Amplicon sequence variant-based meiofaunal community composition revealed by DADA2 tool is compatible with species composition

Zoya Harbuzov [a,b,*], Valeria Farberova [a,b], Moshe Tom [a], Alberto Pallavicini [c], David Stanković [d], Tamar Lotan [b], Hadas Lubinevsky [a]

[a] National Institute of Oceanography, Department of Biology and Biotechnology, Israel Oceanographic & Limnological Research, P.O.B 9753, Haifa 3109701, Israel
[b] Leon H. Charney School of Marine Sciences, Department of Marine Biology, University of Haifa, 199 Aba Koushy Ave., Mount Carmel, Haifa 3498838, Israel
[c] Department of Life Sciences, University of Trieste, Via Licio Giorgieri 5, 34127 Trieste, Italy
[d] National institute of Biology, Department of Organisms and Ecosystems Research, Večna pot 111, 1000 Ljubljana, Slovenia

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ABSTRACT

The present study is aimed at implementing the morphological identification-free amplicon sequence variant (ASV) concept for describing meiofaunal species composition, while strongly indicating reasonable compatibility with the underlying species. A primer pair was constructed and demonstrated to PCR amplify a 470–490 bp 18S barcode from a variety of meiofaunal taxa, high throughput sequenced using the Illumina 300 × 2 bps platform. Sixteen 18S multi-species HTS assemblies were created from meiofaunal samples and merged to one assembly of ~2,150,000 reads. Five quality scores (q = 35, 30, 25, 20, 15) were implemented to filter five 18S barcode assemblies, which served as inputs for the DADA2 software, ending with five reference ASV libraries. Each of these libraries was clustered, applying 3% dissimilarity threshold, revealed an average number of 1.38 ± 0.078 ASVs / cluster. Hence, demonstrating high level of ASV uniqueness. The libraries which were based on q ≤ 25 reached a near-asymptote number of ASVs which together with the low average number of ASVs / cluster, strongly indicated fair representation of the actual number of the underlying species. Hence, the q = 25 library was selected to be used as metabarcoding reference library. It contained 461 ASVs and 342–3% clusters with average number of 1.34 ± 1.036 ASV / cluster and their BLASTN annotation elucidated a variety of expected meiofaunal taxa. The sixteen assemblies of sample-specific paired reads were mapped to this reference library and sample ASV profiles, namely the list of ASVs and their proportional copy numbers were created and clustered.

1. Introduction

Meiofauna is an important size fraction in marine soft substrate habitats, containing a variety of invertebrate groups detailed in0 below Giere, 2009) and references therein. There is no universally accepted meiofaunal size range and the 20–500 μm range was applied here, justified as follows: macrofauna, the larger neighboring size range to meiofauna, is sampled by us in the south-eastern Mediterranean for 30 years and 250 μm was found locally to be the best divider between widely accepted macrofaunal taxa and widely accepted meiofaunal ones. However, few big Nematoda and Harpacticoida widely accepted as meiofauna were > 250 μm (Lubinevsky et al., 2017, 2019) and the upper 500 μm limit used here was intended to include also these few bigger Nematoda and Harpacticoida.

It is widely accepted that the species is the desired taxon for habitat's ecological analysis, assuming that unique set of biotic and abiotic factors affects roughly uniformly the survival of individuals belonging to one species, in contrary to different sets which affect other species. Therefore, the list of species in a studied habitat and their relative abundances, termed here species profile, is the basis for determining ecological characteristics of meiofaunal communities and their relationships to surrounding conditions and permitting also the assignment of functional traits to the elucidated species.

Morphologically identifying meiofauna to species level to be used for ecological analyses, is extremely difficult from several reasons thoroughly discussed in Gielings et al. (2021) and literature therein, and the implementation of molecular-based approach was suggested to overcome this obstacle (Carugati et al., 2015), replacing the term species

* Corresponding author at: National Institute of Oceanography, Israel Oceanographic & Limnological Research, P.O.B 9753, Haifa 3109701, Israel.
E-mail address: zoya.garbu@ocean.org.il (Z. Harbuzov).

with the terms operational taxonomy unit (OTU) or amplicon sequence variant (ASV) used for individuals identified by their molecular barcodes. The term ASV is used when the barcode is assumed to represent a genuine biological sequence free of sequencing errors and assigned to one species, while the term OTU is used when a mixture of false barcodes formed by sequencer errors as well as genuine ones could not be distinguished (Callahan et al., 2017). Partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) (Hebert et al., 2003a, 2003b) and ribosomal RNA genes (e.g. Aylagas et al., 2014; Hadziavdic et al., 2014) are widely used faunal barcodes.

The construction of an OTU / ASV profile from a multi-species environmental sample is termed metabarcoding and its scheme is presented in Fig. 1. Ideally, the resulting OTU / ASV profiles are aimed at including all the species in the sample in quantitative terms. This is a challenging task and its accomplishment could be biased at each stage of the process as recently reviewed (Alberdi et al., 2018; Bruce et al., 2021; Gielings et al., 2021; Pawlowski et al., 2018; Van der Loos and Nijland, 2020). Mere evaluation of community diversity and habitat's environmental status indices (Aylagas et al., 2016; Cordier et al., 2017; Fonseca et al., 2017; Frøslev et al., 2017; Lejzerowicz et al., 2015) could be accomplished using only ASVs. Their annotation to morphological taxa, not necessarily at species level, could allow assignment of additional characteristics to each ASV such as morphology-based feeding guilds and reproduction strategies, habitats of presence during ontogenesis and more, which would enable detailed ecological analysis of a studied marine sedimentary habitat and its biotic-abiotic interrelationships.

