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RESEARCH ARTICLE

Metabarcoding reveals high-resolution biogeographical and metaphylogeographical patterns through marine barriers

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Abstract

Aim: It has been predicted that there should be concordance between biogeographical and phylogeographical processes structuring multi-species regional assemblages. We hypothesise that oceanographic barriers in the marine environment affect concomitantly the distribution and the connectivity of the marine biota, thus producing congruent biogeographical and phylogeographical structures. We also predict that macro- and meio-eukaryotes will be differentially affected by hydrological features.

Location: The Atlanto-Mediterranean transition along the E Iberian coast marked by the Almeria-Oran Front (AOF) and the Ibiza Channel hydrological discontinuities.

Taxon: Eukaryotes.

Methods: A new analytical framework based in the metabarcoding of community DNA and a hypervariable marker is presented. This framework allows the simultaneous detection of multispecies biogeographical and phylogeographical structures. Shallow hard-bottom communities were sampled at 12 sites over the littoral zone and community-DNA metabarcoding was performed using the cytochrome c oxidase I marker. The resulting dataset was analysed at several levels: beta diversity of Molecular Operational Taxonomic Units (MOTUs) as surrogate for species, and Exact Sequence Variants as surrogate for haplotypes. We also assessed genetic differentiation within MOTUs (metaphylogeography). Analyses were performed for the combined dataset and separately for macro- and meio-eukaryotes.

Results: Both hydrological discontinuities had a detectable effect, more marked at all levels for the AOF than for the Ibiza Channel. The MOTU dataset provided more clear-cut patterns than the ESVs. The metaphylogeographical approach provided the highest resolution in terms of differentiating localities and identifying geographical barriers. The separate analyses of macro- and meio-eukaryotes showed a higher differentiation of the latter, both in terms of beta diversity and genetic differentiation.

Main Conclusions: Metabarcoding coupled with metaphylogeography allowed the characterisation of the heterogeneity in community composition and population genetic structure along the Atlanto-Mediterranean transition, coherent with known

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hydrological discontinuities. This methodology unlocks a vast amount of information on the geographical distribution of different components of biodiversity for basic and applied research.

KEYWORDS
benthos, biogeography, cytochrome c oxidase I, eukaryotes, marine barriers, metabarcoding, metaphylogeography

1 | INTRODUCTION

The marine environment, despite its apparent continuity, has physical and oceanographic barriers that determine the distribution of the different biota. The study of marine biogeography is a well-established field, and different regions and provinces have been proposed over the years, from Ekman's seminal review (Ekman, 1953) and Briggs' monograph on zoogeography (Briggs, 1974), to more recent accounts (e.g. Spalding et al., 2007; Toonen et al., 2016). These regions are usually defined by species turnover or changes in species abundances (beta-diversity) concomitant with geographical and oceanographic features. Delimiting homogeneous biogeographical regions has relevance for management, marine reserves' delimitation, evolutionary approaches and socioeconomic issues (Costello et al., 2017; Thiel et al., 2007).

Biogeographical changes are related to eco-evolutionary processes at the species level, and the application of molecular techniques has made these processes tractable (Riddle et al., 2008). In particular, the development of the field of phylogeography (Avise, 2009; Avise et al., 1987) allowed the study of the interplay between genealogies and the spatial distribution of genetic variants within species. A central tenet in comparative phylogeography is that there should be concordance between biogeographical and phylogeographical patterns (Avise, 2000; Edwards et al., 2021), as macroevolution is an extrapolation of microevolution (Avise et al., 1987). Barriers may be reflected, not just in species change, but also in genetic divergence within species due to restricted connectivity coupled with drift/selection. The identification of shared patterns of genetic structure across co-distributed species is crucial to determine drivers of biogeographical processes (Edwards et al., 2021). The reverse is also true, known landscape or seascape features can be used to put forward explanations of phylogeographical processes. This mutual enrichment between molecular genetics and biogeography has promoted the development of an integrated biogeographical approach (McGaughran et al., 2022; Riddle et al., 2008).

Biogeographical breaks have been commonly studied on particular taxa while studies with broad taxonomic coverage are rarer. Costello et al. (2017) provided the most comprehensive analyses of marine realms by compiling data from 65,000 marine species from public databases. Likewise, phylogeographical studies have usually addressed one species at a time, with few instances encompassing up to tens of species (e.g. Cahill et al., 2017; Haye et al., 2014; Kelly & Palumbi, 2010) or reviewing available information from multiple groups (e.g. Pascual et al., 2017; Teske et al., 2011). Most often, however, biogeographical and phylogeographical studies concern macro-organismal components of biodiversity, while the small meio-eukaryotes have been comparatively neglected. Nevertheless, they have a key role in ecosystem functioning and trophic interactions (Losi et al., 2018), and there is evidence of genetic breaks in them (e.g. Derycke et al., 2013). Size determines metabolic scaling and reproductive output, as well as dispersal potential. The latter is purportedly lower for meio- than for macro-eukaryotes (Cerca et al., 2018), rendering the former more prone to the effect of spatial breaks. It is crucial to compare biogeographical patterns across organisms' size classes as their congruence or lack thereof can uncover the underpinning processes (Rappacciuolo et al., 2019; Shade et al., 2018).

The rise of metabarcoding techniques during the last decade provided a new tool for assessing marine diversity in an integrative way, encompassing thousands of organisms from all sizes to efficiently detect biodiversity patterns and processes. In metabarcoding, the so-called Molecular Operational Taxonomic Units (MOTUs) are the conceptual units that group similar sequences, and are commonly generated through clustering algorithms to approach the species-level variability present. Metabarcoding has become an invaluable tool for biomonitoring, impact assessment and detection of introduced species, among others (reviewed in Bowers et al., 2021; Deiner et al., 2017; Miya, 2022; Pawlowski et al., 2022). Likewise, metabarcoding datasets of highly variable markers can be mined for intraspecies genetic diversity (Andújar et al., 2022; Elbrecht et al., 2018; Sigsgaard et al., 2020), thereby opening the field for multispecies phylogeography (metaphylogeography, Turon et al., 2020). For metaphylogeographical analysis, stringent denoising of sequences is necessary, generating hypothetically error-free sequences, called Exact Sequence Variants (ESVs, Andújar et al., 2021; Antich et al., 2022; Callahan et al., 2016; Edgar, 2016). Clustering and denoising are complementary, rather than alternative, methods (Antich et al., 2022).

