Mitochondrial Cytochrome Oxidase Subunit 1: A Promising Molecular Marker for Species Identification in Foraminifera

by . .

Submission date: 03-May-2023 04:38AM (UTC-0400)
Submission ID: 2082910304
File name: OK.2022.fmars-09-809659.pdf (9.58M)
Word count: 10929
Character count: 58869

frontiers in Marine Science

ORIGINAL RESEARCH
published: 02 February 2022
doi: 10.3389/fmars.2022.809659

Mitochondrial Cytochrome Oxidase Subunit 1: A Promising Molecular Marker for Species Identification in Foraminifera

Elsa B. Girard[1,2], Anouk Langerak[1], Jamaluddin Jompa[3], Owen S. Wangensteen[4], Jan-Niklas Macher[1] and Willem Renema[1,2]*

[1] Naturalis Biodiversity Center, Leiden, Netherlands, [2] IBED, University of Amsterdam, Amsterdam, Netherlands, [3] Marine Science Department, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, Indonesia, [4] Norwegian College of Fishery Science, UiT - The Arctic University of Norway, Tromsø, Norway

Traditional morphological methods for species identification are highly time consuming, especially for small organisms, such as Foraminifera, a group of shell-building microbial eukaryotes. To analyze large amounts of samples more efficiently, species identification methods have extended to molecular tools in the last few decades. Although a wide range of phyla have good markers available, for Foraminifera only one hypervariable marker from the ribosomal region (18S) is widely used. Recently a new mitochondrial marker cytochrome oxidase subunit 1 (COI) has been sequenced. Here we investigate whether this marker has a higher potential for species identification compared to the ribosomal marker. We explore the genetic variability of both the 18S and COI markers in 22 benthic foraminiferal morphospecies (orders Miliolida and Rotaliida). Using single-cell DNA, the genetic variability within specimens (intra) and between specimens (inter) of each species was assessed using next-generation sequencing. Amplification success rate was twice as high for COI (151/200 specimens) than for 18S (73/200 specimens). The COI marker showed greatly decreased intra- and inter-specimen variability compared to 18S in six out of seven selected species. The 18S phylogenetic reconstruction fails to adequately cluster multiple species together in contrast to COI. Additionally, the COI marker helped recognize misclassified specimens difficult to morphologically identify to the species level. Integrative taxonomy, combining morphological and molecular characteristics, provides a robust picture of the foraminiferal species diversity. Finally, we suggest the use of a set of sequences (two or more) to describe species showing intra-genomic variability additionally to using multiple markers. Our findings highlight the potential of the newly discovered mitochondrial marker for molecular species identification and metabarcoding purposes.

Keywords: protist, high-throughput sequencing, metabarcoding, intra-genomic variation, benthic foraminifera

OPEN ACCESS

Edited by:
Raphael Morard, University of Bremen, Germany

Reviewed by:
Alexandra Anh-Thu Weber, Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France
Mattia Greco, Institute of Oceanology, Polish Academy of Sciences (PAN), Poland
Agnes Katharina Maria Weiner, Smith College, United States

***Correspondence:**
Elsa B. Girard
elsa.girard@naturalis.nl

Specialty section:
This article was submitted to Marine Evolutionary Biology, Biogeography and Species Diversity, a section of the journal Frontiers in Marine Science

Received: *05 November 2021*
Accepted: *10 January 2022*
Published: *02 February 2022*

Citation:
Girard EB, Langerak A, Jompa J, Wangensteen OS, Macher J-N and Renema W (2022) Mitochondrial Cytochrome Oxidase Subunit 1: A Promising Molecular Marker for Species Identification in Foraminifera. Front. Mar. Sci. 9:809659. doi: 10.3389/fmars.2022.809659

INTRODUCTION

The development of new molecular methods to identify and delimit species as well as to assess community composition, species richness in biodiversity and ecology studies has greatly accelerated in recent years (Hebert et al., 2003; Amaral-Zettler et al., 2009; Pawlowski et al., 2016; Wangensteen and Turon, 2017; van der Loos and Nijland, 2021). Metabarcoding methods based

on high-throughput sequencing enable the identification of a wide range for organisms, especially using mitochondrial markers (Wangensteen and Turon, 2017). Most phyla in different Kingdoms have well defined markers such as the mitochondrial cytochrome oxidase subunit 1 (COI), but this gene has only recently been identified in Foraminifera (Macher et al., 2021b).

Foraminifera are unicellular protists living in aquatic environments, mostly known from marine ecosystems. Additionally to being important carbonate producers, these small organisms are good proxies, for (paleo)climatic changes (Billups and Schrag, 2003; Pawłowska et al., 2020), biochronology (Capotondi et al., 1999; Ueno et al., 2019) and coral reef ecosystem health (Hallock et al., 2003; Oliver et al., 2014; Girard et al., 2022). However, little is known at the whole genome level of Foraminifera. Molecular markers from the ribosomal small-subunit (18S SSU rRNA) and internal transcribed spacer (ITS) regions of the genome have been used to identify known foraminiferal morphospecies and cryptic species (Pawlowski et al., 1994; Morard et al., 2015; Borrelli et al., 2018; Macher et al., 2021a). Nonetheless, some Foraminifera are known to show high levels of genomic polymorphism (Morard et al., 2015), potentially due to the presence of several nuclei per cell (Weber and Pawlowski, 2014). Other taxa show too little variability between species, for example only one single nucleotide polymorphism (SNP) was reported between two Amphisorus species in the ITS gene (Macher et al., 2021a). This makes species identification based on 18S rRNA and ITS markers challenging (Morard et al., 2016; Macher et al., 2021a).

Established molecular barcoding methods commonly use a single marker to characterize species (Hebert et al., 2003). Shorter markers (fragment length < 600 bp) have been proven sufficient to differentiate between species in a wide range of organisms (Meusnier et al., 2008; Leray et al., 2013; Yeo et al., 2020). Many studies also show the importance of combining different markers to get a robust species identification and to better understand evolutionary traits and relationships within and between species (Purty and Chatterjee, 2016; Zhao et al., 2016). Macher et al. (2021b) recently discovered the first mitochondrial COI gene in Foraminifera. COI is a molecular marker commonly used in molecular research on animals (Hebert et al., 2003) and has been shown to be informative on other protists (Nassonova et al., 2010; Pawlowski et al., 2012; Burki et al., 2021). There are many advantages of using a coding region such as COI in addition to a non-coding region such as 18S. This includes less variability in total length, and the use of entropy-ratio metrics based on the variability per codon position (Antich et al., 2021), or the detection of stop codons, to help in distinguishing functional sequences from non-functional pseudogenes and sequencing errors (Andújar et al., 2021).

Here we evaluate the effectiveness of the COI marker for species identification and delimitation of Foraminifera by metabarcoding and compare it with the commonly used nuclear 18S marker. We explore the genetic variability of the 18S and COI markers in 22 large benthic foraminiferal morphospecies from the orders Rotaliida and Miliolida. Using foraminiferal single-cell DNA metabarcoding, we assessed the genetic variability within specimens (intra) and between specimens (inter) of a same species using amplicon sequence variants (ASVs).

MATERIALS AND METHODS

Sampling and Sample Preparation

Samples were collected on the reef flat, the reef slope, and at the reef base from nine islands in the Spermonde Archipelago (Southwest Sulawesi, Indonesia) (**Supplementary Table 1**). The sampling took place between April 23rd and May 8th 2018. A subset of the samples was preserved in 96% ethanol and transported back to the Naturalis Biodiversity Center (NBC), Netherlands. The ethanol-preserved specimens were stored at −20°C at NBC until DNA extraction. One additional sample was taken from the Indo-Pacific coral reef aquarium in Burger's Zoo in Arnhem (Netherlands) on March 18th 2021 (**Supplementary Table 1**). Foraminifera from this sample were kept alive in a 9-L aquarium in the NBC laboratory until DNA extraction. Before DNA extraction, all specimens were separated in individual eppendorf 1.5 mL tubes. The specimens were classified to morphospecies level based on the description from Renema (2018) and Macher et al. (2021a), photographed and cleaned in 70% ethanol with a brush and a needle to remove as much non-foraminiferal material as possible using a stereomicroscope. In total, we selected 200 foraminiferal specimens from two orders (Miliolida and Rotaliida), six families (Alveolinidae, Amphisteginidae, Calcarinidae, Peneroplidae and Soritidae) and 22 morphospecies (*Alveolinella quoyi*, *Amphisorus* SpL, *A.* SpS, *Amphistegina lessonii*, *A. papillosa*, *A. radiata*, *Borelis schlumbergeri*, *Calcarina* sp1, *C. hispida*, *Heterostegina depressa*, *Marginopora vertebralis*, *Neorotalia calcar*, *N. gaimardi*, *Nummulites venosus*, *Operculina ammonoides*, *O. complanata*, *O.* LKI27 type, *Operculinella cumingii*, *Parasorites* sp., *Peneroplis planatus* and *Sorites* sp.).

DNA Extraction and Amplification

Destructive and non-destructive DNA extraction methods were performed on the selected foraminiferal specimens (see **Supplementary Table 1**). After crushing 117 specimens using either tweezers or a mortar and pestle (destructive), we performed single-cell DNA extractions using the QIAamp DNA Micro Kit Tissue (QIAGEN, Hilden, Germany). To preserve morphological features of an additional 83 specimens (non-destructive), we performed the guanidinium isothiocyanate (GITC*) extraction method as in Weiner et al. (2016). DNA was eluted with 50 μL of Milli-Q water. Aliquots of DNA templates were further diluted 10 times before amplification.

Amplifications of targeted COI (Leray region) and 18S (37-41f fragment of the V4 region) markers were performed with foraminiferal specific primers (**Table 1**). The primers were complemented with a Nextera XT tail (Illumina, Inc.) in order to label each sample with a unique barcode. Amplifications were performed by polymerase-chain reaction (PCR) using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, United States). For amplification of the 18S marker, two different reactions (i.e., TaqMan Environmental Master Mix 2.0 and KAPA HiFi HotStart

TABLE 1 | Primer sets used for amplification of the targeted DNA regions COI and 18S.

Marker	Primer name	Primer sequence (5′–3′)	Expected read length	References
18S - F	s14f3	ACGCAMGTGTGAAACTTG	250–330 bp	Holzmann et al., 2003
18S - R	s17	CGGTCACGTTCGTTGC		
COI - F	Foram_COI_fwd1	GWGGWGTTAATGCTGGTYGAAC	300–330 bp	Macher et al., 2021b
COI - R	Foram_COI_rev	RWRCTTCWGGATGWCTAAGARATC		

F, forward primer; R, reverse primer.