DNA for metabarcoding in sedimentary habitat is mainly extracted from two sources: 1) multi-species individuals sorted from a sediment sample. Sorting from large amounts of sediment allows statistically sufficient species representation even from an environment with poor faunal density, enables counting of individuals and even dividing them into taxa at achievable taxonomic levels0 (Fonseca et al., 2017). 2) extraction from unsorted sediment, assumed to contain all the extra and intra-cellular environmental DNA, an approach which is implemented mainly for abundant organisms, because it is limited by the feasible amount of processed sediment (Brannock and Halanych, 2015; Cordier et al., 2017; Fais et al., 2020a; Guardiola et al., 2015; Lejzerowicz et al., 2015).

The well-established COI barcode region has proved to be relatively difficult to amplify in free-living marine nematodes, the major meiofaunal group, because of "rampant gene rearrangement, hypervariation among haplotypes, and frequent recombination in mitochondrial genome" quoting Boufahja et al. (2015) and literature therein and see also Carugati et al. (2015) and references therein. Therefore, the small sub-unit of ribosomal DNA (SSU-rDNA or 18S) was widely applied (Boufahja et al., 2015; Carugati et al., 2015; Dell'Anno et al., 2015; Lee et al., 2017; Macheriotou et al., 2018) and references therein. A series of primer pairs were proposed from a variety of regions along the 18S molecule for amplifying barcodes, and a wide range of barcode lengths were resulted, from 110 bps up to most of the 1700 bps of the entire molecule (Aylagas et al., 2014; Boufahja et al., 2015; Cordier et al., 2017; Fonseca et al., 2017; Gielings et al., 2021; Guardiola et al., 2015; Hadziavdic et al., 2014). Longer barcodes from hypervariable parts of the 18S molecule are assumed to better distinguish species, limited by the need to be compatible with the implemented HTS method.

A variety of approaches were suggested and used for the construction of a comprehensive barcode reference library which would encompass all inhabiting species in a studied soft substrate habitat with the main obstacle of partial knowledge of all the inhabiting species and their species-specific barcodes. Reference libraries were created using comprehensive publicly available sequences (Dell'Anno et al., 2015; Klunder et al., 2019; Leite et al., 2020; Lejzerowicz et al., 2015), local barcodes (Aylagas et al., 2014; Lobo et al., 2017) or fully species-independent OTU / ASV libraries. The later were initially constructed using clustering methods coupled to implementation of semi-arbitrary percentage dissimilarity threshold which does not distinguish ASVs but only OTUs (Fonseca et al., 2017; Guardiola et al., 2016). Denoising methods which specifically reduce sequencing errors (Amir et al., 2017; Callahan et al., 2016; Gaspar and Thomas, 2015) enabled the construction of better species-representing ASV reference libraries.

Metabarcoding utility was tested in meiofaunal communities by several studies (Fais et al., 2020b; Guardiola et al., 2016; Lobo et al., 2017; Macheriotou et al., 2018; Müller et al., 2019; Wangensteen et al., 2018; Weigand and Macher, 2018; Schenk et al., 2020). In addition, simultaneous use of multiple molecular markers, amplified by multiple primer-pairs and/or the combination approach of metabarcoding-ecological network have been applied (e.g. Castro et al., 2021; Cowart et al., 2015; Fais et al., 2020b).

Based on the assumption that there is no biotic or even faunal universal primer pair that could PCR amplify all taxa groups, strongly indicated by the plethora of taxa-specific primers (e.g. Haye et al., 2004; Layton et al., 2016; Mikkelsen et al., 2006; Schroeder et al., 2021 and many others), the demonstrated metabarcoding approach here is meiofauna-specific and aimed only at improving the characterization of meiofaunal communities. The construction of the reference library is based on ASVs derived only from species of the studied region using the

