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Molecular characterization and phylogenetic position of the giant deep-sea oyster *Neopycnodonte zibrowii* Gofas, Salas & Taviani, 2009

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Abstract

The giant deep-sea oyster *Neopycnodonte zibrowii* Gofas, C. Salas & Taviani, 2009 is a keystone deep-sea habitat builder species. Discovered about fifteen years ago in the Azores, it has been described and assigned to the genus *Neopycnodonte* Fischer von Waldheim, 1835 based on morphological features. In this study, we generated DNA sequence data for both mitochondrial (COI and 16S) and nuclear (ITS2 and 28S) markers based on the holotype specimen of *N. zibrowii* to establish a molecular phylogenetic framework for the systematic assessment of this species and to provide a reliable (i.e., holotype-based) reference sequence set for multilocus DNA barcoding approaches. Molecular data provide compelling evidence that the giant deep-sea oyster is a distinct species, rather than a deep-water ecophenotype of *Neopycnodonte cochlear* (Poli, 1795), with extremely high genetic divergence from any other gryphaeid. Multilocus phylogenetic analyses place the giant deep-sea oyster within the clade "*Neopycnodonte*/*Pycnodonte*" with closer affinity to *N. cochlear* rather than to *P. taniguchii* Hayami & Kase, 1992, thus supporting its assignment to the genus *Neopycnodonte*. Relationships within this clade are not well supported because mitochondrial variation is inflated by saturation that eroded phylogenetic signal, implying an old split between taxa within this clade. Finally, the set of reference barcode sequences of *N. zibrowii* generated in this study will be useful for a wide plethora of barcoding applications in deep-sea biodiversity surveys. Molecular validation of recent records of deep-sea oysters from the Atlantic Ocean and the Mediterranean Sea will be crucial to clarify the distribution of *N. zibrowii* and assess the phenotypic variation and ecology of this enigmatic species.

Key Words

Azores, DNA sequences, Gryphaeidae, holotype, molecular systematics, Mollusca, multilocus phylogeny, Natural History Museum

Introduction

Deep-sea is the Earth's largest biome but it is still one of the most underexplored regions (Ramirez-Llodra et al. 2010). Deep-sea biodiversity is mostly unknown due to the extreme environmental conditions that limits sampling capabilities (Rogers et al. 2015; Sinniger et al. 2016; Woodall et al. 2018). Along with advances in exploration technologies (Feng et al. 2022), new molecular technologies such as high-throughput sequencing and the molecular identification of multiple species in environmental DNA (eDNA metabarcoding; Taberlet et al. 2012) have boosted deep-sea biodiversity assessments (Guardiola et al. 2016; Everett and Park 2018). However, the low number of reference sequences taxonomically validated in online repository databases (e.g. GenBank) limits the identifications of MOTUs (Molecular Operational Taxonomic Unit), thus reducing the taxonomic resolution of eDNA studies (Ruppert et al. 2019). Studies using an integrative taxonomic approach − combining molecular, morphological and environmental data − on new deep-sea taxa have been carried out in several groups of organisms, such as Anthozoa (López-González et al. 2022), Mollusca (Xu et al. 2019), and some others (Silva et al. 2016; Błażewicz et al. 2019). However, while molecular data are still not available for a great portion of known

deep-sea biodiversity, deep-sea exploration has continued, contributing to the discovery of new benthic ecosystems and associated communities. Therefore, there is a great need to constantly improve with reliable reference sequences the taxonomic coverage of deep-sea taxa in repository databases.