Metabarcoding has been commonly used for community analysis, but it has seldom been applied to the formal assessment of biogeographical breaks in coastal areas (Gaither et al., 2022). Some instances focused on particular groups of organisms (e.g. protists: Pagenkopp Lohan et al., 2017; ciliates: Santoferrara et al., 2018; vertebrates: Closek et al., 2019; zooplankton: Pitz et al., 2020), while other studies encompassed several groups (Cahill et al., 2018; DiBattista et al., 2022). In all cases so far, however, these contributions analysed changes in alpha- and/or beta-diversity. However, metabarcoding has the potential to uncover not only turnover rates

and abundance changes of taxa, but also to detect phylogeographical patterns of many species simultaneously as related to biogeographical breaks. The combined use of MOTUs as surrogate of species and ESVs within MOTUs as surrogate of haplotypes allows to extract both biogeographical and phylogeographical patterns (Antich et al., 2021; Brandt et al., 2021; Turon et al., 2020), thus widening the scope of studies of marine discontinuities.

The Mediterranean is a well-known sea from the point of view of oceanographic features and biogeographical regions (Bianchi, 2007; Bianchi & Morri, 2000), and the Atlanto-Mediterranean transition is one of the most important biogeographical boundaries worldwide. The westernmost Mediterranean Sea features a sharp transition from Atlantic to Mediterranean waters, both along the N African coast and along the Iberian Peninsula. The latter is marked by two main hydrological features: the Almería-Oran Front (AOF) and the Ibiza Channel (IC). The AOF is a density front where the inflowing Atlantic water is deflected southeastward (Folkard et al., 1994; Tintore et al., 1988). The IC is formed by the interplay between colder and more saline waters of the northern Balearic basin and the warmer and fresher waters of the southern (Algerian) basin (Bouffard et al., 2010; Pinot et al., 2002). In spite of these important hydrological transitions, the biogeography of littoral communities along the Iberian Mediterranean coast has not been comprehensively analysed. Veloy et al. (2022) derived biodiversity indicators from trawled bottoms of this area, but taxonomically broad studies on hard bottom benthic communities are wanting. Most data available come from population genetic studies of particular species (reviewed in Pascual et al., 2017).

In this work, we apply metabarcoding to characterise the inter- and intra-species turnover patterns in hard bottom benthic communities along the Iberian Mediterranean coast. Our hypothesis is that the hydrological discontinuities will affect the distribution patterns of benthic organisms and this effect will be detectable and quantifiable using a multilevel (MOTUs and ESVs) and multi-taxon approach. Furthermore, we test whether the hydrological discontinuities differentially affect macro- and meio-eukaryotes. Finally, we want to ascertain whether genetic differentiation within MOTUs mirrors biogeographical patterns and provides higher resolution to reveal subtle structuration.

2 | MATERIALS AND METHODS

2.1 | Sampling sites

We collected samples from 12 localities along the Mediterranean coast of the Iberian Peninsula (Figure 1). From South to North: Tarifa (TAR), Costa del Sol (SOL), La Herradura (LHE), Granada coast (GRA), Carboneras (CAR), Azohia (AZO), Cape Palos (PAL), Villajoyosa (JOY), Cullera (CLL), Calafat (CAL), Tossa de Mar (TOS) and Roses (ROS). These localities encompass the two targeted oceanographic discontinuities: the AOF, between GRA and CAR and the Ibiza Channel (IC), between JOY and CLL. Accordingly, we grouped locations into three

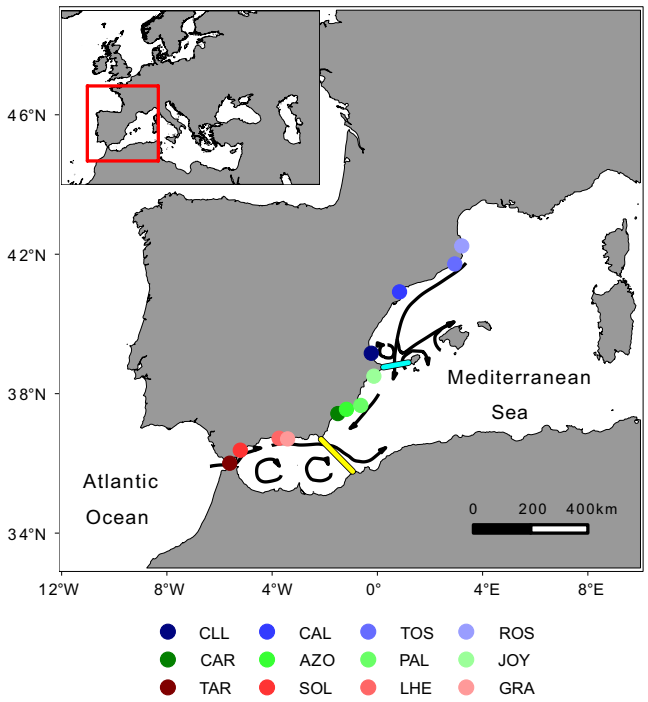


FIGURE 1 Map of the Iberian Mediterranean coast in Western Europe with the sampling localities and the two fronts studied: Ibiza Channel (IC, light blue) and Almeria Oran front (AOF, yellow). Currents are re-drawn from Pascual et al. (2017) and Pinot et al. (2002). Projection: Mercator

regions separated by these potential barriers: southern (TAR, SOL, LHE and GRA), central (CAR, AZO, PAL and JOY) and northern (CLL, CAL, TOS and ROS) regions (Figure 1 and Table S1).

2.2 | Sample collection and laboratory procedures

We targeted the eukaryote component of the photophilous community found between 4 and 8 m of depth in subvertical rocky walls. These communities are dominated by seaweeds with a highly diverse understorey of macro- and meio-eukaryotes. Following Wangensteen et al. (2018), three sample replicates per locality were collected by scraping to bare rocky quadrats of 25 × 25 cm using a hammer and chisel. The material was collected in plastic bags underwater, fixed with 95% ethanol within the hour and stored at −20°C. Sample processing included a size fractionation step in two sizes, large (L, >1 mm) and small (S, between 1 mm and 63 μm) using stainless steel sieves. The two fractions were then homogenised separately with a blender, and 10 g of each was used for DNA extraction with the DNeasy PowerMax Soil Kit (Qiagen). Our initial dataset had thus a total of 72 samples (2 fractions × 3 replicates × 12 localities). All laboratory hardware was rinsed and bleached between samples. Negative controls (3) were prepared by processing charred sand (Wangensteen & Turon, 2017) instead of actual samples.