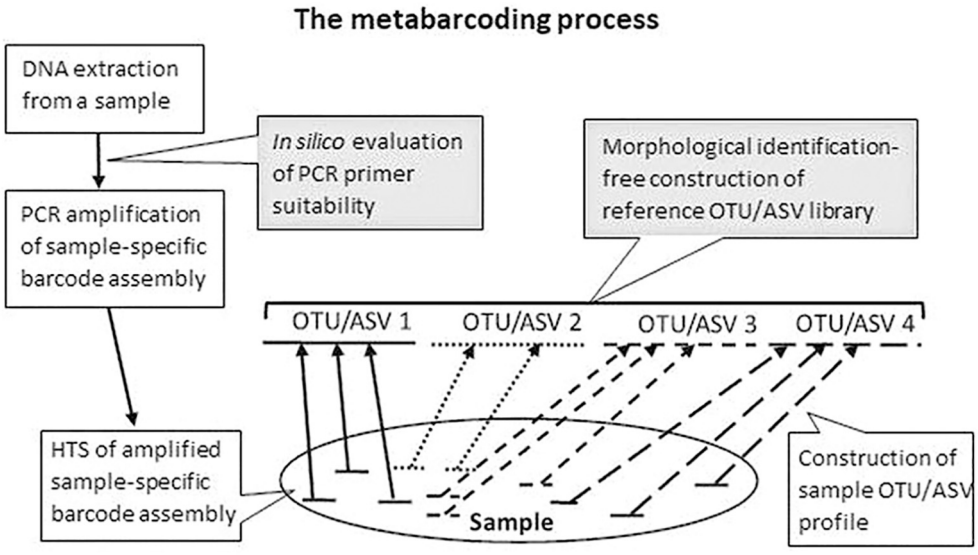

Fig. 1. The metabarcoding process. OTU / ASV 1–4 - reference barcode library, Sample – high throughput sequences (HTS) assembly of the amplified barcode reads from a sample, representing the species composition expressed as proportional copy numbers. Each sample-specific read is mapped to its identical barcode in the reference library and the list of species-specific mapped copy numbers to each reference barcode determines the OTU / ASV profile of the sample. Gray-highlighted boxes mark contributions of this study.

DADA2 denoising protocol (Callahan et al., 2016), initially, without assignment of taxa names to ASVs. This ASV source would avoid false positives which may emerge by using wider library from public sources, assuming that not all species have globally unique barcode for the specifically used sequence.

The major contribution of this methodological study is the suggested molecular procedure aimed at quickly constructing meiofaunal community compositions of poorly studied marine soft substrate habitats, independent of the need for taxonomy expertise on one hand but strongly indicating rough representation of the local species-level composition, hence suitable for immediate performance of ecological studies and environmental monitoring efforts with no delay by taxonomy bottleneck. Species names could be independently assigned to the revealed ASVs at a later stage, corresponding to availability of taxonomists.

2. Materials and methods

2.1. Sampling, sorting and counting

Meiofauna was sub-sampled from a 0.25m^2 box corer (BX-650, Ocean instruments, San Diego, CA) using a 9.4 cm diameter plexiglass core pushed down to the 17 cm horizon of the sediment. The core was horizontally sliced to allow convenient preservation on board by mild suspension in 99% ethanol using a wing mixer to allow efficient ethanol penetration into the sticky mud. In the laboratory, the samples were sonicated for 30 s to reduce sediment aggregation and sieved through 500 and 20 μm sieves. The 20 μm retained individuals were sorted from the remaining sediment by gradient density centrifugation through colloidal silica suspension (Ludox HS-40, Sigma-Aldrich Cat. No. – 420824, density 1.18$g*cm^{-3}$) according to Heip et al. (1985) and Danovaro (2010) and each entire sample was used for further processing and analysis. Five sampling sites within the depth range of 45–360 m which were sampled in October 2017 along the Mediterranean coast of Israel were selected to establish and demonstrate the present approach for constructing sample ASV profiles. A second sampling effort, performed in autumn 2018 served for demonstrating the meiofaunal compositions and densities in the studied region and their division to higher taxonomic groups.

2.2. DNA extraction

Homogenization of samples was performed by the FastPrep® homogenizer and lysing matrix A beads (MP Biomedicals). DNA from whole samples was extracted using the E.Z.N.A.®Mollusc DNA Kit (Omega bio-tek, Cat-D3373). This kit was selected as its lysis buffer contains the cationic detergent cetyl trimethyl ammonium bromide (CTAB) which improves DNA extraction from invertebrate tissues by efficient removal of mucopolysaccharides. DNA levels were measured by fluorometric evaluation (QFX fluorometer, Denovix).

2.3. Initial examination of primers' mismatch level

All unique 18S sequences of the relevant meiofaunal taxa were mined from the public GenBank and aligned according to the different taxa using the Geneious prime software (Biomatters LTD.). An average mismatch level of each of the selected primers with the mined sequences was calculated by the same software through its menus: "Primers – test with saved primers" and its exported summarizing table.

2.4. Construction of an 18S ASV library

The PCR amplification conditions of the target 18S barcode were tested using a range of annealing temperatures and number of amplification cycles. The primers used for the amplifications contained the CS1 and CS2 Illumina hangovers which were required for HTS. The applied PCR conditions were: 95 °C – 2 min; 30–40 cycles (94 °C – 30 s, 46–59 °C – 30 s, 72 °C – 30 s); 72 °C – 3 min. The results of this and later optimization efforts are not the topic of this study. However, the 16 amplicon assemblies resulted from the optimization process were used here as technical samples for demonstrating the suggested metabarcoding process. The amplicons for HTS were cleaned up from 1% agarose gels (NucleoSpin® Gel and PCR Clean-up kit, Macherey-Nagel, Germany) and were HTSed on Illumina 300 × 2 bps platform by a service laboratory.