In this study, we focused on a keystone deep-sea habitat builder species discovered about fifteen years ago in the Azores Archipelago: the giant deep-sea oyster *Neopycnodonte zibrowii* Gofas, C. Salas & Taviani, 2009 (Gryphaeidae Vialov, 1936) (Wisshak et al. 2009b). This reef-forming oyster was first observed during a submersible dive along the Faial Channel (480–500 meter depth) (Wisshak et al. 2009b). Deep-sea reefs of *N. zibrowii* are built by both stacked living and dead specimens on vertical rocky substrate of seamounts, escarpments and in canyons (Beuck et al. 2016), and host peculiar deep-sea communities. Benthic associations between *N. zibrowii* and the cyrtocrinid *Cyathidium foresti* Cherbonnier & Guille, 1972 have been documented in the Atlantic Ocean (Wisshak et al. 2009a), and between *N. zibrowii* and cold-water corals in both the Atlantic Ocean (Van Rooij et al. 2010) and the Mediterranean Sea (Taviani et al. 2017, 2019). Recently, new records and observations on *N. zibrowii* in the Atlantic Ocean allowed updating its ecology and distribution (Beuck et al. 2016). The giant deep-sea oyster has been meticulously described in terms of external morphology, microstructures of shell and anatomy (Wisshak et al. 2009b). The systematic placement of this species in the genus *Neopycnodonte* was based on morphological characteristics such as the circular muscle scar, the enlarged vermiculate chomata (see 'neopycnodontine chomata' in Harry 1985) and the vesicular structures in the inner shell layer. *Neopycnodonte zibrowii* is morphologically different from the only extant congeneric species *Neopycnodonte cochlear* (Poli, 1795) in several characters such as the shell architecture and outline, the absence of the resilifer bulge in the latter species and the shape and thickness of the vesicular microstructures. On the other hand, 15 years on from its discovery, molecular data are still not available for this species, thus limiting the assessment of its phylogenetic position and systematic placement.

The taxonomic assessment of oysters based on morphology can be challenging due to a high shell variability and a low number of diagnostic characters (Lam and Morton 2006; Raith et al. 2015; Salvi et al. 2021). Molecular data have a key role in species delimitation and taxonomic identification of oyster species (Lam and Morton 2003; Bieler et al. 2004; Kirkendale et al. 2004; Al-Kandari et al. 2021; Salvi et al. 2022) and would provide compelling evidence that the giant deep-sea oyster *N. zibrowii* is a distinct species rather than a deep-water ecophenotype of *N. cochlear* (Wisshak et al. 2009b).

In this study, we generated DNA sequence data of the giant deep-sea oyster *N. zibrowii* for both mitochondrial and nuclear markers based on the holotype and performed a multilocus phylogenetic analyses to establish its relationships with other gryphaeids. The main aims of this study are to provide: (i) a molecular phylogenetic framework for the systematic assessment of the giant deep-sea oyster, and (ii) a reliable (i.e., holotype-based) reference sequence set for multilocus DNA barcoding approaches.

Materials and methods

Specimens and sequence data gathering

We gathered tissue samples for molecular analyses from museum collections and by field collection. The holotype of *N. zibrowii* (MNHN-IM-2000-20888) and the specimen of *Hyotissa numisma* (Lamarck, 1819) (MNHN-IM-2013-13700) are deposited at the National Museum of Natural History (MNHN) of Paris, while the specimen of *Pycnodonte taniguchii* Hayami & Kase, 1992 (UF 280382) is preserved in the collection of Florida Museum of Natural History (FLMNH). *Neopycnodonte cochlear* (OS239) was collected during scuba diving off the coast of Civitavecchia (nearby Rome, Italy) and stored in pure ethanol. Total genomic DNA was extracted from adductor muscles following standard high-salt protocols (Sambrook et al. 1989). We amplified two mitochondrial − cytochrome oxidase subunit I (COI) and 16S rRNA (16S) − and two nuclear − 28S rRNA (28S) and ITS2 rRNA (ITS2) − gene fragments by polymerase chain reaction (PCR). Primers and conditions used for the amplification are reported in Table 2. Sequencing of PCR products was carried out by the company Genewiz® (https://www.genewiz.com), using the same primers employed for amplification. Sequences generated from these specimens were complemented with sequences obtained from GenBank for additional gryphaeid species. Localities and GenBank accession numbers of sequences used for molecular analyses are shown in Table 1. GenBank sequences were selected in order to minimise the use of chimeric sequences in concatenated alignments (i.e., sequences of different gene fragments obtained from different voucher specimens), therefore whenever possible for each species we selected mitochondrial (COI and 16S) and nuclear (28S and ITS2) sequences from the same voucher. Three specimens (*Hyotissa hyotis* #2, *Hyotissa imbricata* and *N. cochlear* #1) have GenBank sequences from different vouchers (chimeric concatenated sequences). We validated the taxonomic identification of each of these vouchers based on single-gene NJ trees. First, we built four single-gene datasets (COI, 16S, 28S and ITS2) including all the sequences of Gryphaeidae species in GenBank and our sequences. Then for each marker we selected GenBank sequences that clustered within the same clade of conspecific vouchers we sequenced (*H. hyotis* #1 and *N. cochlear* #1) or that have a congruent phylogenetic placement among the four single-gene datasets (*H. imbricata*) (results not shown).