A fragment of the cytochrome c oxidase I (COI) mitochondrial gene of ca. 313 bp was amplified with the primer set proposed in Wangensteen et al. (2018): forward primer mlCOIintF-XT 5′-GGWACW

RGWTGRACWITITAYCCYCC-3′ and reverse primer jgHCO2198 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′. Primers were tagged with 8-base sample specific tags with at least 3 differences between tags to allow sample demultiplexing after sequencing (Wangensteen et al., 2018). The same tag was added at both primers to detect inter-sample chimeric amplicons. Amplification of COI was done with AmpliTaq Gold DNA polymerase (Applied Biosystems), with 1 ml of each 5 mM forward and reverse 8-base tagged primers, 3 mg of bovine serum albumin and 10 ng of purified DNA in a total volume of 20 ml per sample. The PCR profile consisted of 10 min at 95°C, 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min, and an extension of 5 min at 72°C. One amplification blank was obtained using the PCR mix without addition of DNA template. Library preparation was done with the BIOO NEXTFLEX PCR-Free DNA-Seq Kit (Perkin-Elmer) and sequencing was performed in an Illumina MiSeq V3 run with 2 × 250 bp paired-ends.

2.3 | Bioinformatics pipeline

We processed the sequencing reads following a pipeline based on the 'OBITools' package (Boyer et al., 2016). Illuminapairedend was used to align paired-end reads keeping only those with >40 quality score. Reads were demultiplexed using ngsfilter. Those with mismatched primer tags at any end were discarded. Obigrep and obiuniq were used to perform a length filter (retaining reads 299–320 bp long) and dereplicate sequences. Uchime-denovo algorithm from VSEARCH (Rognes et al., 2016) was used to remove chimeric amplicons.

The downstream processing included clustering sequences into MOTUs with SWARM (with $d = 13$ following Antich et al., 2021). We then removed all MOTUs with less than 5 reads and used ecotag for taxonomic assignment against a local reference database, which is available at https://github.com/uit-metabarcoding/DUFA/ and contains 185,015 COI sequences. We then ran LULU (Frøslev et al., 2017) to remove potentially remaining erroneous MOTUs and manually filtered the MOTU dataset to retain only the marine eukaryotes.

We generated a sequence table for each MOTU using the output information of SWARM that contains a list of all sequences clustered in each MOTU. We denoised the sequences within each MOTU using DnoisE (Antich et al., 2022) to generate a table of ESVs for each MOTU. DnoisE takes into account the natural variability (measured as entropy values) of each codon position for coding genes (such as COI) to improve the denoising algorithm. The entropy values (0.4812, 0.2407, 1.0285 for the first, second and third codon position, respectively) were obtained from the whole dataset before clustering using DnoisE. The stringency parameter (alpha) was set to 4 following (Antich et al., 2022). Final filtering steps were as follows: (i) we removed any ESV for which the abundance in the blanks or negative controls was higher than 10% of its total read abundance; (ii) in each sample, we applied a minimum relative abundance threshold, setting to zero the reads of any ESV with abundance below 0.005% of the total reads of this sample (this was done to eliminate tag-switching between samples); (iii) we eliminated all remaining ESVs with <5 total reads; (iv) we removed sequences with lengths deemed as incorrect: as for most species the length of the fragment used is 313, a correct sequence is expected to have $313 \pm 3 \cdot n$, being n the number of codons added or removed in indels; (v) we finally removed sequences with stop codons and (for Metazoans) sequences with changes in conserved amino acids, since they probably arise from NUMTs, as described in Turon et al. (2020).

After these filtering steps, we obtained a dataset of MOTUs with taxonomic information and a dataset of ESVs (including all ESVs of all MOTUs). This allowed us to perform analyses at both levels: MOTUs (as surrogate of species) and ESVs (as surrogate of haplotypes). For downstream analyses of the whole dataset the relative read abundances of each MOTU or ESV in the two size fractions of each sample were averaged. We also performed separate analyses of MOTUs of two size categories as follows: MOTUs present exclusively in the small fraction (fraction S, less than 1 mm) across samples were labelled as meio-eukaryotes, and those present in the fraction large (L) were labelled as macro-eukaryotes, irrespective of whether they were also found in fraction S of some samples. We assumed that large organisms will generally leave traces in the small fraction, but the contrary will be less common. In this way, we had an approximate size sorting of the organisms and could perform separate analyses of each size category (note that for fractions S of the samples the relative frequencies were recalculated once MOTUs not exclusive of this fraction across samples were excluded).

2.4 | Metaphylogeography dataset

The ESVs obtained in the previous analysis can be used to construct haplotype tables for phylogeographical inference for each MOTU (Antich et al., 2021). We selected only MOTUs that were present in at least two localities of two different regions and with at least two ESVs each to capture potential biogeographical patterns. Relative read frequency data are not an appropriate abundance measure for analyses based on haplotype frequencies (Turon et al., 2020). Following the method tested in Azarian et al. (2021), we used the frequency of occurrence in the three replicates per locality as a proxy for haplotype abundances. Thus, an ESV can have an abundance value between 0 and 3 at a given locality. In this way, we have some information on abundance over and above mere presence–absence data.

2.5 | Analyses

Unless otherwise stated, analyses were performed using the R packages 'vegan' v. 2.5-6 (Oksanen et al., 2019) and 'stats' (R Core Team, 2022). To assess community composition, MOTUs and ESVs were grouped into taxonomic super-groups, the highest category within Eukarya, following Guillou et al. (2013). For metazoans, they

were further sorted into phyla. We then assessed the MOTU diversity (Shannon index computed with diversity function) and richness after rarefying to the lower number of reads (*rrarefy* function) found in each locality for all MOTUs together and for the two size categories (macro- and meio-eukaryotes) separately. Analyses of variance (function *aov* of 'stats' package) were performed to compare these values using region as a fixed factor and locality as a nested random factor, followed by Tukey a posteriori comparisons between regions.

