



Signals from the deep-sea: Genetic structure, morphometric analysis, and ecological implications of *Cylothone braueri* (Pisces, Gonostomatidae) early life stages in the Central Mediterranean Sea

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ABSTRACT

Cylothone braueri (Stomiiformes, Gonostomatidae) is a widely distributed fish inhabiting the mesopelagic zone of marine tropical and temperate waters. Constituting one of the largest biomasses of the ocean, *C. braueri* is a key element in most of the ecological processes occurring in the twilight layer. We focused on the ecological processes linked to early life stages in relation to marine pelagic environmental drivers (temperature, salinity, food availability and geostrophic currents) considering different regions of the Central Mediterranean Sea. A multivariate morphometric analysis was carried out using six parameters with the aim of discerning different larval morphotypes, while a fragment of 367 bp representing the 12S ribosomal RNA gene was used to perform molecular analyses aimed at determining the intraspecific genetic variability. Analysis highlighted two geographically distinct morphotypes not genetically discernible and related to the different nutritional conditions due to spatial heterogeneities in terms of temperature and food availability. The body depth (BD) emerged as an appropriate morphometric parameter to detect the larval condition in this species. Molecular analysis highlighted a moderate genetic divergence in the fish population, showing the recurrence of two phylogroups not geographically separated.

1. Introduction

The mesopelagic zone, defined as the water mass located between 200 and 1000m of depth, is among the widest and at the same time understudied regions of the ocean (St. John et al., 2016). This layer, also called “twilight zone,” is the domain of the mesopelagic community, which constitutes a large, unexploited biomass that includes the most abundant and widespread vertebrates of the Earth (Karuppasamy et al.,

2007). An important component of this community is represented by the mesopelagic fishes, mainly Myctophiformes and Stomiiformes species (Gjøsaeter and Kawaguchi, 1980; Hulley and Prosch, 1987; Sassa et al., 2002), which are numerically the most important fish component of the oceanic tropical and temperate waters in the world and play a key role in the food web of these ecosystems, linking zooplankton to higher trophic levels (Cherel et al., 2008; Williams et al., 2001). With regard to the Mediterranean Sea, *Cylothone braueri* (Stomiiformes, Gonostomatidae)

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is among the most frequent and abundant mesopelagic fish in this bathymetric layer (Aboussouan, 1971; Andersen and Sardou, 1992; Baussant et al., 1993; Goodyear et al., 1972; Jespersen and Taning, 1926; Palma, 1990). This small fish (max SL between 3 and 4 cm) is widely distributed in tropical and temperate marine waters between 300 and 600 m of depth (Olivar et al., 2012; Yoon et al., 2007) and it is one of the main constituents of the biomass characterizing the twilight zone (Sutton et al., 2010). Nevertheless, knowledge about the population structure and the ecological relationship with the marine environmental factors is still poor, especially in the Mediterranean Sea, most likely due to sampling constraints and because this species is largely unexploited.

In this context, the aim of this study is to provide new knowledge on the population of *C. braueri* in the Mediterranean Sea, taking into consideration a morphometric and genetic approach addressed to its planktonic early life stages. Unlike adults, eggs and larvae are typically concentrated in the surface layers of the water column (Olivar et al., 2014) and constitute one of the main component of the offshore larval fish assemblages (Alemany et al., 2006; Cuttitta et al., 2004, 2016a; Oray et al., 2010; Sabatés and Masó, 1990). Thus, plankton samplings and the analysis of the early life stages of this species represent an efficient approach addressed at the study of the fish population. Moreover, the determination of the ecological relationships involving the early life stages are essential for the comprehension of the processes underlying the population dynamics. Indeed, being affected by the highest mortality rates, the early life stage represents the most critical period in the life history of fish (Hjort, 1914). Larval survival is typically strictly dependent to the environment (Pepin and Myers, 1991), which, in affecting growth and mortality patterns, can tune the recruitment of the new generations and in turn impact stock biomass of short-living species such as *C. braueri* (McKelvie, 1989). In this framework, the study of the inter-relationships between the environmental factors and the larval condition, as well as the determination of their genetic structure, are hence essential aspects for the comprehension of the ecological dynamics also involving the adult fraction of this fish population.

