per lineage per my). However, *Microthele* is one of the oldest subgenera in *Holothuria* and even though there is no fossil record for this subgenus, we can speculate based on the few very old fossils for the family Holothuriidae, biogeographic considerations and our own molecular clock results, that this subgenus has an older evolutionary history than the late Cenozoic origin as proposed by Uthicke et al. (2004). An important consideration and caution must be

made here relative to the usage of the evolutionary rates inferred from the divergence after the closure of the Isthmus of Panama. Marko (2002) remarks that the divergence rates can be greatly overestimated when dates corresponding to final closure of the Central American Seaway are used to calibrate the molecular clocks of marine organisms.

The phylogeny presented here may help addressing conservation priorities for harvested species (*H. mammata, H. tubulosa* and *H. polii*). These are taxa from rapidly evolving clades (Erwin, 1991) in comparison with the oldest MS-NEA linages and show phylogenetic distinctiveness (Byrne, 2003), so that their protection would preserve evolutionary processes and environmental systems prone to generate future diversity. Although there is no evident conservation problem for sea cucumbers in the MS and NEA, all initiatives contributing to their knowledge and anticipating to the conservation of these species are important since Holothuriidae is an ancient group with low diversity in the study area.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.08.019.

References

Abdel Razek, F.A., Abdel Rahman, S.H., Mona, M.H., El-Gamal, M.M., Moussa, R.M., 2007. An observation on the effect of environmental conditions on induced fission of the Mediterranean sand sea cucumber, *Holothuria arenicola* in Egypt. Beche-de-mer Inform. Bull. 26, 33–34.

Arndt, A., Marquez, C., Lambert, P., Smith, M.J., 1996. Molecular phylogeny of eastern Pacific sea cucumbers (Echinodermata: Holothuroidea) based on mitochondrial DNA sequence. Mol. Phylogenet. Evol. 6, 425–437.

Aydin, M., 2008. The commercial sea cucumber fishery in Turkey. Beche-de-mer Inform. Bull. 28, 40–41.

Borrero-Pérez, G.H., Pérez-Ruzafa, A., Marcos, C., González-Wangüemert, M., 2009. The taxonomic status of some Atlanto-Mediterranean species in the subgenus *Holothuria* (Echinodermata: Holothuroidea: Holothuriidae) based on molecular evidence. Zool. J. Linn. Soc. 157, 51–69.

Byrne, M., 2003. Phylogenetics and the conservation of a diverse and ancient flora. C. R. Biol. 326, S73–S79.

Clouse, M., Janies, D.A., Kerr, A.M., 2005. Resurrection of *Bohadschia bivittata* from *B. marmorata* (Holothuroidea: Holothuriidae) based on behavioral, morphological, and mitochondrial DNA evidence. Zoology 108, 27–39.

Crame, J.A., 1993. Bipolar molluscs and their evolutionary implications. J. Biogeogr. 20, 145–161.

Erwin, T.L., 1991. An evolutionary basis for conservation strategies. Science 253, 750–752.

Groenevelda, J.C., Gopala, K., Georgeb, R.W., Conrad, A., Matthee, C.A., 2007. Molecular phylogeny of the spiny lobster genus *Palinurus* (Decapoda: Palinuridae) with hypotheses on speciation in the NE Atlantic/Mediterranean and SW Indian Ocean. Mol. Phylogenet. Evol. 45, 102–110.

Harzhauser, M., Piller, W.E., Steininger, F.F., 2002. Circum-Mediterranean Oligo-Miocene biogeographic evolution – the gastropods' point of view. Palaeogeogr. Palaeoclimatol. Palaeoecol. 183, 103–133.

Kamarul Rahim, K., Gires, U., Ridzwan, H., 2006. Paraphyly of the genus *Holothuria* (Aspidochirotida: Holothuriidae) as inferred from 16S mitochondrial rRNA gene sequences. Proceedings of the Eighth National Symposium On Biology: Indigenous Biological Research For National Development.

Kent, D.V., Muttoni, G., 2008. Equatorial convergence of India and early Cenozoic climate trends. Proc. Natl. Acad. Sci. USA 105, 16065–16070.