For biogeographical inference, Bray–Curtis (BC, with four-root transformation of relative read abundance per sample) dissimilarities between samples were calculated using either the MOTU and the ESV dataset. These dissimilarities were used to plot samples in non-metric multidimensional scaling (nmMDS, *metaMDS* function) and to cluster them (*hclust* function). For the MOTU dataset, we also analysed separately the two size categories defined.

For the analysis of metaphylogeographical patterns, we computed a genetic differentiation matrix using the *D* estimator (Jost, 2008) with the function *pairwise_D* from the 'mmod' R package (Winter et al., 2017). *D* values ranged from 0 to 1 (maximal dissimilarity). *D* values were obtained for each MOTU selected for phylogeographical analysis (see above) by performing pairwise comparisons of all localities in which the MOTU was present. Finally, for each pair of localities, the average *D* values across all shared MOTUs were computed and used to construct a genetic dissimilarity matrix. This matrix was used to generate a nmMDS and cluster as before. These analyses were also repeated separately for the macro- and meio-eukaryotes in the selected MOTUs.

A network analysis was performed on the overall *D* matrix with EDENetworks (Kivelä et al., 2015). The program automatically computes the percolation threshold (at which the all-including network breaks down into its main components), and we plotted the network just below this threshold. Finally, we plotted haplotype networks for all selected MOTUs, using the function *haplonet* of the R package 'pegas' (Paradis, 2010).

To further separate the effect of differentiation among localities and of potential breaks, we performed permutational analysis of variance (PERMANOVA) on the three dissimilarity matrices (BC for MOTUs and ESVs, D for genetic differentiation). We performed a two-way analysis using region (fixed factor) and locality (random, nested within region) as factors for the BC matrices. In this way, the effect of the two discontinuities could be assessed once the contribution of differences between localities had been factored out. For the *D* matrix, as we only had one possible comparison among each pair of localities, the analysis included only the region factor. The PERMANOVA module incorporated in the Primer v6 statistical package (Anderson et al., 2008) was used. Tests of multivariate dispersions (permdisp) were run when the factors were significant to determine whether this outcome was a result of different multivariate means or different heterogeneity (spread) of the groups. Permutational pairwise tests were performed to compare levels of the region factor.

Mantel tests (*mantel* function) were performed with the three dissimilarity measures among them and with the logarithm of the shortest distances by sea (obtained using Google Earth) among localities. As localities separated by fronts tended to be also more distant geographically, to disentangle the effects of geographical distance from those of the fronts, the different dissimilarities between adjacent localities were used to assess whether there were peaks in dissimilarity associated with the transition between fronts.

3 | RESULTS

We obtained 16,096,788 reads comprising 4,149,955 unique COI sequences after demultiplexing, quality filtering and chimera removal (available as a Mendeley dataset, https://data.mendeley.com/datasets/5w8drnh7nd). The original raw sequences have been deposited in the NCBI SRA archive (Bioproject: PRJNA890069).

Sequences were clustered with SWARM followed by LULU, resulting in 17,944 MOTUs with 5 or more reads. After taxonomic annotation, we kept only MOTUs assigned to marine eukaryotes. We then obtained the ESVs using DnoisE within MOTUs. After all filtering steps, we retained 18,026 ESVs, 3392 MOTUS and 9,423,471 reads. The list of MOTUs and ESVs is provided as File S1, and the taxonomic and size-class assignments of MOTUs in File S2. As per sample, we had 588 ± 20 (mean ± *SE*) ESVs, 263 ± 10 MOTUs and 130,882 ± 6138 reads.

3.1 | Community composition

Metazoans were the dominant group in all localities both in number of MOTUs and ESVs (Figure S1). They were also the most abundant in relative number of reads, except in JOY, where Rhodophyta were dominant. The latter group was the second most abundant in relative read abundance in all other localities except in TAR where Stramenopiles was the second group. For metazoans (Figure S1), a similar distribution in the number of reads across samples was found, with Porifera, Annelida, Arthropoda and Mollusca being the most abundant groups. MOTU composition across localities was homogeneous at the phylum level, but the composition in terms of ESV was more variable (Figure S1). A total of 2058 MOTUs were classed as macro-eukaryotes, and 1334 as meio-eukaryotes. When separating the two size-categories, differences in composition were apparent: while macro-eukaryotes had a structure similar to the overall community in terms of super-groups, the meio-eukaryotes' composition had a stronger dominance of Metazoa, followed by Stramenopiles and Alveolata (Figure S2). At the level of metazoan phyla, again the macro-eukaryotes' composition was similar to the overall community (albeit with a general reduction in Arthropoda), while the meio-organismal fraction has a different composition, dominated by arthropods and with an increased importance of other groups such as Platyhelminthes (Figure S3).

The values of richness and diversity showed significant differences across regions (ANOVA, $p < 0.001$, Figure 2). Both parameters were lower in the Central than in the Northern or Southern

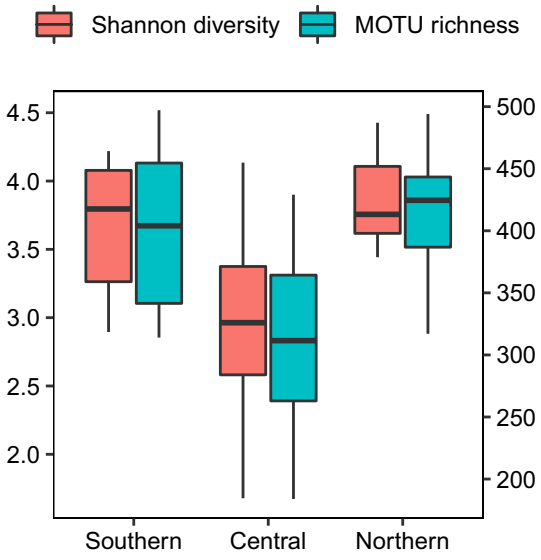


FIGURE 2 Boxplot of the Shannon diversity and rarefied Molecular Operational Taxonomic Unit (MOTU) richness in each region of the Iberian Mediterranean coast for the complete MOTU dataset

region (Tukey tests, all $p < 0.001$), and the latter two did not differ significantly (all $p > 0.30$) (Figure 2). The same pattern of significant differences was found when considering separately macro- and meio-eukaryotes (Figure S4). It can be noted that richness was in general higher for the large size class, while diversity was higher for the smaller component.