Table 1. Details on the species and DNA sequence data used in this study. Asterisks indicate specimens sequenced in this study. GenBank data are as follows: [1]: Matsumoto 2003; [2]: Matsumoto and Hashimoto unpublished; [3]: Kirkendale et al. 2004; [4]: Plazzi and Passamonti 2010; [5]: Kim et al. 2009; [6]: Plazzi et al. 2011; [7]: Li et al. unpublished; [8]: Ren et al. 2016; [9]: Salvi et al. 2014; [10]: Ip et al. 2022.

Specimen	Locality	Genbank accession number			
		COI	16S	28S	ITS2
Hyotissa hyotis #1	Madagascar	GQ166583[6]	GQ166564[6]	–	–
Hyotissa hyotis #2	Singapore (COI); Maldives (16S and ITS2)	OM946450[10]	LM993886[8]	–	LM993876[9]
Hyotissa imbricata	Japan: Okinawa (COI and ITS2); China: Beibu Bay (16S and 28S)	AB076917[1]	KC847136[7]	KC847157[7]	AB102758[2]
Hyotissa numisma #1	Guam	–	AY376598[4]	AF137035[3]	–
Hyotissa numisma #2 *	Papua New Guinea: Rempi Area	–	PP070396	PP070400	–
Neopycnodonte cochlear #1	Italy: Mediterranean Sea (COI, 16S and ITS2)	JF496772[6]	JF496758[6]	–	LM993878[9]
Neopycnodonte cochlear #2 *	Italy: Civitavecchia	PP069758	PP070397	PP070401	PP074322
Neopycnodonte zibrowii *	Azores: Faial Channel	PP069759	PP070398	PP070402	PP074323
Pycnodonte taniguchii #1	Japan: Okinawa	AB076916[1]	–	AB102759[2]	–
Pycnodonte taniguchii #2 *	Indonesia: Sulawesi Island	PP069760	PP070399	PP070403	PP082050
Magallana gigas (outgroup)	Japan (COI, 16S and 28S); South Korea (ITS2)	KJ855241[8]	KJ855241[8]	AB102757[2]	EU072458[5]

Table 2. Primers used in this study: forward primers are listed above and reverse primers below. For the COI and ITS2 gene fragments we designed new primers specific to Ostreoidea Rafinesque, 1815, and we used the following PCR cycling conditions: denaturation step: 94 °C / 3 min; 35 cycles of: 94 °C / 60 s, T° annealing (COI: 49 °C; ITS2: 50 °C) / 60 s, 72 °C / 60 s; final extension: 10 min at 72 °C.

Gene	Primer	Sequence	Reference	Notes
COI	MolI-F	5′ – ATAATYGGNGGNTTTGGNAAYTG – 3′	This study	Dr Zuccon D. (MNHN), pers. comm.
	osHCO998-R	5′ – ACRGTIGCIGCICTRAARTAAGCICG – 3′		Salvi et al., in prep
16S	16Sar-L	5′ – CGCCTGTTTATCAAAAACAT – 3′	Salvi et al. (2010)	
	16Sbr-H	5′ – CCGGTCTGAACTCAGATCAC – 3′		
28S	D1F-OS	5′ – GAGACTACGCCCTGAACTTAAGCAT – 3′	This study	
	D6R-OS	5′ – GCTATCCTGAGGGAAACYTCAGAGG – 3′	Salvi et al. (2022)	
ITS2	its3d-OS	5′ – GGGTCGATGAAGARCGCAGC – 3′	This study	Modified from Oliverio and Mariottini (2001)
	its4r-OS	5′ – CCTAGTTAGTTTCTTTTCCTGC – 3′		