ReadyMix) and their associated PCR program were tested to maximize amplification success. The reactions go as follows: 5 µL of diluted 10x DNA template was mixed to 3 µL Milli-Q water, 10 µL TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA, United States) or KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), 1 µL forward primer 10 µM and 1 µL reversed primer 10 µM for a total volume of 20 µL. The PCR1 program for the Environmental Master Mix was 10 min at 95°C, followed by 35 cycles of 15 s at 95°C, 30 s at 50°C, 40 s at 72°C, followed by 5 min at 72°C. The program for KAPA Mix was 10 min at 95°C, followed by 35 cycles of 45 s at 95°C, 60 s at 57°C, 90 s at 72°C, followed by 5 min at 72°C. See **Supplementary Table 1** on which amplification method was used for each specimen.

For amplification of the COI marker, the protocol was improved from Macher et al. (2021b). 2.5 µL of diluted 10x DNA template was mixed to 11.7 µL Milli-Q water, 2 µL PCR buffer CL 10X (Qiagen), 0.4 µL MgCl2 25 mM, 0.8 µL BSA 10 mg/mL, 0.4 µL dNTP 25 mM, 0.2 µL Taq-polymerase (Qiagen) 5 U/µL, 1 µL forward primer 10 µM and 1 µL reverse primer 10 µM for a total volume of 20 µL. The PCR1 program was 3 min at 96°C, followed by 40 cycles of 15 s at 96°C, 30 s at 50°C, 40 s at 72°C, followed by 5 min at 72°C. A blank was performed with Milli-Q water instead of DNA template for each PCR run to check for potential (cross-)contamination. The quality of the amplification and DNA fragment size were confirmed on an agarose gel electrophoresis [1% agarose in 0.5x TBE buffer with 0.1% SyBr safe DNA gel stain and 1KB plus ladder (Invitrogen, Carlsbad, CA, United States)].

Library Preparation and Sequencing

DNA fragments of successfully amplified specimens were cleaned with NucleoMag NGS-Beads (bead volume at 0.9 times the total volume of the sample, Macherey Nagel, Düren, Germany) using the VP 407AM-N 96 Pin Magnetic Bead Extractor stamp (V&P Scientific, San Diego, CA, United States). Hereafter, the samples were labeled with the MiSeq Nextera XT DNA library preparation kit (Illumina, San Diego, CA, United States). For 18S, 3 µL of cleaned DNA product was mixed to 5 µL Milli-Q water, 10 µL Environmental or KAPA Master Mix, 1 µL NXT_S label and 1 µL NXT_N label for a total volume of 20 µL. The PCR2 program for the Environmental Master Mix was 10 min at 95°C, followed by 8 cycles of 30 s at 95°C, 1 min at 55°C, 30 s at 72°C, followed by 7 min at 72°C. The program for KAPA Mix was 3 min at 95°C, followed by 8 cycles of 20 s at 98°C, 30 s at 55°C, 30 s at 72°C, followed by 5 min at 72°C. For COI, the same reaction was used as for the first amplification, using specific NXT_S and NXT_N labeled primers. The PCR2 program was 3 min at 96°C, followed by 8 cycles of 15 s at 96°C, 30 s at 55°C, 40 s at 72°C, followed by 5 min at 72°C. A blank was performed with Milli-Q water instead of DNA template for each PCR run to check for (cross-)contamination. All blanks were negative. The samples were analyzed with the Agilent 5300 Fragment analyzer with the DNF-910-33 dsDNA Reagent Kit (35–1,500 bp) protocol (Agilent Technologies, Santa Clara, CA, United States) to confirm successful labeling of the DNA fragments. Separately for both markers, the samples were pooled together with QIAgility (Qiagen, Hilden, Germany). The end-pools were cleaned afterward with NucleoMag NGS-Beads, eluted in Milli-Q and DNA concentration measured using Tapestation 4150 (Kit HSD 5000, Agilent Technologies, Santa Clara, CA, United States). The sequencing was performed in two runs (one for each marker) on an Illumina MiSeq V3 PE300 platform (pair-end sequencing 2 × 300 bp) at BaseClear B.V. (Leiden, Netherlands).

Data Quality Filtering

Raw reads from the sequencing runs were merged using FLASH algorithm (settings minimum overlap = 50, maximum overlap = 300, mismatch ratio = 0.2) (Magoè and Salzberg, 2011). The primers were trimmed with cutadapt (minimum bases that need to match = 10, maximum allowed error rate = 0.2, minimum read length = 10) (Martin, 2011). Sequences that have base pairs with a quality score below 30 were filtered out in Usearch (function -fastq_truncqual 30) (Edgar and Flyvbjerg, 2015). We retained sequences within a specific fragment size range depending on the marker used (COI sequences = 300–330 bp, 18S sequences = 250–330 bp) using PRINSEQ (Schmieder and Edwards, 2011). The wide range allowed for 18S is the result of different mean fragment lengths between foraminiferal species. The denoising method UNOISE (settings alpha = 4.0, minimum abundance before clustering = 8) was used to cluster sequences into amplicon sequence variants (ASVs) (Edgar, 2016). From these clusters, an ASV table was produced. ASVs can be interpreted as gene copies present in a specimen. Samples for which less than 10% of the original (raw) reads were retained after bioinformatic processing and which did not exceed 1,000 reads in total were not further analyzed.

The ASV table was carefully screened and controlled in R (R Core Team, 2020). ASVs with a read count below 1.5% of the total sample read count were set to zero to reduce bias due to possible low-key cross-contamination and barcode switching during sequencing. This threshold was selected based on rarefaction curves (see method in section "Data Analysis")

(**Supplementary Figure 1**). ASVs that were at least present in two specimens of a genus (to account for blurry boundaries between morphospecies) were retained for the analysis in order to assess intra- and inter-specimen genetic variability. Additionally, ASVs that were present in only one specimen were retained (for intra-specimen variation analysis). ASVs from species with two or less specimens were also retained.

Data Analysis

To evaluate which threshold the ASVs should be filtered to, rarefaction curves were calculated with the vegan R package [function rarecurve()] (Oksanen et al., 2007). Sequence analysis was performed in Geneious Prime 2019.2.3[1]. Sequences for each marker were aligned with the MAFFT v7.450 algorithm using default settings in Geneious (Katoh and Standley, 2013). Sequences that did not align properly [i.e., created important gap regions (>10 bp)] and did not give any BLAST[2] results somewhat similar to the phylum Insecta, which is a common blast result for Foraminifera COI due to a lack of foraminiferal COI sequences in the reference database (Macher et al., 2021b), were removed from the analysis. Here, such sequences are referred to as "flagged" sequences. Gaps were masked from the alignment at a 50% threshold in Geneious. COI and 18S tree constructions were calculated in IQ-Tree (Trifinopoulos et al., 2016) using default settings with 1,000 iterations for bootstrap support. The trees were midpoint rooted and formatted in R. Additionally, species delimitation by automatic partitioning (ASAP[3]) (Puillandre et al., 2021) was performed to analyze whether the different ASVs clusters align with described morphospecies [settings: Kimura K80 (ts/tv = 2.0)]. ASAP was performed separately for the order Rotaliida and Miliolida, because the algorithm does not accurately delimit species in datasets with sequence clusters that are highly divergent, following Yule speciation model (Puillandre et al., 2021). From the ASAP clustering results, we applied Morard's integrative molecular taxonomic system to screen for cryptic species in our dataset (Morard et al., 2016). In short, Morard's system uses molecular operational taxonomic units (MOTUs) at three different levels by filtering for genetic gaps within species. From the morphospecies level, MOTUs level-1 were identified from a deep molecular gap (known as the "barcoding gap"), MOTUs level-2 identified from a shallow molecular gap and MOTUs level-3 represented by ASVs (the tips of the tree). To apply Morard's system, we assume that specimens from the same morphospecies that share at least one ASV are considered to be the same MOTU level-2, even if they show different intra-specimen variability.

Additional comparative analyses were performed on taxa for which at least five specimens per analyzed marker were available. Barcoding gap analysis was performed using patristic distances (sum of branch length between two nodes in a tree) matrix calculated from a FastTree with default settings in Geneious (Fourment and Gibbs, 2006). Technical replicates from the same specimens and the same sequences were not compared, to avoid the bias toward low patristic distances. Because patristic distances form a bimodal distribution, two statistical tests that do not assume normal distribution of the data were performed. Levene's test (Fox and Weisberg, 2018) and Fligner-Killeen test (Conover et al., 1981) for homogeneity of variances were used to assess whether the genetic variability between both markers is significantly different. These tests were computed in R using the "car" package [functions leveneTest() and fligner.test()] (Fox and Weisberg, 2018). The ASV table, aligned sequences and related R codes are available on GitHub (https://github.com/EBGirard/ForamCOIvs18S).

[1] www.geneious.com
[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi
[3] https://bioinfo.mnhn.fr/abi/public/asap/

RESULTS

To assess intra- and inter-specimen genetic variability of the 18S and COI genes in Foraminifera, 200 specimens from the Spermonde Archipelago (Indonesia) and Burger's Zoo (Netherlands) representing two orders, six families, 13 genera and 22 species were analyzed by single-cell metabarcoding (**Figure 1**). For a more in-depth comparison between the two markers, all species ($n = 7$) with at least five specimens successfully sequenced per marker were selected: four from the order Miliolida (*Amphisorus* SpL, *Amphisorus* SpS, *Marginopora vertebralis, Parasorites* sp.) and three from the order Rotaliida (*Amphistegina radiata, Nummulites venosus, Operculina ammonoides*). Results from the data treatment and quality filtering, more specifically the number of reads and sample analyzed, are found in **Table 2**.

Amplification and Sequencing Success

The amplification protocol for 18S was less successful than the newly developed protocol for COI. Out of 200 extracted foraminiferal specimens, 151 specimens (from 22 species) could be amplified with the COI protocol (75.5%), whereas only a total of 73 specimens (16 species) could be amplified combining the results from both 18S protocols (36.5%) (**Table 2**). For the specimens extracted with Micro kit ($n = 117$), 94 specimens were successfully sequenced with COI marker (80%), whereas 64 were successful with 18S marker (45%). For the specimens extracted with GITC* ($n = 83$), 56 specimens were successfully sequenced with COI marker (67%), whereas only 12 were successful with 18S marker (14%). Additionally, a higher number of samples was retained after data treatment and quality filtering for COI (176/177 samples) compared to 18S (94/120 samples) (**Table 2**).