The 32 resulting FASTQ sequence files (16 forward and 16 reverse) were submitted to GenBank as a Sequence Read Archive (SRA) BioProject PRJNA791542 and merged into two files, forward and reverse on a LINUX platform, using the CAT command. The merged files were filtered using the CUTADAPT software (Martin, 2011) and the filtration included truncation of the poor 3′ side of each sequence using five alternate quality score values, q = 15, 20, 25, 30 and 35, primer removal, and eliminating both short resulted sequences (<250 bps) and sequences with >3 Ns in a row. The five resulting pairs of forward and reverse FASTAQ files were used as inputs for the DADA2 analytical process0 (Callahan et al., 2016), applied through the Qiime2 software plugin (Bolyen et al., 2019). DADA2 created a list of apparently unique ASVs from each of the input FASTQ files and each of the q-related ASV assemblies was considered a potential metabarcoding reference library. Too short sequences were manually removed from each of these reference libraries, realized to be erroneously paired. Annotation of the remaining reads by BLASTN against the nucleotide Genbank standalone database to identify and eliminate non-18S sequences and contaminating mammalian ones, resulted with ~470–490 bp assemblies. P-distance resemblance table of each of the five reference libraries was prepared by the MEGA-X software^0 (Kumar et al., 2018) and served as input for clustering process of the sequences using the PRIMER-v7 software (Clarke et al., 2014; Clarke and Gorley, 2015) through its group average clustering protocol. ASV Clusters were determined using the <3% dissimilarity threshold among cluster members and for each assembly, the number of ASV clusters and the average number of ASVs per cluster were calculated.

OTU assemblies were also constructed from the five, quality score-related paired barcode assemblies, using the VSEARCH software^0 (Rognes et al., 2016) applying dissimilarity threshold of 3%. The following VSEARCH commands were used through the Qiime2 platform: join-pairs - dereplicate-sequences - cluster-features-de-novo. These reference libraries were constructed for comparison with the DADA2 ones to demonstrate the differences between the two strategies for preparing a reference library.

2.5. Construction of ASV sample profiles

HTSs from each of the 16 samples, resulted from the CUTADAPT assembly of q = 25, were paired by the VSEARCH software, using the VSEARCH Join-pairs plugin of Qiime2. Metabarcoding was performed by Geneious Prime through its menus: "Elign/assemble - map to reference", using 3% allowed read dissimilarity and 5% gaps of a maximum of 3 bp per gap, resulted with ASV profiles of each sample.

Similarity among ASV sample profiles using the relative copy numbers was examined by their clustering, using the PRIMER-v7 software. The applied clustering parameters were: square root conversion of the ASV copy numbers, clustering using the Bray-Curtis similarity index and the group average clustering protocol.

All bioinformatic commands used for the various analyses are presented in Supplementary file 1.

3. Results

3.1. Meiofauna abundance in the studied sites

Table 1 presents the abundance and higher taxa division of

Table 1
Abundance of meiofauna sampled in autumn 2018 in three sampling sites (three replicates / site). See stations' designation in Table 4 below. Densities are presented as Ind./70 cm^2 (core area).

Station	Nematoda	Harpacticoida	Polychaeta	Isopoda	Ostracoda	Cumacea	Mollusca
HS122	903 ± 62	158 ± 72	35 ± 2	3 ± 2	2 ± 1	1 ± 1	
HS394	3782 ± 2227	166 ± 196	65 ± 56	3 ± 3	1 ± 1	1 ± 1	3 ± 5
TA76	3020 ± 1707	389 ± 175	94 ± 29	23 ± 10	4 ± 5		

meiofauna sampled in autumn 2018 in our studied region. These results are aimed at generally presenting the taxa and their abundances which were elucidated at our study sites using our sorting method. Only hard-bodied, cuticle-enveloped taxa were revealed. The sequences that were used for the present study were taken in autumn 2017 at the same sites but with no counting and sorting to taxa.

3.2. Design of universal primer pair

Our aim was to design the longest 18S paired-end barcode sequence still compatible with the 300 × 2 bps Illumina sequencing platform. The designed PCR primer pair has to be universal for the target meiofaunal groups, enabling the formation of amplicons from almost all the relevant species-specific DNAs. For initial screening of adequate primer pairs, all the unique sequences of 18S of free-living Nematoda, harparticoid Copepoda, Polychaeta, Isopoda, Ostracoda, Cumacea and also Gastrotricha and Turbellaria which were absent in our samples (Table 1) but are common meiofaunal taxa, were mined from the public sequence databases (supplementary table 1) and were aligned. Iterative visual inspection of relatively uniform sequence regions led to the design of potential primer pairs compatible with the sequencing platform requirements. The selected primer pair with conformed to the length requirements and has the lowest mismatch level produced a ~ 470–490 bp barcode.

The selected universal primer pair was: Forward - 5′-GAGGTAGTGACGAAAAATAAC-3′; Reverse - 5′-CGTTCTTGATTAATGAAAACATTC-3′, and the resulted PCR amplicon was located roughly between base pairs 400–900, region V4 of the ~1700 bp 18S molecule. The compatibility of these primers with 18S sequences from a variety of meiofaunal groups was tested and the results are presented in Table 2, demonstrating an average level of primer mismatch <1 besides the reverse primer in Turbellaria (<1.89).

It has to be noted that part of the mined sequences lacked the area of one of the primers. The results strongly indicated that the selected primer pair is suitable to serve as universal PCR primer pair for meiofauna. This suitability was farther confirmed by examining the annotations of the obtained reference library sequences (see below). This general primer compatibility testing could be performed for any other primer and target taxa.

3.3. Selection of appropriate reference library

Five quality score-dependent DADA2 reference libraries were constructed. An input of 2,143,575 reads was used for the construction of each library and the number of HTSs that passed the quality filtration roughly linearly decreased with increasing **q** value (Fig. 2).