3.2 | Biogeography

Non-metric Multidimensional Scaling using the BC dissimilarity for MOTUs and ESVs provided congruent results (Figure 3a,b). In general, the different localities appeared well separated, with no overlap of the inertia ellipses in the nmMDS plots of MOTUs, while some overlap was found for ESV data between TOS, CAL and AZO. A geographical distribution was apparent, with a differentiation of the southern region from the other two along the first axis. The central and northern region did not form clearly separated clusters for MOTUs, and even less so for ESV data. PERMANOVA analyses (Table S2) of BC dissimilarities showed for MOTUs a significant effect of the differentiation between southern and central regions, and not between central and northern regions. For the ESVs, no significant differentiation associated with regions was detected. In all cases, the nested locality factor explained most of the variation and was highly significant ($p < 0.001$). No dispersion differences were detected for levels of significant factors (permdist tests).

When the ordination of the BC dissimilarities was performed separately for macro and meio-eukaryotes using the MOTU dataset (Figure S5), the three regions appeared well separated for the latter group while for the macro-eukaryotes the picture was similar to the whole community. The BC values were significantly higher (Wilcoxon paired-sample test, $p < 0.001$) for the meio- than the macro-organismal component (0.855 ± 0.004 and 0.780 ± 0.004, mean $\pm$ *SE*, respectively), indicating a higher differentiation in the small-sized organisms. Finally, when comparing adjacent localities, the AOF had the highest mean values of BC dissimilarity for both MOTUs and ESV (Figure S6), while the values between JOY and CLL (bracketing the IC) were lower (ranking fourth in both cases).

3.3 | Metaphylogeography

A total of 437 MOTUs were selected for the metaphylogeographical analysis, with a median of 9 haplotypes (ESVs) each (3 and 60 as 10% and 90% percentiles). Of these, 160 MOTUs were found in at least two localities of each region, of which 12 were found in all localities. Of the 437 MOTUs, 127 were tagged with a species name, 11 had genus and 15 family assignments, while the remaining 284 MOTUs were assigned at order or higher taxonomic rank. In all, 309 of the MOTUs were Metazoa, 72 Rhodophyta, 13 Stramenopiles, 3 Viridiplantae, 2 Alveolata and the remaining 38 were unassigned eukaryotes. The best represented metazoan phylum was Annelida (72 MOTUs), followed by Arthropoda (64 MOTUs), Cnidaria (28 MOTUs), Porifera (20 MOTUs) and Mollusca (17 MOTUs). Haplotype networks for these 437 MOTUs are included in the Mendeley dataset (https://data.mendeley.com/datasets/5w8drnh7nd).

We obtained a dissimilarity matrix with the average *D* values for each pair of localities computed from the shared MOTUs. These values were used to map samples in a nmMDS (Figure 3c), in which the first axis separated the southern region from the other two, which, in turn, formed non-overlapping groups along the second dimension. The cluster analyses showed well-defined groups corresponding to each region. PERMANOVA analyses showed a significant effect of the region factor, and all pairwise comparisons between regions were significant (Table S2). The analysis of *D* dissimilarities from adjacent localities (Figure S6) showed that GRA and CAR (AOF) had the highest average differentiation, followed by JOY-CLL (IC) and AZO-PAL. The lowest differentiation between adjacent localities was found in the northern region (TOS and ROS).

The network analysis using EDENetworks detected the percolation threshold at a *D* value of 0.486. The network obtained just below this threshold ($D = 0.480$, Figure 4) showed a separation between the southern region and the central and northern regions, corresponding to the AOF. In turn, the central and northern regions were connected by a few weak links involving mostly the northernmost central region locality (JOY). The northern region showed strong internal links, particularly between CAL, TOS and ROS. The nodes with the highest betweenness centrality (indicating their importance in connecting other nodes; Kivelä et al., 2015) were JOY (5 links) and CAL (4 links).

We also performed separate analyses for the *D* values of the two size fractions (373 MOTUs were assigned to macro and 64 to meio-eukaryotes). The overall picture was maintained, with the three regions forming separate, non-overlapping groups in the nmMDS (Figure 5a,b).