Genetic Variability

After quality filtering of the ASVs, eight taxa had a single (unique) sequence assigned to COI (i.e., *Amphisorus* SpL, *A.* SpS, *Amphistegina lessonii, A. papillosa, Calcarina* sp1, *Marginopora vertebralis, Operculinella cumingii, Parasorites* sp.) and nine taxa had a set of two characteristic COI sequences present in almost all specimens (**Table 3**), with usually one ASV much more abundant than the other (i.e., *Amphistegina radiata, Borelis schlumbergeri, Calcarina hispida, Neorotalia* spp., *Nummulites venosus, Operculina ammonoides, O. complanata and O.* LKI27 type). *Amphisorus* SpL and *A.* SpS., although morphologically distinct, share the same ASV for the COI marker, but have a

FIGURE 1 | External view of the 22 species barcoded. Order Rotaliida: **(A)** Amphistegina papillosa. **(B)** A. radiata. **(C)** A. lessonii. **(D)** Nummulites venosus. **(E)** Calcarina hispida. **(F)** Calcarina sp1. **(G)** Neorotalia gaimardi. **(H)** N. calcar. **(I)** Heterostegina depressa. **(J)** Operculina ammonoides. **(K)** O. LKI27 type. **(L)** Operculina sp1. **(M)** O. complanata. **(N)** Operculinella cumingii. Order Miliolida: **(O)** Sorites sp. **(P)** Parasorites sp. **(Q)** Amphisorus SpS. **(R)** A. SpL. **(S)** Marginopora vertebralis. **(T)** Borelis schlumbergeri. **(U)** Alveolinella quoyi and **(V)** Peneroplis planatus. The scale bar is 1 mm unless indicated otherwise.

different ASV for the 18S marker. *Peneroplis planatus* has only one ASV in each specimen but a total of two ASVs within the species for the COI marker. *Heterostegina depressa* specimens have two ASVs each, however, both specimens do not share the same two ASVs.

The variability of the 18S marker is greater in most species. It ranges from no ASVs shared between specimens in *A. quoyi* to 10 common, abundant ASVs in *A. radiata* (**Table 3**). Most specimens in a species do not share the same complete set of ASVs, but only few are in common. Besides the rotaliid taxon *N. venosus*, all other taxa for which we obtained both COI and 18S sequences have higher ratios of inter-specimen (between specimens) identical nucleotides and sequence pairwise identity in COI than in 18S (**Table 3** and **Figure 2**). Genetic distance between ASVs were also compared between both markers at each level (intra- and inter-specimen) (**Figure 2** and **Figure 3**). Both statistical tests (Levene and Fligner-Killeen) confirmed that inter-specimen genetic distances in COI are significantly smaller than in 18S for the seven compared species (**Supplementary Table 2**). The COI intra-specimen variability of *A. radiata* was much lower than for 18S, with seven specimens out of eight with only one (unique) ASV, and a single specimen with two ASVs (**Table 3** and **Figure 3**).

Sequences creating an abnormal variability in the datasets were mostly filtered out by applying the 1.5% read count threshold. However, one additional ASV was flagged in

TABLE 2 | Statistics on number of reads, samples and ASVs before and after quality filtering (see also **Supplementary Table 4** for more information on the impact of the quality filtering on the number of reads per sample).

	18S marker	COI marker
Total number of raw reads before quality filtering	10,598,688 reads	11,072,460 reads
Total number of reads after quality filtering	4,032,685 reads	5,103,124 reads
Total number of reads after applying 1.5% cutoff	3,494,557 reads	5,066,907 reads
Total number of samples amplified and sequenced	120 samples	177 samples
Total number of samples retained for analysis	94 samples	176 samples
Total number of ASVs before selection	1,383 ASVs	133 ASVs
Total number of ASVs retained for analysis	237 ASVs	51 ASVs
Total number of ASVs manually flagged	0 ASV	1 ASV
Total number of specimens	67 specimens	150 specimens
Total number of morphospecies	16 morphospecies	22 morphospecies
Total number of morphospecies with at least five specimens	7 morphospecies	13 morphospecies

The number of samples is higher than the number of specimens, because some specimens were amplified more than once (replicates).

the COI dataset and removed manually post processing (**Supplementary Table 3**). It had the desired fragment length, amplified with the right primers but created a 41-bp gap in the alignment with the other two ASVs (between the bases 180 and 220) in two specimens of the species *C. hispida*.

Phylogenetic Reconstruction and Species Delimitation

In both trees, rotaliid specimens cluster well together. With its decreased ASV number at the specimen and the species levels, the COI tree also has a decreased number of outlier sequences (**Figure 4A**). Indeed, the greater variability in 18S creates numerous inconsistencies in the phylogenetic reconstruction from *N. venosus* (specimens 3709, 3711 and 3712), from *Parasorites* sp. (specimen 3723) and from *O. ammonoides* (specimen 3725) (**Figure 4B**). The different morphospecies from the family Calcarinidae do not cluster as well in the 18S tree as in the COI. Miliolid specimens cluster very well in the COI tree, whereas the two alveolinid specimens have such a high variability in the 18S dataset that they do not cluster properly together nor with soritid specimens (**Figure 4**). Disregarding the high inter-specimen variability of certain species, both trees have similarities. For example, *N. venosus* is sister-group to *O. complanata*; *Parasorites* sp. is sister-group to *Amphisorus* spp. and to the clade formed by *M. vertebralis* and *Sorites* sp. Moreover, in both trees, *N. venosus* and *A. quoyi* are the two most variable species.

The ASAP algorithm performed better for delimiting morphospecies with the COI marker (p-value < 0.1) than with the 18S marker (p-value > 0.1) due to the lower variability of the mitochondrial DNA (**Table 4**). Nonetheless, the number of morphospecies was overestimated by the algorithm in both cases. The genetic distance threshold in Miliolida is smaller than in Rotaliida in both markers (**Table 4**). When applying Morard's integrative molecular taxonomic system on the datasets, we observed that COI and 18S are generally congruent in the estimated numbers of MOTUs level-2 and level-1 (**Table 5**). Seven species show divergences that could be interpreted as multiple MOTUs level-2. More interestingly, *A. quoyi* and *N. venosus* have two and three clusters, respectively, that appear to align with the concept of MOTUs level-2 and level-1 defined by Morard et al. (2016) in both markers. There are some exceptions, for example, *Calcarina* sp1 and *O. ammonoides* have two clusters aligning with MOTUs level-2 in the 18S dataset, but not in the COI one. Overall, most species do not show barcoding gaps that would result in multiple MOTUs level-1 based on Morard's system (**Table 5**).

DISCUSSION

Only one widely used molecular marker, the 18S rRNA, has a large reference database and is available for species identification in foraminifera (Ertan et al., 2004; Morard et al., 2015; Holzmann and Pawlowski, 2017; Borrelli et al., 2018). Additional nuclear markers have been studied, such as the ITS (Macher et al., 2021a), which is also often used for molecular analyses of protists in general (Thornhill et al., 2007; Pawlowski et al., 2016), the ribosomal large subunit (Pawlowski et al., 1994), actin (Flakowski, 2005), tubulin (Takishita et al., 2005) and multiple genes based on expressed sequence tags data (Sierra et al., 2013). Recently, Macher et al. (2021b) established a barcoding protocol for foraminiferal mitochondrial cytochrome c oxidase I (COI), based on Rotaliida and Miliolida sequences. Here, we use high-throughput sequencing to assess intra- and inter-specimen genetic variability of two foraminiferal molecular markers, in order to test the potential of the newly discovered COI compared to the widely used 18S.

Amplification Methodology

The low amplification success rate from the 18S maker, compared to the COI, might be linked to suboptimal amplification protocol, either from the ratio between DNA template and reagents, the type of reagents (i.e., mastermix choice) or inadequate amplification program. Nested or semi-nested PCRs have often been used to amplify ribosomal regions (e.g., Holzmann et al., 2003; Holzmann and Pawlowski, 2017; Morard et al., 2018) and might provide better amplification results. Since the COI marker amplified well and the targeted fragment for both markers was about the same size (up to 330 bp), the preservation of the samples in ethanol and the extraction protocol were likely not the issue.

High Molecular Diversity in Large Benthic Foraminifera

We performed metabarcoding with high sequencing depth on single specimens and found a high genetic variability in the foraminiferal taxa studied. Genetic variability differed between

TABLE 3 | Species genetic variability, intra-specimen and inter-specimen.

Morphospecies	Marker	Total number of specimens	Highest number of ASVs in a specimen	Lowest intra-specimen percent of identical nucleotide	Total number of ASVs	Number of ASVs shared among all specimens	Inter-specimen percent of identical nucleotide	Inter-specimen percent of pairwise identity
Alveolinella quoyi	COI	18	2	66.9	4	0	54.5	73.7
	18S	2	11	48.2	14	0	49.4	72.7
***Amphisorus* SpL**	COI	11	1	NA	1	1	100.0	100.0
	18S	15	3	61.3	11	1	49.9	72.2
***Amphisorus* SpS**	COI	10	1	NA	1	1	100.0	100.0
	18S	6	1	NA	2	0	96.9	96.9
Amphistegina lessonii	COI	2	1	NA	1	1	100.0	100.0
Amphistegina papillosa	COI	1	1	NA	1	NA	NA	NA
Amphistegina radiata	COI	8	2	99.4	2	1	99.4	99.4
	18S	5	19	72.1	51	2	71	87.2
Borelis schlumbergeri	COI	1	2	99.4	2	NA	NA	NA
Calcarina sp1	COI	2	1	NA	1	1	100.0	100.0
	18S	2	5	70	7	0	69.7	86.6
Calcarina hispida	COI	11	2	99.1	2	1	99.1	99.1
	18S	2	11	95.5	12	6	95	98.5
Heterostegina depressa	COI	10	2	99.1	4	0	98.5	99
Marginopora vertebralis	COI	6	1	NA	1	1	100.0	100.0
	18S	5	6	50.6	10	2	48.7	71.6
Neorotalia calar	COI	2	2	99.1	2	2	99.1	99.1
	18S	1	12	81.8	12	NA	NA	NA
Neorotalia gaimardi	COI	8	2	99.1	2	1	99.1	99.1
	18S	2	13	76.1	21	5	75.8	94.7
Nummulites venosus	COI	11	6	52.6	18	0	40.2	82.5
	18S	8	11	49.6	36	0	45.4	75.7
Operculina ammonoides	COI	17	2	99.1	2	1	99.1	99.1
	18S	9	11	50.7	31	0	50.4	85
Operculina complanata	COI	2	2	99.1	2	1	99.1	99.1
	18S	1	9	67.8	9	NA	NA	NA
Operculina LKI27 type	COI	1	2	99.1	2	NA	NA	NA
	18S	1	14	96.9	14	NA	NA	NA
Operculina sp1	COI	1	2	99.1	2	NA	NA	NA
Operculinella cumingii	COI	1	1	NA	1	NA	NA	NA
	18S	1	6	99.1	6	NA	NA	NA
***Parasorites* sp.**	COI	10	1	NA	1	1	100.0	100.0
	18S	6	5	54	9	1	52.1	73.7
Peneroplis planatus	COI	6	1	NA	2	0	99.7	99.7
Sorites sp.	COI	12	2	98.1	2	0	98.1	98.1
	18S	1	1	NA	1	NA	NA	NA