The number of created ASVs for each quality score-dependent library is presented in Fig. 3. Unlike the number of input HTSs, both the number of ASVs and more emphasized, the number of 3% dissimilarity clustered ASVs, nearly approached an asymptote.

The average number of ASV / 3% dissimilar clustered ASVs, in relation to the applied quality score is presented in Fig. 4, averaging 1.38 ± 0.078 ASVs / cluster across libraries, with no statistical difference between the various values (Kruskal-Wallis rank sum test; $P = 0.32$). The near-asymptote shape of the ASV and clustered ASV curves in Fig. 3 and the low average number of sequences per 3% clustered ASVs (Fig. 4) led to the conclusion that a total of ~340–380 species-specific barcodes, the range of the asymptotic part of the clustered ASV curve in Fig. 3, were PCR amplified from our meiofaunal samples.

The distribution pattern of the ASVs per cluster elucidated an overwhelming majority of clusters represented by one ASV and few with higher numbers (presented only for q = 25, Fig. 5), indicated also by the relatively high standard deviations in Fig. 4.

The small average number of ASVs per cluster indicated also that within the limits of the tested quality scores, the DADA2 protocol, efficiently but not perfectly, distinguished unique ASVs. Consequently, reference library which emerged from the read assembly created by q = 25 was preferred to be used by the metabarcoding process due to the asymptotic number of ASVs on one hand and the low probability of

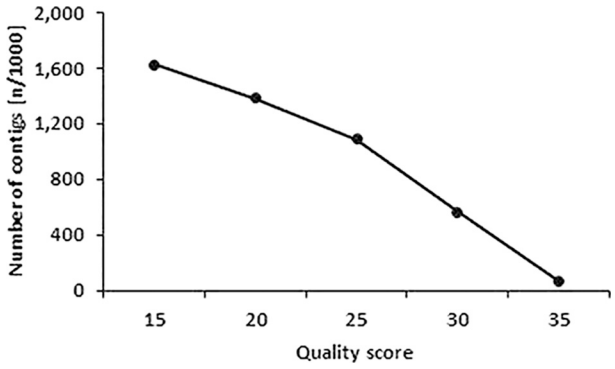

Fig. 2. The effect of the the applied quality score during the CUTADAPT filtration procedure on the number of reads which passed the filtration thresholds. The original assembly included 2,143,575 reads.

Table 2
Testing the similarity between the universal primers and 18S sequences of a variety of meiofaunal groups.

Taxon	Number of examined sequences	Number of sequences aligned by the forward primer	Average mismatch of the forward primer [bps]	Number of sequences aligned by the reverse primer	Average mismatch of the reverse primer [bps]
Polychaeta	490	472	0.44 ± 0.8	433	0.52 ± 0.8
Nematoda	203	203	0.09 ± 0.3	203	0.08 ± 0.4
Harpacticoida	69	69	0.22 ± 0.4	69	0.03 ± 0.02
Ostracoda	37	37	0.08 ± 0.4	16	0.9 ± 0.6
Isopoda	17	17	0.12 ± 0.5	17	0.12 ± 0.5
Cumacea	9	8	0.75 ± 0.9	8	0.75 ± 1.4
Gastrotricha	241	238	0.19 ± 0.5	159	0.29 ± 0.6
Turbellaria	151	151	0.96 ± 1.7	137	1.89 ± 1.12

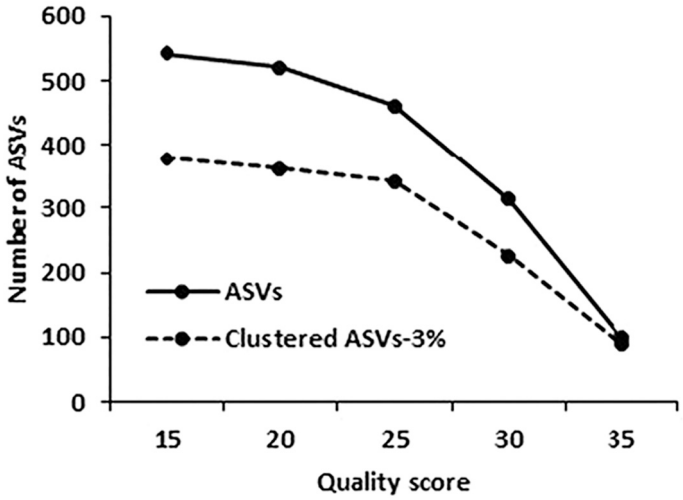

Fig. 3. The effect of the the applied quality score and its underlying number of reads (Fig. 2) during the CUTADAPT filtration procedure on the number of ASVs and clustered ASVs (<3% dissimilarity) resulted from the DADA2 procedure.

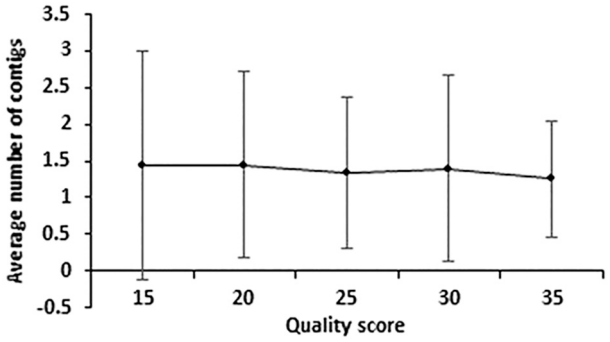

Fig. 4. The effect of the the applied quality score during the CUTADAPT filtration procedure on the average number of unique ASVs / ASV cluster (<3% dissimilarity).