With these goals in mind, we took advantage of two quasi-simultaneous ichthyoplankton surveys carried out in two environmentally different regions of the Central Mediterranean Sea: the Strait of Sicily and the Central-Eastern Tyrrhenian Sea. Regarding the Strait of Sicily, some previous studies recognized the family *Gonostomatidae*, almost entirely represented by *C. braueri*, as one of the main components of the larval fish assemblages distributed in the offshore zone (Cuttitta et al., 2016b, 2018). Taking into account several environmental parameters (e.g. temperature, chl-a and dissolved oxygen concentration, zooplankton wet weight) these authors identified bathymetry as the main driver controlling the spatial distribution of larval assemblages in the studied areas, although in the Strait of Sicily a fundamental role is also played by oceanographic physical forcings (e.g. wind stress, currents, fronts, eddies), due to the advection of neritic and offshore assemblages linked to the local pronounced mesoscale oceanographic structures. The present study represents the first attempt aimed at describing the ecology of the *C. braueri* early life stage in Central Mediterranean Sea, and uses additional information from the Central-Eastern Tyrrhenian Sea, an area not investigated in the previous above cited papers.

Based on the available information, the identification of the effects of key marine parameters linked to the hydrodynamic of the surface layers on the larval condition, on larval mortality rate and on recruitment remains an interesting research subject to be further explored. In order to improve the knowledge on these research issues, we used a multidisciplinary approach aimed at: i) determining the spatial distribution and the dispersion of the early life stage of *C. braueri* in two separated areas of the Central Mediterranean Sea, the first one previously explored (Strait of Sicily) and the second one not yet explored (Central-Eastern Tyrrhenian Sea) in previous studies; ii) assessing the effect of the environmental factors occurring in the surface layers on the spatial distribution, as well as on the larval conditions in the two areas; iii) obtaining

new findings on the fish populations taking into account both environmental and potential genetic heterogeneity on a wide spatial scale. In particular, the multivariate morphometric technique has been used in order to identify potential larval morphotypes and to obtain information on the larval condition in both study areas. The analysis of the environmental parameters and the genetic technique allowed to discuss the morphological findings in order to shed light on the source of variability underlying the larval fish population and to provide new knowledge of the genetic structure of the *C. braueri* in the Mediterranean waters.

2. Material and methods

2.1. Ichthyoplankton surveys

Ichthyoplankton data were collected during two oceanographic cruises carried out on board the R/V “Urania” in the Strait of Sicily and Central-Eastern Tyrrhenian Sea in summer 2013. Surveys were conducted consecutively, from June 26th to July 16th in the Strait of Sicily and from July 17th to July 25th July in Central-Eastern Tyrrhenian Sea. During the two cruises, 234 and 76 stations were sampled, respectively, following a systematic sampling on a regular grid of stations ($1/10^\circ \times 1/10^\circ$ in both surveys along the continental shelf and $1/5^\circ \times 1/5^\circ$ further offshore only in Strait of Sicily) (Fig. 2). Based on morphological features of the coastal zone, the two sampling zones were subdivided in sub-areas, which formed the basis for comparisons in the subsequent analysis. In particular, in the Strait of Sicily, four sub-areas were identified: “West”, “Middle”, and “East” for the inshore zone (bottom depth < 200 m), and “Offshore” for the deeper zone. For the Central-Eastern Tyrrhenian Sea, three sub-areas were identified: “North-West”, “Middle” and “South-East” in correspondence of the gulfs of Gaeta, Napoli and Salerno, respectively (see also Figs. 2 and 3). Planktonic sampling was conducted using Bongo 40 (two mouths of 40 cm in diameter equipped with 200 μ m mesh size nets) oblique hauls towed at the speed of 2 knots. The nets were hauled from within 5 m above the sea bottom to the surface, or from 100 m to the surface in the deeper stations. In each net mouth, calibrated flow-meters were mounted in order to calculate the volume of filtered water (m^3). To preserve plankton samples, a borax-buffered solution of 4% formaldehyde and/or a solution of 95% ethanol were used according to the applied subsequent analyses. To identify early life stages of *C. braueri*, all samples were observed under a microscope in a land-based laboratory and fish larvae were sorted from the rest of the plankton following Whitehead et al. (1984).