*Specimens with five or more specimens for both markers (marked in bold) were further analyzed for comparison between markers. See **Figure 1** for morphological traits.*

analyzed markers and species. Some species were well identified and showed low intra- and inter-specimen variability (e.g., *M. vertebralis* and *Parasorites* sp.), resulting in a single amplicon sequence variant (ASV) per species. Other species show a variability exceeding the commonly applied 97% operational taxonomic unit (OTU) threshold, with up to 59.8% of non-identical sites in *N. venosus*. Similar patterns were observed in the SSU rRNA of other benthic foraminifera (Pillet et al., 2012; Weber and Pawlowski, 2014) and amoebozoa protists like *Ripella* spp., overestimating the number of species because of intragenomic variability (Kudryavtsev and Gladkikh, 2017).

The genetic variability in both markers is highlighted by the species delimitation analysis that overestimated the number of species in our dataset for both markers (Thornhill et al., 2007). Highly variable sequences remaining in the dataset after quality filtering might be the result of non-functional pseudogenes encoded in the genome (Thornhill et al., 2007; Guillou et al., 2013; Graham et al., 2021). If not flagged, pseudogenes may participate in increasing the amount of intra-genomic variation (Schultz and Hebert, 2021), especially in markers with non-coding regions such as 18S. The observed intra-genomic variation in single specimens may also be caused by the presence of

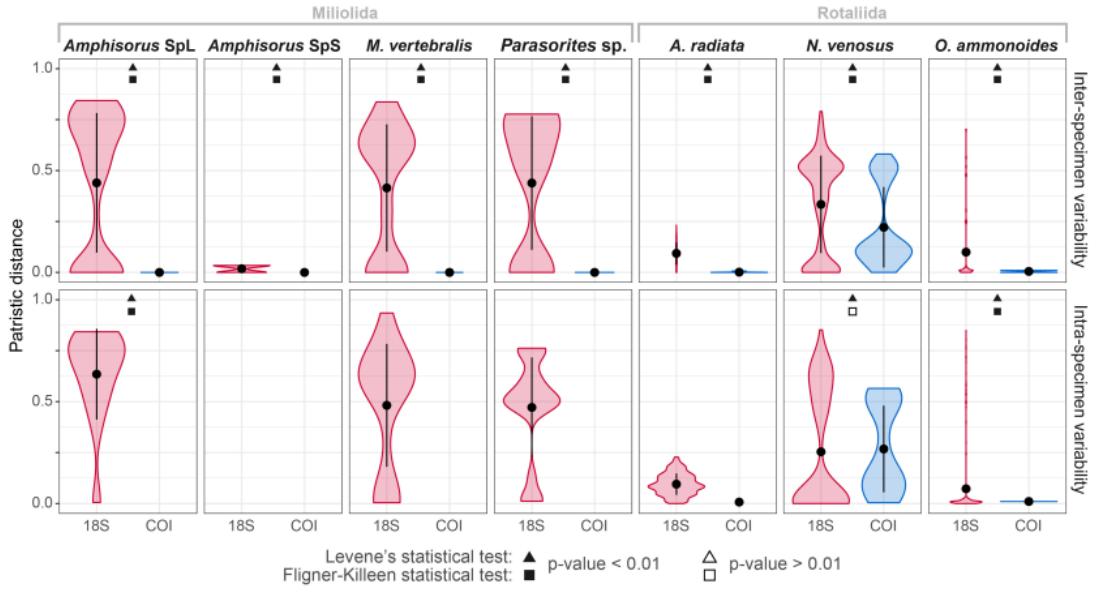

FIGURE 2 | Comparison of the intra- and inter-specimen genetic variability between COI and 18S markers based on patristic distances. The black circles represent the mean distance and the vertical black line the standard deviation from the mean. Statistical Levene's test (triangles) and Fligner-Killeen test (squares) were performed for each species and each variability level (intra- or inter-specimen). Complete statistical results in **Supplementary Table 2**. Significant differences in variance between both markers are indicated with black-filled shapes, whereas not significant results are indicated with white-filled shapes. The absence of boxplot in a facet means that there is no genomic variation (where the total number of ASVs = 1).

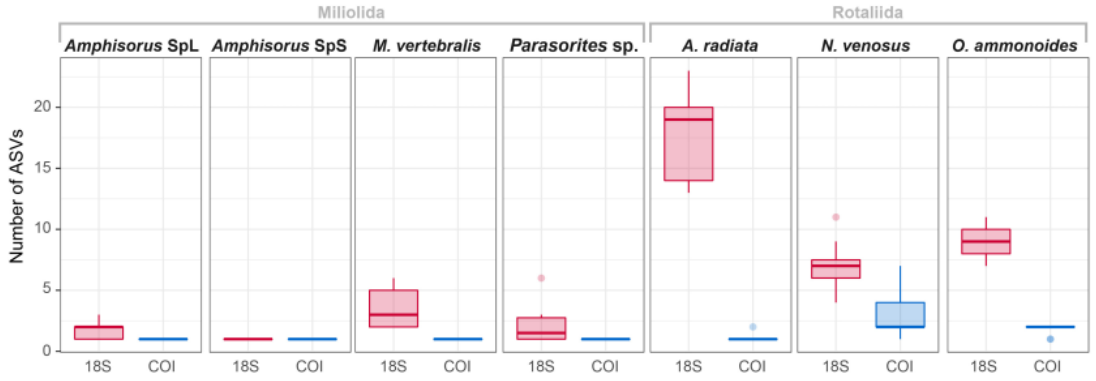

FIGURE 3 | Overview of the number of ASVs in each specimen for 18S and COI markers.

multiple nuclei within the cell (Zhao et al., 2019), a high number of gene copies in the genome (Kudryavtsev and Gladkikh, 2017; Milivojević et al., 2021), or hybridization events (Pillet et al., 2012). Indeed, foraminifera can reproduce by multiple fission, which results in specimens at a multinucleate agamont stage (Bé and Anderson, 1976; Lee et al., 1979; Parfrey and Katz, 2010; Milivojević et al., 2021). The presence of high intra-genomic variability in some of the studied species may also be the results of cryptic speciation. Cryptic species in benthic and planktonic foraminifera have been identified multiple times (e.g., Pawlowski and Holzmann, 2002, 2009; Majewski et al., 2015; Morard et al., 2016). Morard et al. (2016) developed an integrative molecular taxonomic system for community metabarcoding in order to identify biological species and cryptic diversity in planktonic foraminifera. We tried to apply Morard's system to our datasets even though our experimental design did not correspond to an eDNA sequencing output. The two species showing the most inter-specimen variability in both markers, *A. quoyi* and *N. venosus*, also clustered in what could be interpreted as multiple MOTUs

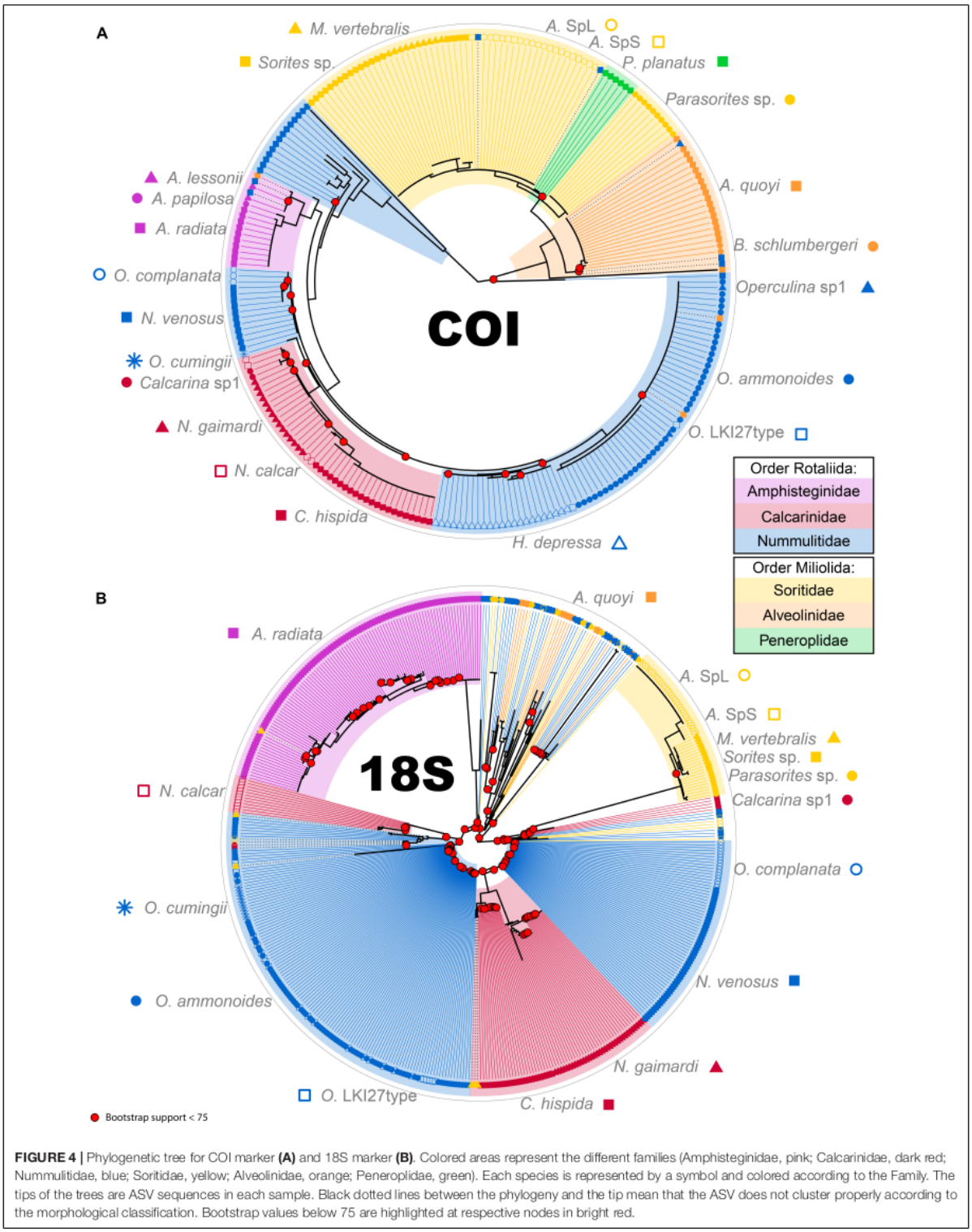

FIGURE 4 | Phylogenetic tree for COI marker **(A)** and 18S marker **(B)**. Colored areas represent the different families (Amphisteginidae, pink; Calcarinidae, dark red; Nummulitidae, blue; Soritidae, yellow; Alveolinidae, orange; Peneroplidae, green). Each species is represented by a symbol and colored according to the Family. The tips of the trees are ASV sequences in each sample. Black dotted lines between the phylogeny and the tip mean that the ASV does not cluster properly according to the morphological classification. Bootstrap values below 75 are highlighted at respective nodes in bright red.