Phylogenetic analyses

Newly generated sequences for each marker were used as query in BLAST searches (blastn algorithm) using default settings to evaluate contaminants and to confirm the identification of the specimens from family to species level. Multiple sequence alignments of each marker were performed with MAFFT v.7 (Katoh et al. 2019) using the G-INS-I iterative refinement algorithm for the COI and the E-INS-i iterative refinement algorithm for the rRNA markers. GBlocks (Castresana 2000) was used to remove poorly aligned and ambiguous position of the hypervariable regions of the rRNA alignments using a relaxed selection of blocks (Talavera and Castresana 2007). Single-gene alignments were concatenated using the software SequenceMatrix (Vaidya et al. 2011).

Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. We used the oyster *Magallana gigas* (Thunberg, 1793) as outgroup based on previous phylogenetic studies (Tëmkin 2010; Plazzi et al. 2011). ML analyses were performed in the W-IQ-TREE web server v.1.6.12 [http://iqtree.cibiv.univie.ac.at/; (Trifinopoulos et al. 2016)] based on a partitioned substitution model. For each gene partition, the best substitution model was calculated by the ModelFinder module (Kalyaanamoorthy et al. 2017) using an edge-linked model and the BIC criterion (COI: TPM2u+F+G4; 16S: HKY+F+G4; 28S: TN+F+G4; ITS2: K2P+G4). ML analysis was performed with 1,000 pseudo-replicates of ultrafast bootstrapping [uBS; (Minh et al. 2013)]. Bayesian analyses (BA) were carried out with MrBayes v.3.2.7 (Ronquist et al. 2012), using the substitution models selected by ModelFinder for each gene partition. We ran two Markov chains of two million generations each, with a sample frequency of 200 generations. Convergence of the runs (ESS values > 200) were checked with Tracer 1.7 (Rambaut et al. 2018) after a burn-in of 25%. Nodal support was estimated as Bayesian posterior probability (BPP). FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize both ML and BI trees.

Genetic divergence between species at each marker (COI, 16S, 28S and ITS2) were calculated using both uncorrected genetic distance (*p*-distance) and genetic distance corrected under the Kimura 2-paramer model (K2P-distance) using the software Mega11 and the option "*Compute Between Groups Mean Distance*" (Tamura et al. 2021).

Results

BLAST searches using mitochondrial sequences (COI and 16S) of the newly sequenced specimens of *H. numisma*, *N. cochlear* and *P. taniguchii* confirmed the taxonomic identifications of these species (sequence identity of 99–100%). BLAST searches using the mitochondrial sequences generated from the holotype of *N. zibrowii* recovered as best hits sequences belonging to Gryphaeidae species (COI: sequence identity of 73.2%/72.5%/73.1% with GenBank sequences of *Hyotissa* sp./*Neopycnodonte* sp./*Pycnodonte* sp. respectively; 16S: sequence identity of 87.3%/87.5% with GenBank sequences of *Hyotissa* sp./*Neopycnodonte* sp. respectively). This confirms the lack of contamination during the amplification and the affiliation of this species to Gryphaeidae.

The concatenated dataset included 2409 positions (COI: 455, 16S: 449, 28S: 1078, ITS2: 427 positions) and among the 828 variable positions 436 were phylogenetically informative (i.e., parsimony informative). Maximum likelihood and Bayesian trees show two main clades: one including *Hyotissa* species (uBS = 95; BPP = 1), and the other one including *Pycnodonte* and *Neopycnodonte* species (uBS = 83; BPP = 0.94) (Fig. 1). *Neopycnodonte zibrowii* is nested within the second clade with a sister relationship with *N. cochlear* (uBS = 56; BPP = 0.72), whereas *P. taniguchii* is sister to *Neopycnodonte* species.