2.2. The environmental parameters and multivariate modeling

Temperature and salinity profiles of the water column were recorded in each sampling station using a CTD SBE 911 plus probe mounted on a General Oceanics rosette, also equipped with a Turner fluorometer (mod. Aquatracka) for chlorophyll-a profiling. Then, mixed layer temperature (MLT), mixed layer salinity (MLS) and mixed layer chlorophyll-a concentration (CHLa) have been calculated as the mean value of the first 20 m, i.e. the mixed layer detected from the profiles in both areas, with the aim at characterizing the environmental conditions of the surface layers where most of the larvae of this species, as well as other



Fig. 1. Morphometric parameters collected on *Cyclothone braueri* at larval stage: standard length (SL), body depth (BD), head length (HL), eye diameter (ED), mouth length (ML) and pre-anal length (PL).

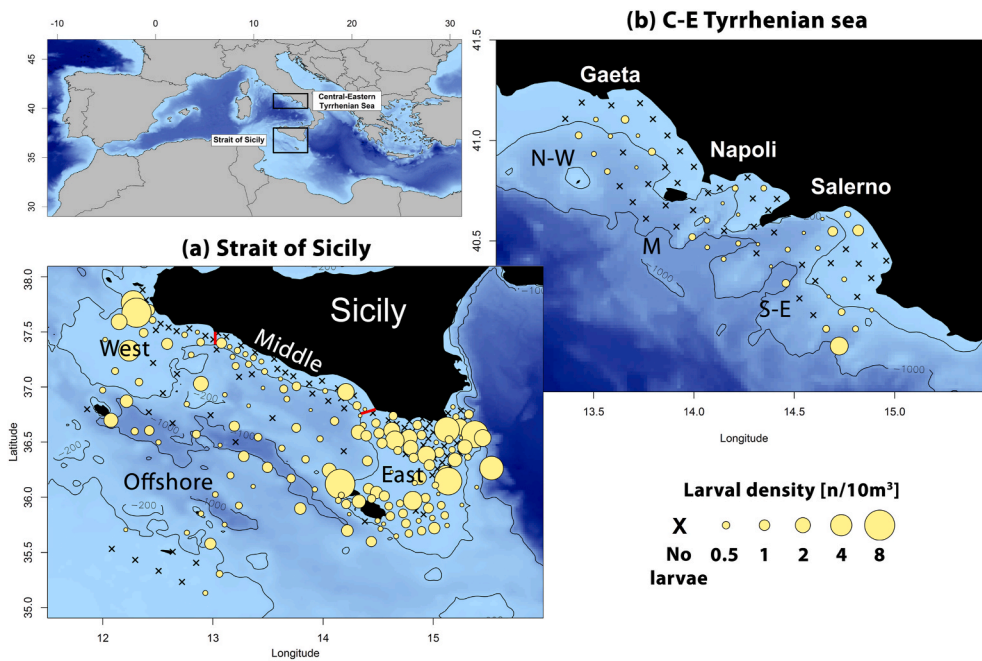


Fig. 2. Spatial distribution of *Cyclothone braueri* at larval stage in the Strait of Sicily (a) and in the Central-Eastern Tyrrhenian Sea (b). In the Strait of Sicily (a), four sub-areas were identified based on the morphology of the continental shelf: West, Middle and East areas within the isobath of 200 m and separated by the red lines; the Offshore zone beyond the 200 m. In the Central-Eastern Tyrrhenian Sea, three sub-areas were identified in correspondence with the Gulf of Gaeta (North-West), the Gulf of Naples (Middle) and the Gulf of Salerno (South-East). Isobaths of 200 and 1000m are reported in both maps. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fish species, reside as they feed on phytoplankton and zooplankton (Olivar et al., 2014). In this context, the CHLa parameter represents an indication of the photosynthetic planktonic component and has been considered as a good proxy of the food availability.