Kerr, A.M., Janies, D.A., Clouse, R.M., Samyn, Y., Kuszak, J., Kim, J., 2005. Molecular phylogeny of coral-reef sea cucumbers (Holothuriidae: Aspidochirotida) based on 16Smt rDNA sequence. Mar. Biotechnol. 7, 53–60.

Marko, P., 2002. Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panama. Mol. Biol. Evol. 19, 2005–2021.

O'Loughlin, P.M., Paulay, G., VandenSpiegel, D., Samyn, Y., 2007. New *Holothuria* species from Australia (Echinodermata: Holothuroidea: Holothuriidae), with comments on the origin of deep and cool holothuriids. Memoirs of Museum Victoria 64, 35–52.

Palumbi, S.R., 1996. Nucleic acids II: The polymerase chain reaction. In: Hillis, D.M., Moritz, C.M., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Associates Inc., Sunderland, MA, pp. 205–247.

Pawson, D.L., Shirley, T.C., 1977. Occurrence of the subgenus *Holothuria* (*Holothuria*) in the Gulf of Mexico (Echinodermata: Holothuroidea). Proc. Biol. Soc. Wash. 90, 915–920.

Pérez-Ruzafa, A., Entrambasaguas, L., Bacallado, J.J., 1999. Fauna de equinodermos (Echinodermata) de los fondos rocosos infralitorales del archipiélago de Cabo Verde. Rev. Acad. Canar. Cienc. 11, 43–62.

Reich, M., 2004. Aspidochirote holothurians (Echinodermata) from the Middle Triassic of southern Germany. In: Heinzeller, T., Nebelsick, J. (Eds.), Proceedings of the 11th International Echinoderm Conference. Balkema, Netherlands, pp. 485–486.

Ribera, I., Vogler, A.P., Balke, M., 2008. Molecular phylogeny and diversification of diving beetles (Coleoptera, Dytiscidae). Cladistics 24, 563–590.

Rowe, F.W.E., 1969. A review of the family Holothuriidae (Holothurioidea: Aspidochirotida). Bull. Br. Mus. Nat. Hist. (Zool.) 18, 7–170.

Sambrook, E., Fritsch, F., Maniatis, T., 1989. Molecular Cloning. Cold Spring Harbour press, New York.

Samyn, Y., Appeltants, W., Kerr, A.M., 2005. Phylogeny of *Labidodemas* and the Holothuriidae (Holothuroidea: Aspidochirotida) as inferred from morphology. Zool. J. Linn. Soc. 144, 103–120.

Samyn, Y., Vandenspiegel, D., Massin, C., 2006. A new Indo-West Pacific species of *Actinopyga* (Holothuroidea: Aspidochirotida: Holothuriidae). Zootaxa 1138, 53–68.

Siesser, W.G., 1980. Late Miocene origin of the Benguela upwelling system of northern Namibia. Science 208, 283–285.

Solís-Marín, F.A., Billet, D., Preston, J., Rogers, A., 2004. Mitochondrial DNA sequence evidence supporting the recognition of a new North Atlantic *Pseudostichopus* species (Echinodermata: Holothuroidea). J. Mar. Biol. Assoc. UK 84, 1077–1084.

Thandar, A.S., 1988. A new subgenus of *Holothuria* with a description of a new species from the south-east Atlantic Ocean. J. Zool. 215, 47–54.

Toral-Granda, V., Lovatelli, A., Vasconcellos, M. (Eds.). 2008. Sea cucumbers. A global review of fisheries and trade. FAO Fisheries and Aquaculture Technical Paper. No. 516. Rome.

Uthicke, S., O'Hara, T.D., Byrne, M., 2004. Species composition and molecular phylogeny of the Indo-Pacific teatfish (Echinodermata: Holothuroidea) bêche-de-mer fishery. Mar. Freshwater Res. 55, 837–848.

Uthicke, S., Purcell, S., Blockmans, B., 2005. Natural hybridization does not dissolve species boundaries in commercially important sea cucumbers. Biol. J. Linn. Soc. 85, 261–270.