TABLE 4 | ASAP results performed separately on the orders Rotaliida and Miliolida representing best the dataset.

Marker	Order	# morpho-species	# species based on ASAP algorithm	ASAP score	*p*-value	Threshold distance
COI	Miliolida	8	10	6.00	0.0782	0.009411
	Rotaliida	12*	20	7.00	0.0756	0.014101
18S	Miliolida	6	30	15.00	0.3850	0.038994
	Rotaliida	9**	27	15.00	0.5980	0.050352

**Operculina sp1, O. LKI27 type and O. ammonoides are considered as the Operculina ammonoides-group species since they have the same ASVs in COI.*
***Operculina LKI27 type and O. ammonoides are considered as the Operculina ammonoides-group species since they have very little variation between the species in 18S.*

level-1 according to Morard's system (Morard et al., 2016). We refrain from interpreting these levels of genetic variability until more data on variability within species and between populations is available.

Another hypothesis is the possible horizontal gene transfer that should not be disregarded, since about 1% in the protist genome can be inherited from horizontal gene transfer processes (Van Etten and Bhattacharya, 2020). Specific groups of foraminifera commonly live in symbiosis with prokaryotes and eukaryotes, e.g., the so-called large benthic foraminifera that obligatory house micro-algal symbionts (Prazeres and Renema, 2019 and references therein) and other groups that thrive in anaerobic conditions (Orsi et al., 2020) that might be the result of such a biological process (Keeling and Palmer, 2008). Species that have closely related ASVs to other unrelated taxa, e.g., *N. venosus* and *A. quoyi*, might also be the result of possible horizontal gene transfer during close cohabitation in densely packed communities. However, this phenomenon has not yet been observed in foraminifera (Pillet and Pawlowski, 2013). Cross-contamination is also a possibility, but precautions were strictly applied during laboratory work and appropriate data filtering was performed.

Our results show that 14 out of 22 taxa consistently had two or more mitochondrial ASVs associated with a single specimen. The number of ASVs for the ribosomal gene in a single specimen can be very high, for example, up to 10 ribosomal ASVs are equally abundant and shared between two or more *A. radiata* specimens. This highlights the need to rethink the method of a "single sequence" for species identification and delimitation. The extreme intragenomic variability observed in the foraminiferal 18S marker is not a unique phenomenon. It has been observed in marine ciliates (Zhao et al., 2019), ichthyosporeans (Lohr et al., 2010) and radiolarians (Decelle et al., 2014). Zhao et al. (2019) suggests the use of a 97% similarity cutoff to delimit marine ciliate OTUs aiming to remove the intragenomic polymorphism. We rather suggest the use of a set of sequences based on ASVs or approaches like ZOTUs (Edgar, 2016) to identify species at the molecular level in addition to using a combination of different markers, with two or more ASVs per marker. This proposition is even more worthwhile for markers that have a hypervariable component, such as 18S in Foraminifera. How using sets of sequences can be applied to metabarcoding and bulk sample analysis is a question that will need further targeted mock community analyses.

Effectiveness of Cytochrome Oxidase Subunit 1 vs. 18S Marker in Species Identification

The COI and 18S datasets enabled us to identify specimens that are slightly morphologically different, but were misclassified

TABLE 5 | Morard's integrative molecular taxonomic system results at each MOTUs level, for each species and each marker.

Morphospecies	Marker	MOTUs level-3 (ASVs)	MOTUs level-2	MOTUs level-1
Alveolinella quoyi	COI	4	2	2
	18S	14	2	2
***Amphisorus* SpL**	COI	1	1	1
	18S	11	1	1
***Amphisorus* SpS**	COI	1	1	1
	18S	2	1	1
Amphistegina lessonii	COI	1	1	1
Amphistegina papillosa	COI	1	1	1
Amphistegina radiata	COI	2	1	1
	18S	51	1	1
Borelis schlumbergeri	COI	2	1	1
Calcarina sp1	COI	1	1	1
	18S	7	2	1
Calcarina hispida	COI	2	1	1
	18S	12	1	1
Heterostegina depressa	COI	4	2	1
Marginopora vertebralis	COI	1	1	1
	18S	10	1	1
Neorotalia calar	COI	2	1	1
	18S	12	1	1
Neorotalia gaimardi	COI	2	1	1
	18S	21	1	1
Nummulites venosus	COI	18	3	3
	18S	36	3	3
Operculina ammonoides	COI	2	1	1
	18S	31	2	2
Operculina complanata	COI	2	1	1
	18S	9	1	1
Operculina LKI27 type	COI	2	1	1
	18S	14	1	1
Operculina sp1	COI	2	1	1
Operculinella cumingii	COI	1	1	1
	18S	6	1	1
***Parasorites* sp.**	COI	1	1	1
	18S	9	1	1
Peneroplis planatus	COI	2	2	1
Sorites sp.	COI	2	2	2
	18S	1	1	1

Specimens with five or more specimens for both markers (marked in bold) were further analyzed for comparison between markers.

during the morphospecies classification step (i.e., *Calcarina* sp1, *N. calcar* and *B. schlumbergeri*) (see **Figure 1**). Specimens from these three species had different mitochondrial and ribosomal sets of ASVs compared to their closely related taxon, which alerted us of their possible misclassification. It was clear from the COI dataset due to the decreased number of ASVs and genomic variability. The calcarinid 18S dataset blurred the molecular differences between species, but working with a set of ASVs instead of a single sequence has proven itself worthy to distinguish the misclassified species. Based on the latter findings, the inter-specimen variability observed in *H. depressa* and *P. planatus* hints at possible undiscovered biological species within these groups. The variability in *H. depressa* might be linked to its geographical molecular diversity, because the specimens come from an aquarium zoo where the populations are heterogeneous (Janse et al., 2008). Additionally, the taxonomy and morphological traits of the genus *Peneroplis* is yet unresolved (Langer et al., 2009; Consorti et al., 2020). Additional mitochondrial genomic data can therefore provide key insights on where to look for characteristic morphological traits in difficult taxonomic groups (Macher et al., 2021a). On the contrary, some taxa show more morphological than mitochondrial molecular variations. This is indeed the case for *Amphisorus* spp. that share the same unique COI sequence, but are distinguishable with the 18S fragment we sequenced. This example highlights the need to use multiple markers for robust molecular species identification. Another example is the *Operculina ammonoides* group, where specimens were classified in three morphological categories (i.e., *O. ammonoides*, *O.* LKI27type, *O.* sp1). Environmental drivers, such as hydrodynamics energy and depth, likely influenced the calcification pattern of their test, but the interaction between genetics and environmental pressures is still misunderstood (Pecheux, 1995; Oron et al., 2018).

Overall, for its decreased intragenomic variability and higher amplification success rate, the foraminiferal COI region is a great additional marker to combine with the 18S marker for molecular species identification. The same conclusion was obtained for ciliate protists (Zhao et al., 2016). The absence of several versions of this mitochondrial marker, indeed, renders its effectiveness over the ribosomal ones. The genetic code for the foraminiferal mitochondrion of the orders Miliolida and Rotaliida aligns well with translation **Table 4** (Macher, personal communication), common in rhizarians (Wideman et al., 2020), but is yet unstudied in other foraminiferal groups. Since the COI marker was developed and tested mainly on Rotaliida and Miliolida specimens, complementary analyses on other foraminiferal groups, such as monothalamids, should be performed to confirm the potential of the COI marker on the whole phylum Foraminifera.

Beyond Species Identification

Metabarcoding methods have been used to identify foraminiferal community composition in water (Morard et al., 2018) and sediment samples (Frontalini et al., 2020; Li et al., 2020; Cavaliere et al., 2021). A recent study, however, unraveled extreme variations in the number of SSU rRNA gene copies in the genome of planktonic foraminifera (Milivojević et al., 2021). Additionally, metabarcoding results of the SSU rRNA do not always align well with morphospecies delimitation (Morard et al., 2016). With its multiple advantages (e.g., less intragenomic variability, consistent fragment length, higher potential for quality filtering) (Andújar et al., 2021; Antich et al., 2021), the mitochondrial marker is very promising for foraminiferal community metabarcoding. Together with previous studies, we call for cautions when using ribosomal markers in metabarcoding of protists as it likely leads to an exaggerated estimation of the number of species in environmental samples (Gribble and Anderson, 2007; Kudryavtsev and Gladkikh, 2017).

In light of our results, the foraminiferal COI set of sequences provides a great addition to the 18S reference sequence database for a more robust species identification at the molecular level. This newly discovered mitochondrial marker has great potential in molecular foraminiferal research that, we believe, will successfully support future metabarcoding projects toward environmental biomonitoring. Since this is a new marker, interpretation of metabarcoding data is still limited by the small number of available references.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OK415028-OK415275; https://www.ncbi.nlm.nih.gov/genbank/, OK634032-OK634088; https://www.ncbi.nlm.nih.gov/, PRJNA767744.