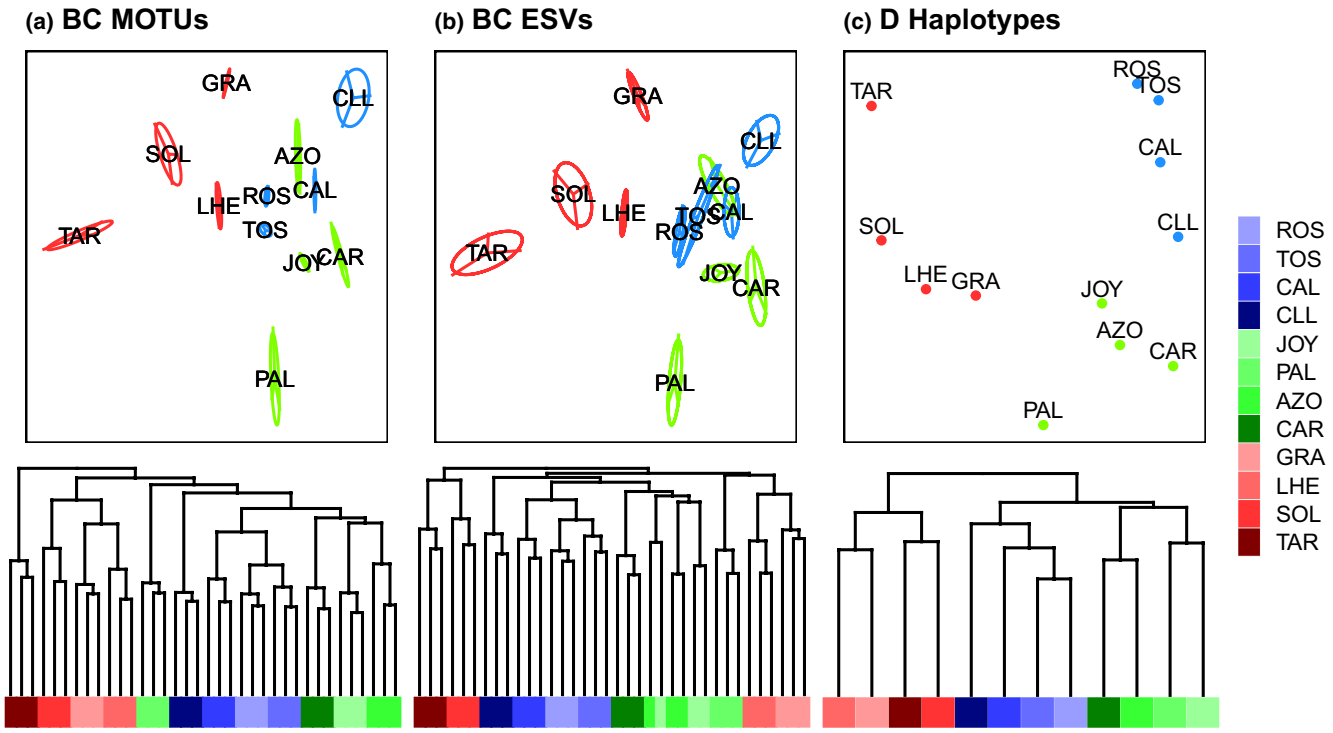
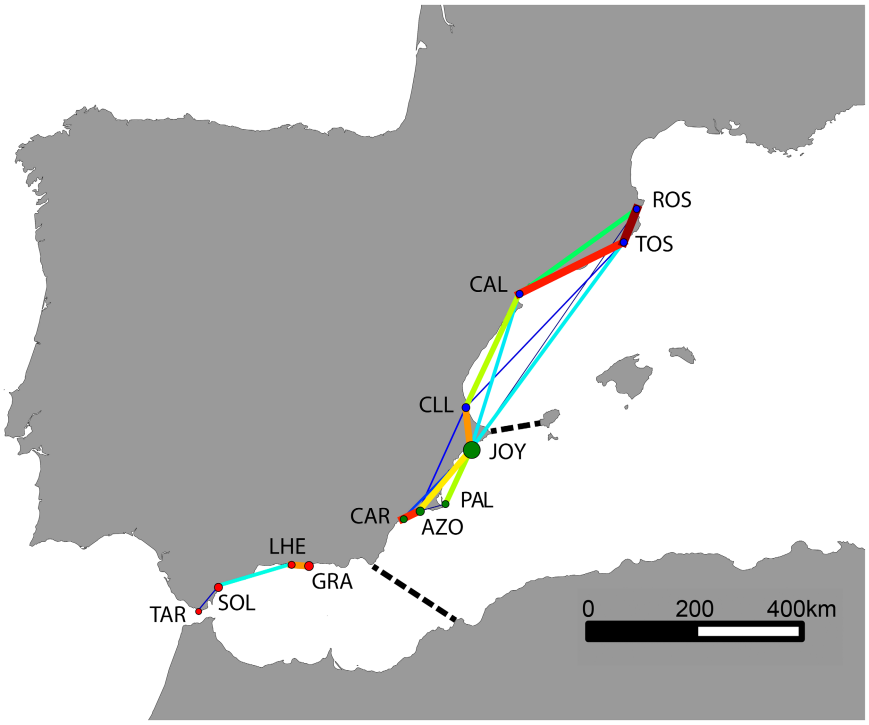


FIGURE 3 Non-metric Multidimensional Scalings and clusters of eukaryote samples from the Iberian Mediterranean coast using Bray–Curtis dissimilarities for all Molecular Operational Taxonomic Unit (MOTUs) (a), all Exact Sequence Variants (ESVs) (b) and mean *D* dissimilarities for haplotypes within MOTUs selected for phylogeographical analyses (c). Samples grouped by locality. Regions are represented by colours (northern, blues; central, greens; southern, reds)

FIGURE 4 Network analysis using EDENetworks with *D* values superimposed to the map of the Iberian Mediterranean coast. *D* values computed for the eukaryote MOTUs selected for metaphylogeographical analyses. Wider lines and warmer colours represent stronger connections and thinner lines and colder colours represent weaker connections. The size of the locality symbols is proportional to the betweenness centrality of the nodes. The two breaks are represented in dashed lines; Ibiza Channel (IC) between CLL and JOY and Almeria-Oran Front (AOF) between CAR and GRA. Projection: Mercator



However, the cluster analysis showed that the central and northern regions formed a group for macro-eukaryotes while for meio-eukaryotes the central region clustered more closely with southern localities. As with the BC dissimilarities, the genetic differentiation values using *D* were significantly higher (Wilcoxon paired-sample test, $p < 0.001$) for the meio-eukaryotes than for the macro-eukaryotes (0.641 ± 0.018 and 0.508 ± 0.008, mean $\pm$ *SE*, respectively). The three variables considered (average BC dissimilarity between localities calculated with the

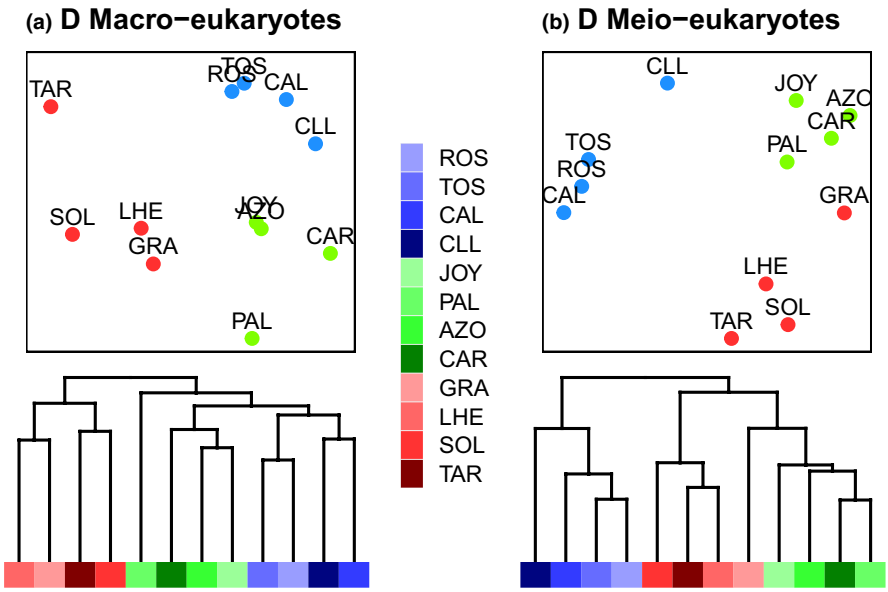


FIGURE 5 Non-metric multidimensional Scalings and clusters of samples from the Iberian Mediterranean coast obtained using mean *D* dissimilarities for haplotypes within Molecular Operational Taxonomic Units (MOTUs) of macro- (a) and meio-eukaryotes (b) selected for phylogeographical analyses. Regions are represented by colours (northern, blues; central, greens; southern, reds)