In addition, daily data of geostrophic current velocities (0.125×0.125 degree of spatial resolution) computed from Altimeter satellite gridded Sea Level Anomalies (SLA), as distributed by Copernicus marine environment monitoring service (CMEMS, <http://marine.copernicus.eu/>), were used in order to obtain a mean field of the surface currents occurring during the period of the oceanographic surveys. The information obtained allowed us to recognize the main mesoscale oceanographic structures that can affect the environmental conditions and the spatial distribution of the early life stages. Moreover, the value of the geostrophic current velocity (GCV) has been extracted from the satellite daily data in the position nearest to each plankton station and in correspondence with its sampling date.

Thereafter, MLT, MLS, CHLa and GCV parameters have been used in the subsequent multivariate analyses. In this framework, generalized additive models (GAMs; Hastie and Tibshirani, 1990) were applied using the R package “mgcv” (Wood, 2011), to investigate on the effect of the environmental parameters on the variability in *C. braueri* larval abundance in the two study areas. The larval density (number of larvae $\cdot m^{-3}$) has been log-transformed in order to meet the normality assumption and used as response variable in the model. Gaussian distribution and “identity” link between the response variable and the systematic part of the model was considered. The potential non-linear relationships between covariates and the dependent variables were investigated by cubic regression splines. Before applying the GAMs, we analyzed the presence of any outliers to be deleted, the collinearity among independent variables and the distribution of the input parameters. In this regard, we transformed CHLa into natural logarithm in order to achieve uniform distribution, useful for improving the model application. The R package “MuMIn” (Barton, 2020) has been used for the estimation of the predictor importance. For this purpose, the model averaging procedure based on the Akaike’s Information Criterion (AICc; Akaike, 1998) has been applied considering a set of models that include all possible combinations of the covariates. A model selection has been carried out considering $\Delta AICc < 4$ as a selection criterion (Burnham et al., 2011). Then, the relative importance of each predictor has been derived as the sum of Akaike weights (Σw ; Burnham and Anderson, 2002) over the

selected models including the explanatory variable under estimation. It follows that Σw values range between 1 (the predictor is included in all selected models) and 0 (predictor is excluded in all selected models).

2.3. – Morphometric and statistical analysis

In order to assess morphological differences among individuals collected in different areas, multivariate morphometric analyses were applied on a total of 623 larvae (501 collected in the Strait of Sicily; 122 collected in the Central-Eastern Tyrrhenian Sea). Larval specimens were first individually selected under a binocular stereo microscope and photographed. The following morphometric parameters were acquired by software Image Pro Plus 6.0 (Image Cybernetics, RoperIndustries, SilverSpring, MD, USA; image analysis and processing): Standard Length (SL), Body Depth at cleithrum (BD), Head Length (HL), Eye Diameter (ED), and Mouth Length (ML) (Fig. 1). In order to increase the accuracy of the study, we also considered the Pre-anal Length (PL: distance from the extreme front end of the upper jaw to the end of the digestive tract). These measures were size-normalized using the procedure presented by Thorpe (1975) and generalized by Leonart et al. (2000), to remove the allometric effect of the SL on the other parameters. This procedure completely removes the information related to size, scaling all other morphometric parameters and standardizing the original values to that they would have considering a reference size that has been set as the mean SL of the whole dataset (7.9 ± 2.5 mm). This procedure allowed us to obtain a comparable morphometric dataset considering the sampling regions and the related sub-areas.