AUTHOR CONTRIBUTIONS

WR, OW, J-NM, EG, and AL participated in the method development. WR and JJ organized fieldwork. WR collected the samples and identified the specimens. EG and AL performed the experiment and wrote the manuscript. EG analyzed the data. WR, J-NM, and OW revised and provided feedback on the manuscript. All authors approved the final version of the manuscript.

FUNDING

This research was funded by the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie grant agreement (4D-REEF, No 813360). Fieldwork in 2018 was supported by NWO-VIDI (#16.161.301).

ACKNOWLEDGMENTS

We would like to thank all persons who participated in the field campaign in 2018 for collecting the samples. We would also like to thank Elza Duijm, Frank Stokvis, and Roland Butôt for their help at the NCB lab. We would further like to thank the Indonesian authorities for providing the research visa and permits (research permit holder: WR; SIP

no: 115/SIP/FRP/E5/Dit.KI/IV/2018) to conduct the research activities in the Spermonde Archipelago, in collaboration with Hasanuddin University (UNHAS, Makassar). We would like to thank the reviewers for their time and constructive input on our manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.809659/full#supplementary-material

REFERENCES

Amaral-Zettler, L. A., McCliment, E. A., Ducklow, H. W., and Huse, S. M. (2009). A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PLoS One* 4:e6372. doi: 10.1371/journal.pone.0006372

Andújar, C., Creedy, T. J., Arribas, P., López, H., Salces-Castellano, A., Pérez-Delgado, A. J., et al. (2021). Validated removal of nuclear pseudogenes and sequencing artefacts from mitochondrial metabarcode data. *Mol. Ecol. Resour.* 21, 1772–1787. doi: 10.1111/1755-0998.13337

Antich, A., Palacin, C., Wangensteen, O. S., and Turon, X. (2021). To denoise or to cluster, that is not the question: optimizing pipelines for COI metabarcoding and metaphylogeography. *BMC Bioinformatics* 22:177. doi: 10.1186/s12859-021-04115-6

Bé, A. W., and Anderson, O. R. (1976). Gametogenesis in planktonic Foraminifera. *Science* 192, 890–892. doi: 10.1126/science.946914

Billups, K., and Schrag, D. P. (2003). Application of benthic foraminiferal Mg/Ca ratios to questions of Cenozoic climate change. *Earth Planet. Sci. Lett.* 209, 181–195. doi: 10.1016/S0012-821X(03)00067-0

Borrelli, C., Hou, Y., Pawlowski, J. W., Holzmann, M., Katz, M. E., Chandler, G. T., et al. (2018). Assessing SSU rDNA barcodes in Foraminifera: a case study using bolivina quadrata. *J. Eukaryot. Microbiol.* 65, 220–235. doi: 10.1111/jeu.12471

Burki, F., Sandin, M. M., and Jamy, M. (2021). Diversity and ecology of protists revealed by metabarcoding. *Curr. Biol.* 31, R1267–R1280. doi: 10.1016/j.cub.2021.07.066

Capotondi, L., Maria Borsetti, A., and Morigi, C. (1999). Foraminiferal ecozones, a high resolution proxy for the late Quaternary biochronology in the central Mediterranean Sea. *Mar. Geol.* 153, 253–274. doi: 10.1016/S0025-3227(98)00079-6

Cavaliere, M., Barrenechea Angeles, I., Montresor, M., Bucci, C., Brocani, L., Balassi, E., et al. (2021). Assessing the ecological quality status of the highly polluted Bagnoli area (Tyrrhenian Sea, Italy) using foraminiferal eDNA metabarcoding. *Sci. Total Environ.* 790:147871. doi: 10.1016/j.scitotenv.2021.147871

Conover, W. J., Johnson, M. E., and Johnson, M. M. (1981). A comparative study of tests for homogeneity of variances, with applications to the outer continental shelf bidding data. *Technometrics* 23, 351–361. doi: 10.1080/00401706.1981.10487680

Consorti, L., Kavazos, C. R. J., Ford, C., Smith, M., and Haig, D. W. (2020). High productivity of Peneroplis (Foraminifera) including aberrant morphotypes, in an inland thalassic salt pond at Lake Macleod, Western Australia. *Mar. Micropaleontol.* 160:101919. doi: 10.1016/j.marmicro.2020.101919

Decelle, J., Romac, S., Sasaki, E., Not, F., and Mahé, F. (2014). Intracellular diversity of the V4 and V9 regions of the 18S rRNA in marine protists (radiolarians) assessed by high-throughput sequencing. *PLoS One* 9:e104297. doi: 10.1371/journal.pone.0104297

Edgar, R. C. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv [Prerpint]* doi: 10.1101/081257

Edgar, R. C., and Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* 31, 3476–3482. doi: 10.1093/bioinformatics/btv401

Ertan, K. T., Hemleben, V., and Hemleben, C. (2004). Molecular evolution of some selected benthic Foraminifera as inferred from sequences of the small subunit ribosomal DNA. *Mar. Micropaleontol.* 53, 367–388. doi: 10.1016/j.marmicro.2004.08.001

Flakowski, J. (2005). Actin phylogeny of Foraminifera. *J. Foraminiferal Res.* 35, 93–102. doi: 10.2113/35.2.93

Fourment, M., and Gibbs, M. J. (2006). PATRISTIC: a program for calculating patristic distances and graphically comparing the components of genetic change. *BMC Evol. Biol.* 6:1. doi: 10.1186/1471-2148-6-1

Fox, J., and Weisberg, S. (2018). *An R Companion to Applied Regression*. New York, NY: SAGE Publications.

Frontalini, F., Cordier, T., Balassi, E., Armynot du Chatelet, E., Cermakova, K., Apothéloz-Perret-Gentil, L., et al. (2020). Benthic foraminiferal metabarcoding and morphology-based assessment around three offshore gas platforms: congruence and complementarity. *Environ. Int.* 144:106049. doi: 10.1016/j.envint.2020.106049

Girard, E. B., Estradivari, Ferse, S., Ambo-Rappe, R., Jompa, J., and Renema, W. (2022). Dynamics of large benthic foraminiferal assemblages: A tool to foreshadow reef degradation? *Sci. Total Environ.* 811:151396. doi: 10.1016/j.scitotenv.2021.151396

Graham, N. R., Gillespie, R. G., and Krehenwinkel, H. (2021). Towards eradicating the nuisance of numts and noise in molecular biodiversity assessment. *Mol. Ecol. Resour.* 21, 1755–1758. doi: 10.1111/1755-0998.13414

Gribble, K. E., and Anderson, D. M. (2007). High intraindividual, intraspecific, and interspecific variability in large-subunit ribosomal DNA in the heterotrophic dinoflagellates Protoperidinium, Diplopsalis, and Preperidinium (Dinophyceae). *Phycologia* 46, 315–324. doi: 10.2216/06-68.1

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., et al. (2013). The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Res.* 41, D597–D604. doi: 10.1093/nar/gks1160

Hallock, P., Lidz, B. H., Cockey-Burkhard, E. M., and Donnelly, K. B. (2003). Foraminifera as bioindicators in coral reef assessment and monitoring: the FoRAM Index. *Environ. Monit. Assess.* 81, 221–238. doi: 10.1023/A:1021337310386

Hebert, P. D. N., Cywinska, A., Ball, S. L., and deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270, 313–321. doi: 10.1098/rspb.2002.2218

Holzmann, M., and Pawlowski, J. (2017). An updated classification of rotaliid Foraminifera based on ribosomal DNA phylogeny. *Mar. Micropaleontol.* 132, 18–34. doi: 10.1016/j.marmicro.2017.04.002

Holzmann, M., Habura, A., Giles, H., Bowser, S. S., and Pawlowski, J. (2003). Freshwater foraminiferans revealed by analysis of environmental DNA samples. *J. Eukaryot. Microbiol.* 50, 135–139. doi: 10.1111/j.1550-7408.2003.tb00248.x

Janse, M., Wensing, J., Gieling, H., and de Jongh, T. (2008). "Ecological management of a large coral reef eco-display at Burgers' Zoo, Arnhem, The Netherlands," in *Advances in Coral Husbandry in Public Aquariums Public Aquarium Husbandry Series*, eds R. J. Leewis and M. Janse (Arnhem: Burgers' Zoo), 293–303.

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Keeling, P. J., and Palmer, J. D. (2008). Horizontal gene transfer in eukaryotic evolution. *Nat. Rev. Genet.* 9, 605–618. doi: 10.1038/nrg2386

Kudryavtsev, A., and Gladkikh, A. (2017). Two new species of Ripella (Amoebozoa, Vannellida) and unusual intragenomic variability in the SSU rRNA gene of this genus. *Eur. J. Protistol.* 61, 92–106. doi: 10.1016/j.ejop.2017.09.003

Langer, M. R., Makled, W. A., Pietsch, S. J., and Weinmann, A. (2009). Asynchronous calcification in juvenile megalospheres: an ontogenetic window into the life cycle and polymorphism of Peneroplis. *J. Foraminiferal Res.* 39, 8–14. doi: 10.2113/gsjfr.39.1.8

Lee, J. J., McEnery, M. E., Kahn, E. G., and Schuster, F. L. (1979). Symbiosis and the evolution of larger Foraminifera. *Micropaleontology* 25, 118–140. doi: 10.2307/1485262

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., et al. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Front. Zool.* 10:34. doi: 10.1186/1742-9994-10-34

Li, Q., Lei, Y., Morard, R., Li, T., and Wang, B. (2020). Diversity hotspot and unique community structure of Foraminifera in the world's deepest marine blue hole - Sansha Yongle Blue Hole. *Sci. Rep.* 10:10257. doi: 10.1038/s41598-020-67221-0

Lohr, J. N., Laforsch, C., Koerner, H., and Wolinska, J. (2010). A Daphnia parasite (Caullerya mesnili) constitutes a new member of the Ichthyosporea, a group of protists near the animal-fungi divergence. *J. Eukaryot. Microbiol.* 57, 328–336. doi: 10.1111/j.1550-7408.2010.00479.x

Macher, J.-N., Wideman, J. G., Girard, E. B., Langerak, A., Duijm, E., Jompa, J., et al. (2021b). First report of mitochondrial COI in Foraminifera and implications for DNA barcoding. *Sci. Rep.* 11:22165. doi: 10.1038/s41598-021-01589-5

Macher, J.-N., Prazeres, M., Taudien, S., Jompa, J., Sadekov, A., and Renema, W. (2021a). Integrating morphology and metagenomics to understand taxonomic variability of Amphisorus (Foraminifera, Miliolida) from Western Australia and Indonesia. *PLoS One* 16:e0244616. doi: 10.1371/journal.pone.0244616