The COI genetic distances (K2P/*p*-distance) between *N. zibrowii* and *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 35.8%/28.2% and 35%/27.6% (Table 3). The 16S genetic distances (K2P/*p*-distance) between *N. zibrowii* and either *N. cochlear* or *P. taniguchii* are 13.5%/12.1% (Table 3). The mean interspecific genetic distances (K2P/*p*-distance) among the six gryphaeid species are 33.7% ± 4.6% / 26.8% ± 3% at the COI and 15.5% ± 4.6%/13.7% ± 3.7% at the 16S. The 28S genetic distances (K2P/*p*-distance) between *N. zibrowii* and *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 2.5%/2.4% and 9%/8.4% (Table 4). The ITS2 genetic distances (K2P/*p*-distance) between *N. zibrowii* and *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 15.8%/14.9% and 38.2%/29.6% (Table 4). The mean interspecific genetic distances (K2P/*p*-distance) among the six gryphaeid species are 5.5% ± 2.5% / 5.1% ± 2.2% at the 28S and 27.4% ± 16.8% / 22.0% ± 11.9% at the ITS2.

Discussion

Benthic organisms such as oysters, with extensive phenotypic variation and few diagnostic characters, are prone to misidentification in morphological assessments. The utility of molecular characters for taxonomic identification