MOTUs and ESVs, and genetic distance *D*) had a high correlation as measured with Mantel tests (all Mantel $r > 0.750$, $p < 0.001$). Likewise, the three of them showed a significant correlation with the logarithm of geographical distance (all Mantel $r > 0.400$, $p < 0.001$).

4 | DISCUSSION

Metabarcoding of highly diverse shallow benthic communities, using a broadly used mitochondrial marker (COI), retrieved both biological and genetic diversity from the Atlanto-Mediterranean transition along the eastern Iberian coast. This study is the first to explore the effects of barriers to gene flow in the marine realm simultaneously with biogeographical patterns using metabarcoding data and encompassing different groups of eukaryotes. Both the biogeographical and the phylogeographical perspectives showed similar patterns of community differentiation but with different resolution. The different approaches reveal important information at several levels of biological organisation.

4.1 | Biogeographical patterns

Along the 1200 km of the Iberian coast, we retrieved a high diversity of taxa (3392 MOTUs in total) in all localities. The communities were dominated by metazoans in both number of MOTUs and relative read abundance, with Porifera, Cnidaria, Annelida and Arthropoda being the most abundant phyla. When macro- and meio-eukaryotes were considered separately, the former presented patterns of taxa composition similar to the total community, but metazoans represented almost all the assigned meio-eukaryotes. In addition, Porifera and Cnidaria were negligible in this size fraction while they were dominant among the macro-eukaryotes. Overall, about 25% of metazoan MOTUs did not match with any phyla, emphasising the importance of completing current reference databases (Mugnai et al., 2021; Wangensteen et al., 2018).

Geophysical barriers play a crucial role in population differentiation even in apparently continuous marine environments. In this study, three regions were considered, separated by two known hydrological discontinuities, the AOF and the IC, determined by water masses and marine currents (Folkard et al., 1994; Pinot et al., 2002). We detected a pattern of significantly impoverished richness and diversity in the central region. This pattern was consistent also for the two size fractions considered. Our findings contrast with previous reports of a latitudinal gradient of biodiversity in this area, with decreasing values from south to north (Veloy et al., 2022). That study was based on demersal communities of fish, crustaceans and cephalopods on trawling grounds, and may not be extrapolated to littoral hard substratum communities. Caution is therefore necessary when generalising observed patterns. In our case, it appears that the communities on the south (with strong Atlantic influence) and those on the north (bathed by water masses coming from the Gulf of Lyons) may sustain diverse, well-adapted assemblages, while the intermediate zone may have conditions not optimal for any of the two species-guilds.

Our results using BC dissimilarities showed that localities from the southern region were well separated from those of central and northern regions. In addition, the localities of GRA and CAR, separated by the AOF, showed the highest values of BC dissimilarity of all comparisons of adjacent localities. On the contrary, the IC was not so clear a divide. The pattern that emerges is thus one of a marked biogeographical structure at both sides of the AOF. This divide is a geostrophical front that separates Atlantic waters entering through the Gibraltar Strait from Mediterranean waters, thus marking the main boundary in the Atlanto-Mediterranean transition (Folkard et al., 1994; L'Helguen et al., 2002; Tintore et al., 1988). In spite of its importance, there is to date no comprehensive analysis of its effect in species beta diversity in shallow benthic communities, with studies restricted in general to biodiversity indicators in commercially exploited communities and taxa (reviewed in Veloy et al., 2022).

When we analysed separately macro- and meio-eukaryotes, the same general pattern arose, albeit the central and northern regions did

not overlap in the nmMDS for the latter. The dissimilarities detected were also significantly higher for the meio-eukaryotes. All this seems to indicate that the small-sized organisms may have sharper biogeographical boundaries. Meiobenthos and macrobenthos are strongly coupled via biological and ecological interactions (Giere, 2009), and more so in rocky bottoms, where macro-eukaryotes shape the seascape and provide habitat (Losi et al., 2018). However, dispersal is assumed to be more restricted for meio-eukaryotes, often lacking active dispersal mechanisms (Cerca et al., 2018). The distribution of small-sized eukaryotes responds to small-scale habitat heterogeneity, while macro-eukaryotes are influenced by larger-scale processes (Armenteros et al., 2019; Gallucci et al., 2020). This can explain the higher differentiation detected with the meio-eukaryotes between localities.

While both descriptors (MOTUs and ESVs) unveiled basically the same general pattern, there were nevertheless differences, with ESVs showing overlap of some localities in the nmMDS configuration and more mixed clusters. The PERMANOVA analyses showed a significant differentiation between the southern and central localities with the MOTU dataset, which was not found with ESVs. Recent articles have discussed the relative merits of using MOTUs and/or ESVs as the unit for metabarcoding studies (Antich et al., 2021; Brandt et al., 2021; Glassman & Martiny, 2018). These works emphasise that using ESVs as a standard unit of analysis, as suggested previously (Callahan et al., 2017), may be valid for ribosomal markers but not when studying eukaryotes with highly variable markers such as COI. The analysis of MOTU-level turnover is the metabarcording equivalent to the standard biogeographical species-level analysis. On the other hand, using ESVs instead is equivalent to analyse haplotype turnover. If we use the ESVs without the MOTU context, we would be lumping together biogeographical (interspecific) and phylogeographical (intraspecific) information. We suggest that, when working with highly variable markers, diversity patterns are best studied with a hierarchical approach using MOTUs as a proxy for species, and ESVs within MOTUs as a proxy for intraspecies variability (metaphylogeographical approach).