With the aim of exploring the morphometric patterns between and within regions and relating them to environmental and genetic variability, the same set of univariate and multivariate analyses was performed on three morphometric datasets: i) the entire dataset collected in the Central Mediterranean Sea; ii) a subset regarding the Strait of Sicily; and iii) a subset regarding the Central-Eastern Tyrrhenian Sea.

For each of these subsets, univariate Kruskal-Wallis rank sum test was used on single standardized morphometric variables in order to detect significant differences among groups (i.e. regions (i) or sub-areas within the region (ii and iii)).

Regarding the multivariate analysis performed on the size-corrected morphometric parameters, the Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) was implemented using 999

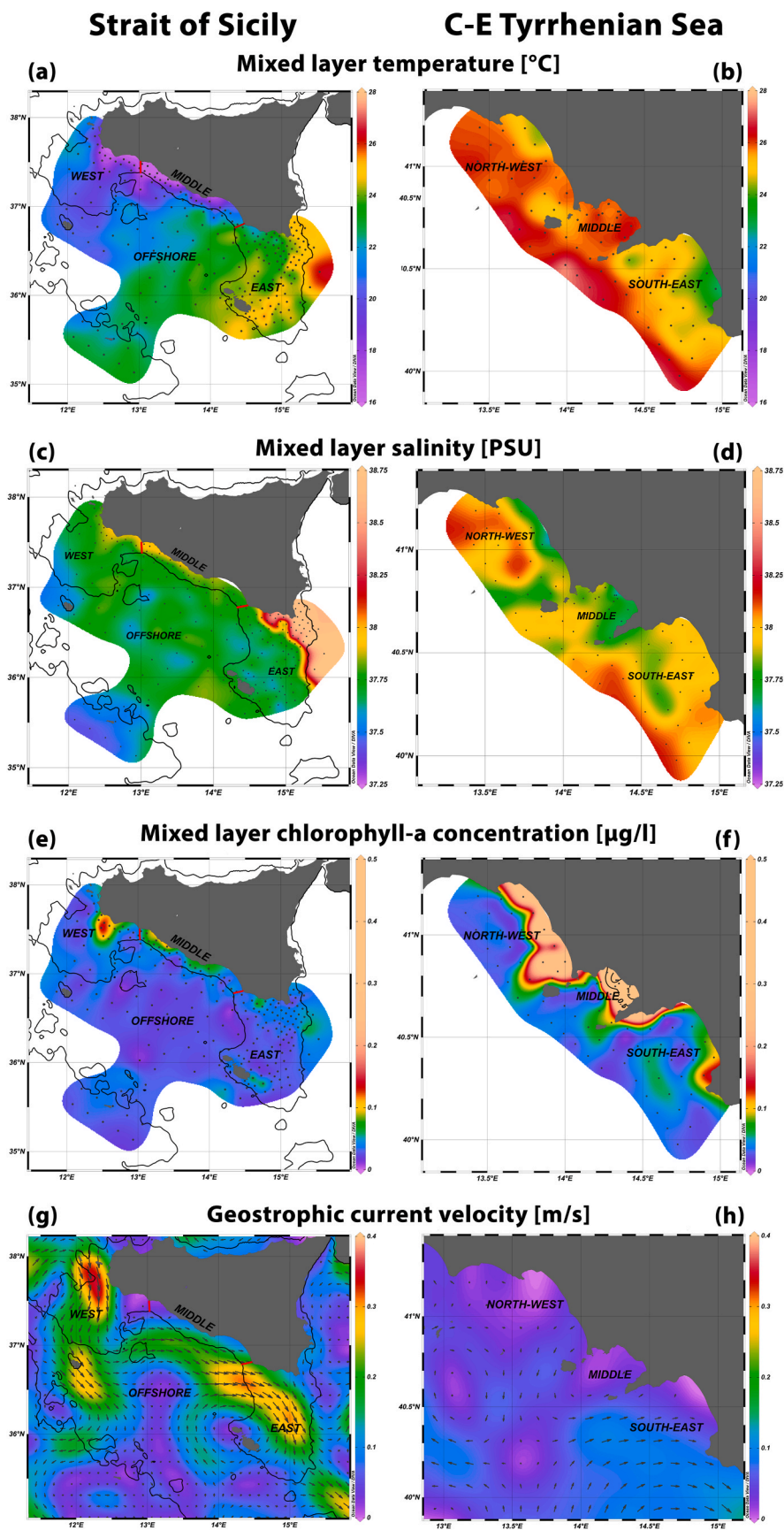


Fig. 3. Spatial distribution of mixed layer temperature (a–b), mixed layer salinity (c–d), mixed layer chlorophyll-a concentration (e–f) and geostrophic current velocity (g–h) occurring during the sampling period (summer 2013) in the two considered study areas: the Strait of Sicily (left panels) and Central-Eastern Tyrrhenian Sea (right panels).

permutations in order to verify the presence of significant differences among regions (i). When the number of the groups was higher than two (i.e. for the comparison among sub-areas of the same region (ii and iii)), a pairwise PERMANOVA with Bonferroni correction on the p-value has been used.

With the aim of determining the distribution of the standardized morphometric parameters and identifying the most important variable that accounts for the discrimination among regions and subareas, the Random Forest classification technique (RF; Breiman, 2001) was used. RF models have been implemented considering all sub-areas in the dataset i) (i.e. the four sub-areas within the Strait of Sicily and three sub-areas located in the Central-Eastern Tyrrhenian Sea), or the sub-areas located within the reference region, as the cases ii) and iii)). In the RF, as in all machine learning models, class imbalance constitutes a difficulty for most learning algorithms that assume an approximately balanced data distribution among groups. As in this case the number of the collected and measured specimens was unequal among classes (the geographical (sub-areas), the Synthetic Minority Over-sampling Technique (SMOTE) (Chawla et al., 2002) was used in order to balance classes before performing RF method. Classification