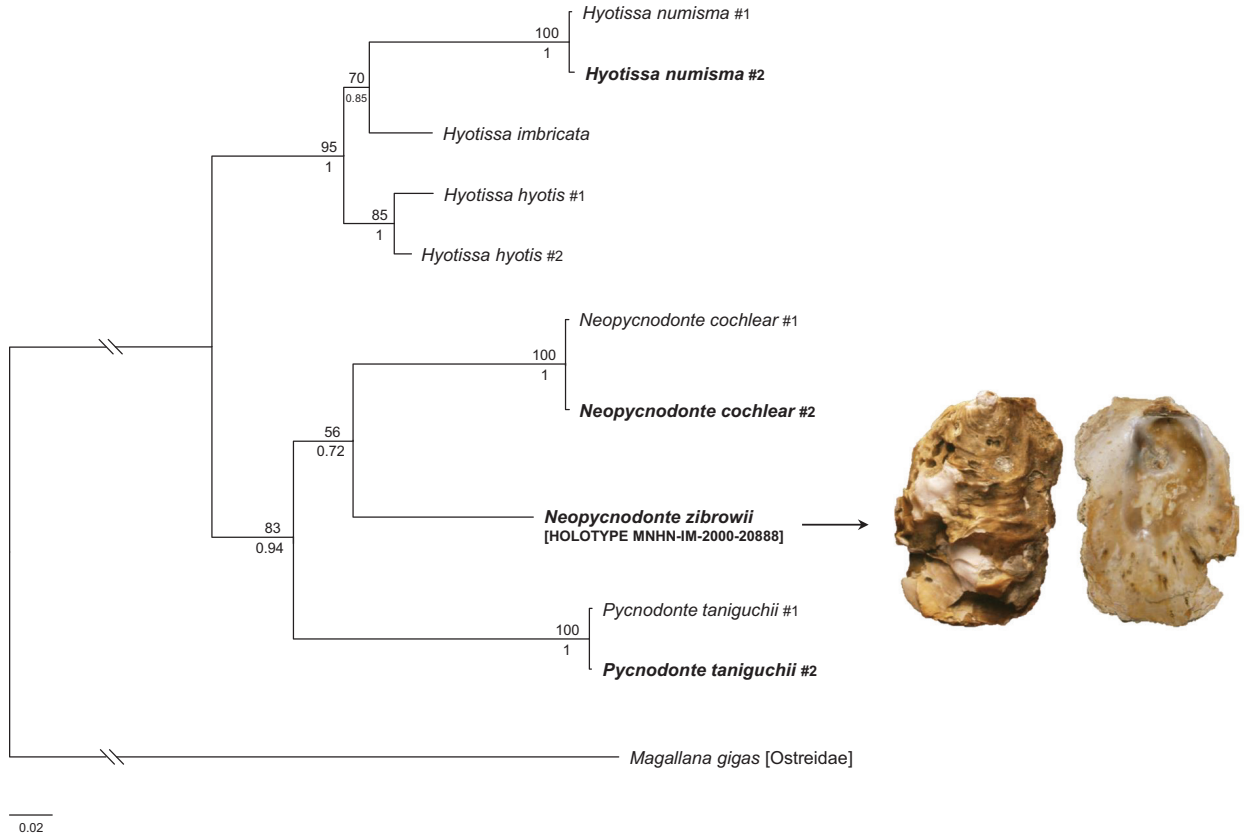

Figure 1. Bayesian phylogenetic tree of six Gryphaeidae species based on COI, 16S, 28S and ITS2 markers. Nodal supports indicate the values of uBS (upper) and the BPP (lower). The tree is rooted with *Magallana gigas* which belongs to the sister family Ostreidae Rafinesque, 1815. Specimens sequenced in this study are highlighted in bold.

Table 3. Mean genetic distance based on COI (lower triangular matrix) and 16S (upper triangular matrix) DNA sequences, calculated using the K2P model (first value) and uncorrected (*p*-distance: value inside brackets). The COI and 16S dataset are composed by 2 sequences for each species, except for *N. zibrowii* (one sequence for each marker), *H. imbricata* (one COI and one 16S sequence) and *P. taniguchii* (one 16S sequence), see Table 1; n. a.: not available.

	Neopycnodonte zibrowii	***Neopycnodonte cochlear***	***Pycnodonte taniguchii***	***Hyotissa hyotis***	***Hyotissa numisma***	***Hyotissa imbricata***
Neopycnodonte zibrowii	–	13.5% (12.1%)	13.5% (12.1%)	15.1% (13.4%)	23.5% (19.9%)	14.9% (13.3%)
Neopycnodonte cochlear	35.8% (28.2%)	–	11.2% (10.3%)	15.5% (13.8%)	22.9% (19.5%)	14.9% (13.4%)
Pycnodonte taniguchii	35.0% (27.6%)	35.4% (28.1%)	—	14.9% (13.3%)	22.3% (19.0%)	14.2% (12.7%)
Hyotissa hyotis	33.3% (26.7%)	39.6% (30.5%)	32.7% (26.5%)	–	15.6% (13.9%)	5.3% (5.1%)
Hyotissa numisma	n. a.	n. a.	n. a.	n. a.	–	15.1% (13.6%)
Hyotissa imbricata	34.0% (27.2%)	37.0% (28.8%)	31.8% (25.8%)	22.4% (19.1%)	n. a.	–

Table 4. Mean genetic distance based on 28S (lower triangular matrix) and ITS2 (upper triangular matrix) DNA sequences, calculated using the K2P model (first value) and uncorrected (*p*-distance: value inside brackets). The 28S and ITS2 dataset are composed by 2 sequences for each species, except for *N. zibrowii* (one sequence for each marker), *H. hyotis* (one ITS2 sequence and no 28S sequence), *N. cochlear* (one 28S sequence) and *P. taniguchii* (one ITS2 sequence), see Table 1. The ITS2 sequence of *H. imbricata* was not used for genetic distance calculation because it was too short; n. a.: not available.

	Neopycnodonte zibrowii	***Neopycnodonte cochlear***	***Pycnodonte taniguchii***	***Hyotissa hyotis***	***Hyotissa numisma***	***Hyotissa imbricata***
Neopycnodonte zibrowii	–	15.8% (14.9%)	38.2% (29.6%)	23.8% (20.4%)	n. a.	n. a.
Neopycnodonte cochlear	2.5% (2.4%)	–	43.5% (33.0%)	20.9% (19.4%)	n. a.	n. a.
Pycnodonte taniguchii	9.0% (8.4%)	4.4% (4.2%)	–	48.4% (35.4%)	n. a.	n. a.
Hyotissa hyotis	n. a.	n. a.	n. a.	–	n. a.	n. a.
Hyotissa numisma	6.8% (6.5%)	4.4% (4.2%)	7.3% (6.9%)	n. a.	–	n. a.
Hyotissa imbricata	6.8% (6.5%)	4.6% (4.4%)	7.7% (6.3%)	n. a.	1.2% (1.2%)	–

and systematic assessment of these organisms cannot be overstated and has been proven over and over by studies on true oysters (Lam and Morton 2006; Raith et al. 2015; Salvi et al. 2021), pearl oysters (Cunha et al. 2011), tree oysters (Garzia et al. 2022) as well as gryphaeid oysters (Li et al. 2023). Conchological convergence, phenotypic plasticity, and the occurrence of cryptic species make molecular taxonomic validation of new oyster species necessary to accurately estimate the diversity of these taxa.