Magoè, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Majewski, W., Bowser, S. S., and Pawlowski, J. (2015). Widespread intra-specific genetic homogeneity of coastal Antarctic benthic Foraminifera. *Polar Biol.* 38, 2047–2058. doi: 10.1007/s00300-015-1765-1

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Meusnier, I., Singer, G. A. C., Landry, J.-F., Hickey, D. A., Hebert, P. D. N., and Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics* 9:214. doi: 10.1186/1471-2164-9-214

Milivojević, T., Rahman, S. N., Raposo, D., Siccha, M., Kucera, M., and Morard, R. (2021). High variability in SSU rDNA gene copy number among planktonic Foraminifera revealed by single-cell qPCR. *ISME Communications* 1:63. doi: 10.1038/s43705-021-00067-3

Morard, R., Darling, K. F., Mahé, F., Audic, S., Ujiié, Y., Weiner, A. K. M., et al. (2015). PFR^2: a curated database of planktonic Foraminifera 18S ribosomal DNA as a resource for studies of plankton ecology, biogeography and evolution. *Mol. Ecol. Resour.* 15, 1472–1485. doi: 10.1111/1755-0998.12410

Morard, R., Escarguel, G., Weiner, A. K. M., André, A., Douady, C. J., Wade, C. M., et al. (2016). Nomenclature for the nameless: a proposal for an integrative molecular taxonomy of cryptic diversity exemplified by planktonic Foraminifera. *Syst. Biol.* 65, 925–940. doi: 10.1093/sysbio/syw031

Morard, R., Garet-Delmas, M.-J., Mahé, F., Romac, S., Poulain, J., Kucera, M., et al. (2018). Surface ocean metabarcoding confirms limited diversity in planktonic Foraminifera but reveals unknown hyper-abundant lineages. *Sci. Rep.* 8:2539. doi: 10.1038/s41598-018-20833-z

Nassonova, E., Smirnov, A., Fahrni, J., and Pawlowski, J. (2010). Barcoding amoebae: comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose amoebae. *Protist* 161, 102–115. doi: 10.1016/j.protis.2009.07.003

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., et al. (2007). *The Vegan Package*. Available online at: https://cran.r-project.org/web/packages/vegan/index.html (accessed August 1, 2021).

Oliver, L. M., Fisher, W. S., Dittmar, J., Hallock, P., Campbell, J., Quarles, R. L., et al. (2014). Contrasting responses of coral reef fauna and foraminiferal assemblages to human influence in La Parguera, Puerto Rico. *Mar. Environ. Res.* 99, 95–105. doi: 10.1016/j.marenvres.2014.04.005

Oron, S., Abramovich, S., Almogi-Labin, A., Woeger, J., and Erez, J. (2018). Depth related adaptations in symbiont bearing benthic Foraminifera: new insights from a field experiment on Operculina ammonoides. *Sci. Rep.* 8:11175. doi: 10.1038/s41598-018-27838-8

Orsi, W. D., Morard, R., Vuillemin, A., Eitel, M., Wörheide, G., Milucka, J., et al. (2020). Anaerobic metabolism of Foraminifera thriving below the seafloor. *ISME. J.* 14, 2580–2594. doi: 10.1038/s41396-020-0708-1

Parfrey, L. W., and Katz, L. A. (2010). Dynamic genomes of eukaryotes and the maintenance of genomic integrity. *Microbe Magazine* 5:156.

Pawłowska, J., Wollenburg, J. E., Zajączkowski, M., and Pawlowski, J. (2020). Planktonic Foraminifera genomic variations reflect paleoceanographic changes in the Arctic: evidence from sedimentary ancient DNA. *Sci. Rep.* 10:15102. doi: 10.1038/s41598-020-72146-9

Pawlowski, J., and Holzmann, M. (2002). Molecular phylogeny of Foraminifera a review. *Eur. J. Protistol.* 38, 1–10. doi: 10.1078/0932-4739-00857

Pawlowski, J., and Holzmann, M. (2009). "Diversity and geographic distribution of benthic Foraminifera: a molecular perspective," in *Protist Diversity and Geographical Distribution*, eds W. Foissner and D. L. Hawksworth (Dordrecht: Springer Netherlands), 83–94. doi: 10.1007/978-90-481-2801-3_7

Pawlowski, J., Audic, S., Adl, S., Bass, D., Belbahri, L., Berney, C., et al. (2012). CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. *PLoS Biol.* 10:e1001419. doi: 10.1371/journal.pbio.1001419

Pawlowski, J., Bolivar, I., Fahrni, J., and Zaninetti, L. (1994). Taxonomic identification of Foraminifera using ribosomal DNA sequences. *Micropaleontology* 40, 373–377.

Pawlowski, J., Lejzerowicz, F., Apotheloz-Perret-Gentil, L., Visco, J., and Esling, P. (2016). Protist metabarcoding and environmental biomonitoring: time for change. *Eur. J. Protistol.* 55, 12–25. doi: 10.1016/j.ejop.2016.02.003

Pecheux, M. J.-F. (1995). Ecomorphology of a recent largeforaminifer, Operculina ammonoides. *Geobios Mem. Spec.* 28, 529–566. doi: 10.1016/S0016-6995(95)80209-6

Pillet, L., and Pawlowski, J. (2013). Transcriptome analysis of foraminiferan Elphidium margaritaceum questions the role of gene transfer in kleptoplastidy. *Mol. Biol. Evol.* 30, 66–69. doi: 10.1093/molbev/mss226

Pillet, L., Fontaine, D., and Pawlowski, J. (2012). Intra-genomic ribosomal RNA polymorphism and morphological variation in Elphidium macellum suggests inter-specific hybridization in Foraminifera. *PLoS One* 7:e32373. doi: 10.1371/journal.pone.0032373

Prazeres, M., and Renema, W. (2019). Evolutionary significance of the microbial assemblages of large benthic Foraminifera. *Biol. Rev. Camb. Philos. Soc.* 94, 828–848. doi: 10.1111/brv.12482

Puillandre, N., Brouillet, S., and Achaz, G. (2021). ASAP: assemble species by automatic partitioning. *Mol. Ecol. Resour.* 21, 609–620. doi: 10.1111/1755-0998.13281

Purty, R. S., and Chatterjee, S. (2016). DNA barcoding: an effective technique in molecular taxonomy. *Austin J. Biotechnol. Bioeng.* 3:1059.

R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Renema, W. (2018). Terrestrial influence as a key driver of spatial variability in large benthic foraminiferal assemblage composition in the Central Indo-Pacific. *Earth Sci. Rev.* 177, 514–544. doi: 10.1016/j.earscirev.2017.12.013

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27, 863–864. doi: 10.1093/bioinformatics/btr026

Schultz, J., and Hebert, P. (2021). Do pseudogenes pose a problem for metabarcoding marine animal communities? *Authorea [Preprints]* doi: 10.22541/au.163277441.14875855/v1

Sierra, R., Matz, M. V., Aglyamova, G., Pillet, L., Decelle, J., Not, F., et al. (2013). Deep relationships of Rhizaria revealed by phylogenomics: a farewell to Haeckel's Radiolaria. *Mol. Phylogenet. Evol.* 67, 53–59. doi: 10.1016/j.ympev.2012.12.011

Takishita, K., Inagaki, Y., Tsuchiya, M., Sakaguchi, M., and Maruyama, T. (2005). A close relationship between Cercozoa and Foraminifera supported by phylogenetic analyses based on combined amino acid sequences of three cytoskeletal proteins (actin, alpha-tubulin, and beta-tubulin). *Gene* 362, 153–160. doi: 10.1016/j.gene.2005.08.013

Thornhill, D. J., Lajeunesse, T. C., and Santos, S. R. (2007). Measuring rDNA diversity in eukaryotic microbial systems: how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. *Mol. Ecol.* 16, 5326–5340. doi: 10.1111/j.1365-294X.2007.03576.x

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., and Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* 44, W232–W235. doi: 10.1093/nar/gkw256

Ueno, K., Ha, T. T. N., and Iryu, Y. (2019). Foraminiferal biochronology of the triassic hoang mai formation, central vietnam. *J. Foraminiferal Res.* 49, 339–354. doi: 10.2113/gsjfr.49.3.339

van der Loos, L. M., and Nijland, R. (2021) Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. *Mol Ecol.* 30, 3270–3288. doi: 10.1111/mec.15592

Van Etten, J., and Bhattacharya, D. (2020). Horizontal gene transfer in eukaryotes: not if, but how much? *Trends Genet.* 36, 915–925. doi: 10.1016/j.tig.2020.08.006

Wangensteen, O. S., and Turon, X. (2017). "Metabarcoding Techniques for assessing biodiversity of marine animal forests," in *Marine Animal Forests: The Ecology of Benthic Biodiversity Hotspots*, eds S. Rossi, L. Bramanti, A. Gori, and C. Orejas Saco del Valle (Cham: Springer International Publishing), 445–473. doi: 10.1007/978-3-319-21012-4_53

Weber, A. A.-T., and Pawlowski, J. (2014). Wide occurrence of SSU rDNA intragenomic polymorphism in Foraminifera and its implications for molecular species identification. *Protist* 165, 645–661. doi: 10.1016/j.protis.2014.07.006

Weiner, A. K. M., Morard, R., Weinkauf, M. F. G., Darling, K. F., André, A., Quillévéré, F., et al. (2016). Methodology for single-cell genetic analysis of planktonic Foraminifera for studies of protist diversity and evolution. *Front. Mar. Sci.* 3:255. doi: 10.3389/fmars.2016.00255

Wideman, J. G., Monier, A., Rodríguez-Martínez, R., Leonard, G., Cook, E., Poirier, C., et al. (2020). Unexpected mitochondrial genome diversity revealed by targeted single-cell genomics of heterotrophic flagellated protists. *Nat. Microbiol.* 5, 154–165. doi: 10.1038/s41564-019-0605-4

Yeo, D., Srivathsan, A., and Meier, R. (2020). Longer is not always better: optimizing barcode length for large-scale species discovery and identification. *Syst. Biol.* 69, 999–1015. doi: 10.1093/sysbio/syaa014

Zhao, F., Filker, S., Xu, K., Li, J., Zhou, T., and Huang, P. (2019). Effects of intragenomic polymorphism in the SSU rRNA gene on estimating marine microeukaryotic diversity: a test for ciliates using single-cell high-throughput DNA sequencing. *Limnol. Oceanogr. Methods* 17, 533–543. doi: 10.1002/lom3.10330