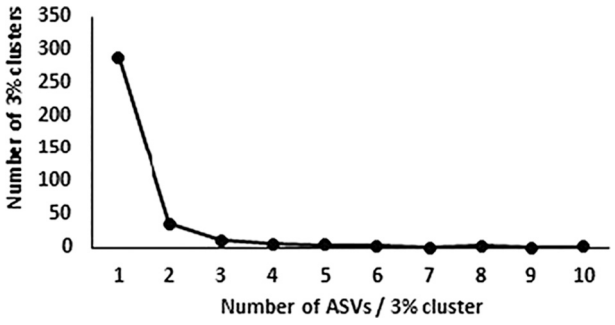

Fig. 5. The distribution of the number of ASVs / 3% cluster in the q = 25 reference library.

erroneous bases of q = 25 (0.33%) on the other hand.

OTU reference libraries were also constructed using the five CUTADAPT filtered barcode assemblies using 3% dissimilarity threshold. The number of OTUs resulted from each assembly is presented in Fig. 6 elucidating lack of OTU asymptote with decreasing q values.

The results of annotating the selected reference library to GenBank nucleotide collection (nt/nr) is presented in Table 3. Annotations of the reference library ASVs using public databases were assigned to the sequences only at family and above taxon level. Genera and species names were assigned only for sequences that were validated by obtaining

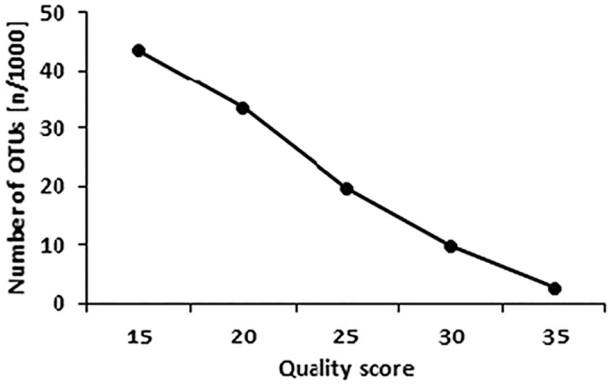

Fig. 6. The effect of the the applied quality score during the CUTADAPT filtration procedure on the number of OTUs resulting from clustering procedure applying 3% dissimilarity threshold and performed using the VSEARCH software.

Table 3
Annotation of the sequences of the reference library. In parentheses, number of elucidated families.

Taxonomic group	Number of ASVs
Nematoda (27)	197
Annelida (17)	56
Copepoda (13)	35
Ostracoda (4)	11
Others	43
Total	342

identical sequences from barcoded identified individuals, done outside the scope of this article. Nematoda from a variety of families dominate the annotations, followed by Annelida, almost all of them Polychaeta, Copepoda, mainly benthic families and a variety of other meiobenthic groups: Ostracoda, Nemertea, Rotifera, Bryozoa, Mollusca, Platyhelminthes, Hemichordata, Echinodermata, Cnidaria, Phoronida, Gnathostomulida, Xenacoelomorpha, Kinorhyncha and Brachiopoda.

3.4. *Construction of sample ASV profiles and their comparison*

Sixteen samples were used as technical samples for demonstrating our metabarcoding approach. They were sampled from five natural sites

and their replicate assemblies were created by several PCR runs using the five DNA templates. Reads from each sample that were filtered by CUTADAPT with q = 25 were paired and mapped to the reference library. An average of 86.4 ± 5.2% of the paired and filtered reads from each sample (Table 4) were mapped to 337 out of 342 ASV clusters of the reference library. The number of mapped reads was quite variable among samples (Table 4). The clustering of the various sample profiles using proportional copy numbers is presented in Fig. 7.

4. Discussion

The major contribution of the present study is the suggested molecular approach for quickly constructing ASV meiofaunal community reference library in poorly faunistically studied marine soft substrate habitats, independent of the immediate need for taxonomy expertise. The library strongly indicated compatibility with the local species-level composition, hence, preventing delay of ecological studies and environmental monitoring efforts which require determination of community compositions.

Twelve articles, listed in Gielings et al. (2021), used the DADA2 tool to reveal ASVs from HTS assemblages without examining compatibility with the actual number of species. DADA2 developers (Callahan et l 2016, 2017) demonstrated their ability to distinguish real biological sequences (ASVs) from an OTU assembly by using a machine learning process of the Illumina error rate for each processed assembly. However, they mentioned that distinction of particular ASV depends on a minimum of OTU copies clustered with this ASV. Indeed, the numbers of ASVs increased with increasing number of amplicons (Fig. 2) because assumedly more ASVs reached the minimum OTU number required for their distinction. However, above a certain number of participating reads, a near-asymptote ASV number was formed (Fig. 3) assumedly representing the barcodes of most PCR-amplified species. Clustering of the reference library at 3% dissimilarity threshold demonstrated mostly one ASV / cluster (Figs. 4, 5), further indicating the validity of one-ASV-one-species concept. The number of amplicons participating the DADA2 analysis increased here by gradually reducing the sequencing quality of the participating amplicons, enabled the selection of the asymptote-approaching reference library, which concurrently used a reasonable sequencing quality score. Another way to gradually increasing the number of DADA2 input reads, which was not applied by us yet, is the construction of a very broad OTU assembly using reasonable quality score and gradually increasing the number of DADA2 participating reads until reaching an asymptote. Modifying of the reference library due to spatially and temporally broadening of the sampling effort may be accomplished by repeating the present process.