4.2 | Phylogeographical perspective

Geophysical barriers determine not only species turnover, but also population differentiation across the marine littoral zone. Phylogeography analyses the geographical distribution of genetic lineages, linking geography and genealogy (Avise, 2009). In this study, we performed a genetic dissimilarity analysis of 437 MOTUs. Our results showed high values of genetic dissimilarity when comparing samples from different localities. However, dissimilarities between localities of the same region were smaller (0.470±0.004, mean± *SE*) than those between regions (0.560±0.002) indicating that gene flow is higher within than between regions. In addition, the three regions appeared well separated in nmMDS ordinations and clusters, and significant differences between all regions were found. Furthermore, a network analysis reflected disconnected networks in the southern and the central plus northern regions. Among the latter, the links between regions were feeble. The JOY locality had connections with another 5 localities and featured the highest betweenness centrality in the whole network, thus constituting a hotspot for genetic connectivity in the area. If we perform the network analysis without JOY, the central and northern regions appear disconnected (results not shown). Rather than a clearcut divide, the network analysis indicated that the IC is placed in a transition zone connected to both sides.

Phylogeographical studies rely on species that are easy to sample, being therefore restricted in general to macro-organisms, commercially interesting species, or flagship iconic species. Small organisms are only rarely studied due to the difficulty of sampling individuals and identifying them. The contrast between apparent wide distributions and small dispersal capabilities became known as the 'meiofauna paradox' (Giere, 2009) which, upon closer scrutiny, was largely explained by deficient morphological identification and the presence of cryptic clades (Cerca et al., 2018; Giere, 2009). This is therefore a field where molecular techniques, such as the metaphylogeography approach, can be of great value to assess marine connectivity. There is a lack of information on whether phylogeographical patterns of marine macro-eukaryotes are coherent with those of meio-eukaryotes. Our results identified the same general pattern in both groups, but meio-eukaryotes had a significantly higher degree of genetic differentiation (*D* values ca. 26% higher) than the larger size class, consistent with their more restricted dispersal. At the same time, clustering analyses showed that meiobenthos separated more clearly the northern from the other two regions, indicating a stronger effect of the IC barrier on this size-class. There are few comparative studies of gene flow in marine meio-eukaryotes (e.g. Derycke et al., 2013), and none altogether in the area studied. A complex interplay of habitat characteristics and life-history traits seems to shape the genetic structure in these organisms (Derycke et al., 2013), but the overall pattern found in our study is one of marked genetic differentiation among localities.

Phylogeographical structure and species beta diversity are two complementary dimensions of integrative biogeography in a broad sense (Riddle et al., 2008). However, the former is much harder information to acquire. Phylogeographical marine breaks have been usually studied on a single species basis, sampling populations and analysing a set of genetic markers, depending on the study. Multispecies studies are rare and include up to tens of species (e.g. Haye et al., 2014; Kelly & Palumbi, 2010). Alternatively, meta-analyses of published data can be used to make inferences (Arranz et al., 2022; Dawson, 2014; Pascual et al., 2017). Metaphylogeography is a new way to study population genetic differentiation for the whole community using metabarcoding data. We used in this work only basic analyses (population differentiation and network analyses), but the whole panoply of phylogeographical analytical methods such as mismatch analyses, gene trees or coalescence analyses can be applied depending on the question of interest. This new tool has the potential to detect subtle patterns of genetic connectivity with a relatively low sampling effort and targeting a huge amount of taxa of any size. As pointed out by Zizka et al. (2020), the study of haplotypic diversity can provide crucial information on the state of the ecosystem and predict which populations are more sensitive to environmental

changes. Moreover, the study of barriers affecting gene flow is mandatory to manage not only biodiversity but also genetic diversity (Sandström et al., 2019), and metaphylogeography can become a key tool to achieve integrated management programmes.

5 | CONCLUSIONS

The simultaneous study of biogeographical and phylogeographical patterns captured important information at different levels of biological organisation. There was an overall pattern of high structure between localities and a significant relationship with geographical distance. Superimposed to this pattern, the AOF had a strong structuring effect in most analyses, confirming expectations. On the other hand, the Ibiza Channel (IC) barrier had a minor effect, detected only with the genetic differentiation analyses (metaphylogeography). Meio-eukaryotes showed higher differentiation than macro-eukaryotes, both in terms of β-diversity and genetic differentiation, thus suggesting that they can capture subtler structuration of biodiversity.

The distribution of species can be determined by a broad range of biotic and abiotic factors, leading to differences in community composition. However, these factors can have an effect not only on the species distribution but also determine shifts in haplotype frequencies within species. We suggest to use MOTUs as the unit for species turnover analysis and ESVs within MOTUs for phylogeographical analysis when using metabarcoding data.

Metabarcoding coupled with metaphylogeography provides a new tool to integrate the simultaneous analysis of species turnover and genetic differentiation, unlocking a vast amount of information on the geographical distribution of biodiversity for basic and applied research.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

The original raw sequences have been deposited in the NCBI SRA archive (Bioproject: PRJNA890069). A fasta file with the unique sequences after pairing, demultiplexing and dereplicating, a table with the sample tags needed to demultiplex the raw data and a pdf file with the networks of the selected MOTUs for phylogeographical analyses are available as a Mendeley dataset: https://data.mendeley.com/datasets/5w8drnh7nd.

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BIOSKETCHES

The authors' team is keen on applying molecular tools for the assessment of the biodiversity of the marine benthos. In particular, they have developed the metabarcoding approach for the study of hard bottom benthic communities, contributing new methods and software. The team also uses routinely population genetics and genomics to infer connectivity among populations of selected species and to understand their adaptive responses to anthropogenic impacts. They are also focused in analysing invasion biology of benthic invertebrates from genetic and ecological perspectives.

Author contributions: A.A., C.P., X.T. and O.S.W. designed the research. A.A., C.P. and X.T. collected the samples. A.A. and J.Z. performed the laboratory work. A.A., X.T. and O.S.W. did bioinformatic and statistical analyses. A.A. prepared figures and tables and drafted the manuscript. All authors revised and contributed to the paper.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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