Our molecular phylogenetic results clearly demonstrate that *N. zibrowii* is a distinct species with extremely high genetic divergence from any other gryphaeid at all the markers analysed (Tables 3, 4). *Neopycnodonte zibrowii* is nested within the clade "*Neopycnodonte*/*Pycnodonte*" with closer affinity to *N. cochlear* rather than *P. taniguchii* (Fig. 1), and thus supporting its assignment to the genus *Neopycnodonte* Fischer von Waldheim, 1835 based on morphological features (Wisshak et al. 2009b). Phylogenetic relationships within this clade are not well-supported, like in a previous phylogenetic study including *N. cochlear* and *P. taniguchii* and based on COI and 28S markers (Li et al. 2021). However, the extended dataset of our study improved nodal support and allowed us to clarify the source of phylogenetic uncertainty. Indeed, at both mitochondrial markers, values of pairwise genetic distance between *N. cochlear* / *N. zibrowii* / *P. taniguchii* are similar and remarkably high (COI: 35.0–35.8%; 16S: 11.2–13.5%); whereas at nuclear markers the genetic distance between *P. taniguchii* and *N. zibrowii* is two-three times higher than between the latter and *N. cochlear* (Table 4). Such a pattern suggests that mitochondrial variation is inflated by saturation that eroded phylogenetic signal, implying an old split between taxa within this clade. Wisshak et al. (2009b) highlighted a low number of morphological and ecological differences between the genus *Neopycnodonte* and *Pycnodonte* Fischer von Waldheim, 1835 and pointed out the need for a systematic revision of the genera. Our results highlight that nuclear data will have a key role in further systematic assessment of these genera.

The availability of taxonomically validated reference sequence is a premise for DNA barcoding and metabarcoding approaches for large-scale, fast, and cost-effective molecular taxonomic identification (Hebert et al. 2003; Moritz and Cicero 2004; Schindel and Miller 2005; Salvi et al. 2020). The mitochondrial COI and 16S are the most common markers in DNA barcoding studies on Ostreidae and Gryphaeidae (Lam and Morton 2003, 2004, 2006; Kirkendale et al. 2004; Liu et al. 2011; Hsiao et al. 2016; Salvi et al. 2021). However, also nuclear rRNA markers such as 28S (Mazón-Suástegui et al. 2016) and ITS2 (Salvi et al. 2014; Salvi and Mariottini 2017) have proven useful for molecular taxonomic identification of oysters. Moreover, rRNA markers are frequently selected as target genes in eDNA metabarcoding projects (Ruppert et al. 2019). In this respect, the set of four reference (holotype-based) barcode sequences of *N. zibrowii* provided in this study will be useful for a wide plethora of barcoding applications in deep-sea biodiversity surveys. During the last decade, deep-sea oysters from a mounting number of regions across the Atlantic Ocean and the Mediterranean Sea have been morphologically identified as *N. zibrowii*: from Bay of Biscay (Van Rooij et al. 2010), Gulf of Cadiz (Gofas et al. 2010), Celtic Sea (Johnson et al. 2013), Angola and Mauritania (Beuck et al. 2016), southern Sardinia (Taviani et al. 2017), Sicilian Channel (Rueda et al. 2019) and Gulf of Naples (Taviani et al. 2019). Molecular validation of these records will be crucial to clarify the distribution of *N. zibrowii* and assess the phenotypic variation and ecology of this enigmatic species. Finally, given the high prevalence of cryptic species in oysters, it is not unlikely that future molecular assessments of deep-sea oysters will disclose new species.

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