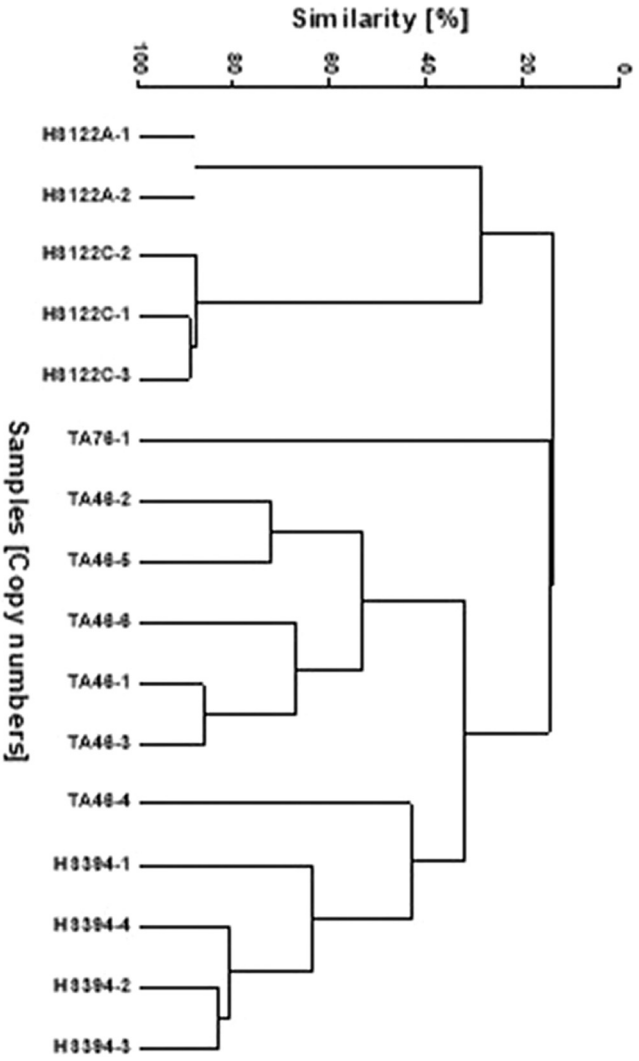

Fig. 7. Comparison among sample profiles using the proportional copy numbers. The designations of the sample names were composed of the perpendicular to the coast transects, HS – Haifa transect and TA – Tel Aviv transect, the bottom depth in meters and the serial number of replicate PCRs done with each sample DNA as template.

Table 4
Sample data including number of reads that were filtered using q = 25, their percentage mapping to the reference library with mismatch threshold of 3% and the number of 3% clustered ASVs in each sample.

[1]Sample name	Total number of paired reads (q = 25)	% aligned to the reference library	Number of aligned ASVs
HS122A-1	44,922	83.6	59
HS122A-2	59,206	75.6	62
HS122C-1	52,398	92.3	43
HS122C-2	50,580	80.2	52
HS122C-3	71,261	86.6	37
HS394–1	32,794	90.8	116
HS394–2	25,961	88.9	161
HS394–3	11,720	83.6	156
HS394–4	28,962	83.9	149
TA46–1	85,727	91.2	143
TA46–2	118,352	83.8	118
TA46–3	18,691	91.1	126
TA46–4	30,456	86.7	73
TA46–5	272,903	93.7	105
TA46–6	15,914	90.2	152
TA76–1	41,122	79.8	86
Average	60,061 ± 63,229	86.4 ± 5.2	

[1] The designations of the sample names were composed of the perpendicular to the coast transects, HS – Haifa transect and TA – Tel Aviv transect, the bottom depth in meters and the serial number of replicate PCRs done with each sample DNA as template.

The still slow increase of ASVs even after reaching near-asymptote state (Fig. 3) is assumedly a result of insufficiency of the 2.15 million reads used here but alternatively it may be a result of residual error rate which is not compensated by DADA2 and continue to create new ASVs from erroneous reads. This assumed residual error may also explain another phenomenon, occurred later in the analytical process. Although the barcode reference library and the samples HTS barcode assemblies emerged from the same source of sequences, 5 ASVs of the reference library, out of 342, were not aligned to any sample read and may be erroneous OTUs and not genuine species.

The 3% dissimilarity threshold among barcodes used here is widely accepted as a species-distinguishing dissimilarity, although deviations from this value are frequent and also make sense, as not all species and all barcodes have the same variability level. However, it is assumed to improve the estimation of ASVs number, partially compensating barcode variability. Dissimilarity levels of 0–5% for DADA2 results in the

course of similar testing were applied by Frøslev et al. (2017) and it could also be done in future studies by us or elsewhere. It is assumed by us that applying higher dissimilarity percentage would emphasize the ASV asymptote on one hand, but would increase the average number of ASVs per cluster on the other hand.