Zhao, Y., Yi, Z., Gentekaki, E., Zhan, A., Al-Farraj, S. A., and Song, W. (2016). Utility of combining morphological characters, nuclear and mitochondrial genes: an attempt to resolve the conflicts of species identification for ciliated protists. *Mol. Phylogenet. Evol.* 94, 718–729. doi: 10.1016/j.ympev.2015.10.017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors WR.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Mitochondrial Cytochrome Oxidase Subunit 1: A Promising Molecular Marker for Species Identification in Foraminifera

ORIGINALITY REPORT

19%	14%	14%	9%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.nature.com Internet Source	2%
2	Submitted to Leiden University Student Paper	2%
3	Submitted to Broward Community College Student Paper	2%
4	Submitted to Macquarie University Student Paper	1%
5	Elsa B. Girard, Estradivari, Sebastian Ferse, Rohani Ambo-Rappe, Jamaluddin Jompa, Willem Renema. "Dynamics of large benthic foraminiferal assemblages: A tool to foreshadow reef degradation?", Science of The Total Environment, 2022 Publication	1%
6	wrap.warwick.ac.uk Internet Source	1%
7	link.springer.com Internet Source	1%

8	pdfs.semanticscholar.org Internet Source	<1 %
9	cris.leibniz-zmt.de Internet Source	<1 %
10	Submitted to University of Wales, Bangor Student Paper	<1 %
11	www.mdpi.com Internet Source	<1 %
12	www.vliz.be Internet Source	<1 %
13	escholarship.org Internet Source	<1 %
14	peerj.com Internet Source	<1 %
15	Andrés Carrazco-Montalvo, Patricio Ponce, Stephany D. Villota, Emmanuelle Quentin et al. "Establishment, Genetic Diversity, and Habitat Suitability of Aedes albopictus Populations from Ecuador", Insects, 2022 Publication	<1 %
16	www.esp.org Internet Source	<1 %
17	eprints.gla.ac.uk Internet Source	<1 %
	usir.salford.ac.uk	

18	Internet Source	<1%
19	archimer.ifremer.fr Internet Source	<1%
20	commons.ln.edu.hk Internet Source	<1%
21	Submitted to Liverpool John Moores University Student Paper	<1%
22	nbn-resolving.de Internet Source	<1%
23	Jan-Niklas Macher, Dimitra Maria Bloska, Maria Holzmann, Elsa B. Girard, Jan Pawlowski, Willem Renema. "Mitochondrial cytochrome c oxidase subunit I (COI) metabarcoding of Foraminifera communities using taxon-specific primers", PeerJ, 2022 Publication	<1%
24	Submitted to West Coast University Student Paper	<1%
25	d-nb.info Internet Source	<1%
26	Sayaka Takahashi, Masayuki K. Sakata, Toshifumi Minamoto, Reiji Masuda. "Comparing the efficiency of open and enclosed filtration systems in environmental	<1%

	DNA quantification for fish and jellyfish", PLOS ONE, 2020 Publication	
27	bg.copernicus.org Internet Source	<1 %
28	bjo.bmj.com Internet Source	<1 %
29	data.ingv.it Internet Source	<1 %
30	pubmed.ncbi.nlm.nih.gov Internet Source	<1 %
31	Caroline F. Dawber. "Constraints on glaciation in the middle Eocene (46–37 Ma) from Ocean Drilling Program (ODP) Site 1209 in the tropical Pacific Ocean", Paleoceanography, 04/27/2011 Publication	<1 %
32	fhpkat.kobv.de Internet Source	<1 %
33	research.bangor.ac.uk Internet Source	<1 %
34	"Full Issue PDF", Phytobiomes Journal, 2019 Publication	<1 %
35	9pdf.net Internet Source	<1 %

36	Chiara Borrelli, Yubo Hou, Jan W. Pawlowski, Maria Holzmann, Miriam E. Katz, G. Thomas Chandler, Samuel S. Bowser. " Assessing SSU rDNA Barcodes in Foraminifera: A Case Study using ", Journal of Eukaryotic Microbiology, 2018 Publication	<1 %
37	Philippe Borsa, Collin Taylor Williams, Ashlie Jane McIvor, Thierry B. Hoareau, Michael L. Berumen. "Neotype designation and re-description of Forsskål's reticulate whipray Himantura uarnak", Marine Biodiversity, 2021 Publication	<1 %
38	ddd.uab.cat Internet Source	<1 %
39	theses-test.ncl.ac.uk Internet Source	<1 %
40	www.um.edu.mt Internet Source	<1 %
41	Langer, Gerald, Aleksey Sadekov, Silke Thoms, Antje Mewes, Gernot Nehrke, Mervyn Greaves, Sambuddha Misra, Jelle Bijma, and Henry Elderfield. "Li partitioning in the benthic foraminifera Amphistegina lessonii", Geochemistry Geophysics Geosystems, 2015. Publication	<1 %
	dipot.ulb.ac.be	

42	Internet Source	<1%
43	orca.cardiff.ac.uk Internet Source	<1%
44	pure.hw.ac.uk Internet Source	<1%
45	research-repository.st-andrews.ac.uk Internet Source	<1%
46	serval.unil.ch Internet Source	<1%
47	ubffm.hds.hebis.de Internet Source	<1%
48	ueaeprints.uea.ac.uk Internet Source	<1%
49	www.barcodeoflife.org Internet Source	<1%
50	www.pure.ed.ac.uk Internet Source	<1%
51	www.zora.uzh.ch Internet Source	<1%
52	Elsa B. Girard, Estradivari, Sebastian Ferse, Rohani Ambo-Rappe, Jamaluddin Jompa, Willem Renema. "Dynamics of large benthic foraminiferal assemblages: A tool to	<1%

foreshadow reef degradation?", Science of The Total Environment, 2021
Publication

---

53 Liang Peng, Chen-Fei Liu, Hong Wu, Hai Jin, Xiao-Yan Deng, Li-Ting Zeng, Yi Xiao, Cong Deng, Zhi-Kai Yang. "Complete Genome Sequencing and Comparative Analysis of the Clinically-Derived Apiotrichum mycotoxinivorans Strain GMU1709", Frontiers in Cellular and Infection Microbiology, 2022 <1 %
Publication

---

54 Menke, Sebastian, Wasimuddin, Matthias Meier, JÃ¶rg Melzheimer, John K. E. Mfune, Sonja Heinrich, Susanne Thalwitzer, Bettina Wachter, and Simone Sommer. "Oligotyping reveals differences between gut microbiomes of free-ranging sympatric Namibian carnivores (Acinonyx jubatus, Canis mesomelas) on a bacterial species-like level", Frontiers in Microbiology, 2014. <1 %
Publication

---

55 Michael Cysouw, Søren Wichmann, David Kamholz. "A critique of the separation base method for genealogical subgrouping, with data from mixe-zoquean", Journal of Quantitative Linguistics, 2006 <1 %
Publication

56	Owen S. Wangensteen, Emma Cebrian, Creu Palacín, Xavier Turon. "Under the canopy: Community-wide effects of invasive algae in Marine Protected Areas revealed by metabarcoding", Marine Pollution Bulletin, 2018 Publication	<1 %
57	Shahed Uddin Ahmed Shazib, Peter Vďačný, Marek Slovák, Eleni Gentekaki, Mann Kyoon Shin. "Deciphering phylogenetic relationships and delimiting species boundaries using a Bayesian coalescent approach in protists: A case study of the ciliate genus Spirostomum (Ciliophora, Heterotrichea)", Scientific Reports, 2019 Publication	<1 %
58	Urania Christaki, Savvas Genitsaris, Sébastien Monchy, Luen L. Li, Sara Rachik, Elsa Breton, Télesphore Sime-Ngando. "Parasitic Eukaryotes in a Meso-Eutrophic Coastal System with Marked Phaeocystis globosa Blooms", Frontiers in Marine Science, 2017 Publication	<1 %
59	Vasco Elbrecht, Sarah J. Bourlat, Thomas Hörren, Angie Lindner et al. "Pooling size sorted Malaise trap fractions to maximize taxon recovery with metabarcoding", PeerJ, 2021	<1 %

Publication

60	Vera M. A. Zizka, Matthias F. Geiger, Thomas Hörren, Ameli Kirse et al. " Repeated subsamples during extraction reveal increased diversity estimates in metabarcoding of Malaise traps ", Ecology and Evolution, 2022 Publication	<1 %
61	Yurena Arjona, Paula Arribas, Antonia Salces‐Castellano, Heriberto López, Brent C. Emerson, Carmelo Andújar. "Metabarcoding for biodiversity inventory blind spots: a test case using the beetle fauna of an insular cloud forest", Molecular Ecology, 2022 Publication	<1 %
62	bdj.pensoft.net Internet Source	<1 %
63	doi.org Internet Source	<1 %
64	epub.ub.uni-greifswald.de Internet Source	<1 %
65	jyx.jyu.fi Internet Source	<1 %
66	nora.nerc.ac.uk Internet Source	<1 %
67	www.biorxiv.org Internet Source	

<1%

68 Kate F. Darling, Magali Schweizer, Karen Luise Knudsen, Katharine M. Evans et al. "The genetic diversity, phylogeography and morphology of Elphidiidae (Foraminifera) in the Northeast Atlantic", Marine Micropaleontology, 2016
Publication
<1%

69 Quentin Blandenier, Enrique Lara, Edward A.D. Mitchell, Daniel M.C. Alcantara et al. "NAD9/NAD7 (mitochondrial nicotinamide adenine dinucleotide dehydrogenase gene)—A new “Holy Grail” phylogenetic and DNA-barcoding marker for Arcellinida (Amoebozoa)?", European Journal of Protistology, 2017
Publication
<1%

70 Roberto Sierra, Florian Mauffrey, Joana Cruz, Maria Holzmann et al. "Taxon-rich transcriptomics supports higher-level phylogeny and major evolutionary trends in Foraminifera", Molecular Phylogenetics and Evolution, 2022
Publication
<1%

Exclude quotes	On	Exclude matches	< 5 words
Exclude bibliography	On		

Mitochondrial Cytochrome Oxidase Subunit 1: A Promising Molecular Marker for Species Identification in Foraminifera

GRADEMARK REPORT

FINAL GRADE

/0

GENERAL COMMENTS

Instructor

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13

PAGE 14