Clustering of the five assemblies by the VSEARCH software with gradually increasing amplicon numbers, applying a 3% dissimilarity threshold and not considering sequencer error rate, revealed ~20,000 OTUs with q = 25 with no asymptote (Fig. 6). It is assumed to result from the gradually increasing number of sequencing errors with no relation to the real number of species, emphasizing the benefit of denoising and the efficiency of DADA2.

A variety of 18S barcodes, amplified by several primer pairs are present in the meiofaunal literature, mainly for regions V1–2, V4 and V9 (Gielings et al., 2021, and literature therein). Comparative examination of primer performance depends on the set of the tested sequences and different geographical regions or a variety of mock collections of 18S may result with different sets of 18S sequences, differently amplified by PCR. Therefore, it is our opinion that selecting the most appropriate primer pair for a specific study have to be done using DNA extracts of the studied communities. The approach presented here enables the construction of an appropriate ASV assemblies using each of the tested primer pairs and then, comparing the resulting ASV varieties, higher taxa compositions as elucidated by annotation and ASV uniqueness.

A new meiofaunal-specific primer-pair was added to the arsenal of meiofaunal 18S primer list. Its resulted barcode is located in the V4 region of the molecule and is relatively long (470–490 bps) in comparison to other applied barcodes from the same region (Broman, 2019; Brandt et al., 2020; Clark et al., 2020; Laroche et al., 2020; Leasi et al., 2021; Pearman et al., 2020). Actually, it is almost the longest possible paired-end barcode using the Illumina 300 × 2 bps sequencing platform. It is our assumption that the longer the barcode, it is potentially more variable among species, permitting more detailed species-specific distinction. Indeed, the library was indicated to cover the vast majority of the local meiofaunal community, with a variety of meiofaunal phyla and families, permitting high resolution distinction among meiofaunal profiles at different sampling sites. Comparison to other primer-pairs was not done here.

Although a mild mechanical suspension of each slice of mud was applied on board to allow fast contact of the ethanol fixative with the sampled individuals, only hard-bodied species were revealed in the sorted samples and only few ASVs were annotated to soft-bodied meiofaunal species. Consequently, identifying the presence of soft-bodied taxa in the studied region would require detailed examination of selected samples using stronger fixatives, staining with strong dyes of organismal tissues even if not compatible with downstream molecular procedures and observing the samples under binocular before further sorting stages.

The annotation of the reference library to public databases at family level and above, provided a way to assign additional biological features to the ASVs, such as feeding guilds based on established classifications like those of polychaeta (Jumars et al., 2015) or of nematoda (Jensen, 1987) and similarly other biological traits that could be identified at family level and above.

Clustering of sample profiles, applying the widely used Bray-Curtis index is affected by the absolute numbers of individuals in the various samples. The total roughly predetermined number sample HTS reads which is further randomly reduced by the quality filtration process are not related to the absolute number of individuals in the sample. In addition, the ASV copy number proportion among ASVs in each sample is distorted because of slightly different efficiency of the universal primers for each of the species during PCR amplification and also from the different number of 18S copies per individual in each of the species present in the sample (Van der Loos and Nijland, 2020). The sorting and counting of individuals before DNA extraction may be used for reducing these sources of error by normalizing the copy numbers to the actual number of sampled individuals (Table 1). Annotation of the reference library to higher taxa level (Table 3) and division of the counted individuals into similar higher taxa (Table 1) would enable normalizing separately each higher taxonomy group (e.g. Nematoda, Polychaeta, etc.), further increasing the accuracy and leaving the bias only to within-group level.

It is our opinion that the fast construction of meiofaunal sample profile at roughly species-like level using metabarcoding, with its inherent error is preferred in comparison to the practically non-realistic dependence on morphological identification. In addition, the repeatability of the generic barcode-dependent sample profile, which rely on stable ASV sequences, bypasses poor taxonomy knowledge of certain groups and disputes among taxonomists, both causing changes of taxonomic status of species with time, and complicating broad temporal comparisons.

The sample HTS used for pairing was filtered by q = 25, similar to the quality score of the sequences applied by the DADA2 analysis. However, this is not obligatory. The pairing of single-end HTSs is done in the region of relatively poor base calls, the 3′ end of the read. However, the pairing improves the base identification at that poor region, and lower q could be used, increasing the number of applied reads during the mapping process and fortifying the statistical significance of the metabarcoding.

5. Conclusions

To summarize, a fast and efficient morphological identification-free approach for analyzing community structure of marine meiofauna was presented here, strongly indicating the relationship between ASVs and actual number of local species. A novel primer pair was constructed, resulting with relatively long barcode suitable for meiofaunal communities. A normalization method of the copy number by real individual counts was suggested. Morphological identifications and their accompanied barcodes could be gradually added later from public databases or from in-house Sanger sequencing, not delaying initiation of ecological studies.

Author contributions

ZH is the leading researcher and the study is part of her PhD thesis, ZH and VF carried out the experimental work, data analysis was performed by ZH, VF and MT and was assisted by AP and DS. The writing was done by ZH and was reviewed and corrected by AP and DS, and by HL, MT and TL, ZH PhD supervisors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw sequence data on which this study relies was deposited in GenBank, SRA BioProject PRJNA791542. GenBank mined data and bioinformatic procedures are supplementary files of the manuscript.

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