trees were constructed using a bootstrap aggregating algorithm (Breiman, 1996; Breiman et al., 1984) that allows for a reduced variance of predicted values and decreased risk of overfitting. Consequentially, each tree was built on a random sub-sampled training dataset while the subsequent predictions were carried out considering the remaining data (called Out-Of-Bag, OOB) and allowing an unbiased estimate of the classification error. Prediction performance of the model is additionally improved by introducing a further source of diversity by a random restriction of the predictor variables used in each split (Breiman, 2001). Optimal model parameters were identified setting up a grid of tuning parameters in order to maximize correct predictions, using the OOB estimate of misclassification rates as a measure of model performance. As a consequence of this approach, 2500 trees and two random variables were considered at each split. The resulting confusion matrix and the classification error were used to analyze the model performance in terms of correct classification among groups. The predictor's contribution to the fitted model was used in order to identify the most important variables for the correct classification of the groups. In this framework, a Mean Decrease in Accuracy (MDA) was defined as the normalized difference of the two classification accuracies: one considering the original predictor and one taking into account a randomly permuted predictor (Liaw and Wiener, 2002). Therefore, variable importance was evaluated in dependence of their impact on the model predictions in terms of MDA.

With the aim of relating results of the morphometric analysis to environmental conditions occurring in the sampling sites, a canonical correlation analysis (CCA) was performed between standardized morphometric parameters and environmental factors recorded in the two regions. Regarding the morphometric dataset, an average value for each standardized morphometric parameter was calculated from the larvae examined at each sampling station in order to compare them to environmental conditions occurring in the surface layer. The resulting CCA ordination diagram for the first two axes was generated to display the projections of the larval observations collected in the different sampling sites and the direction and magnitude of the relationships between the environmental factors and the morphometric parameters. In the diagram, the environmental factors have been indicated by arrow lines and the length of the arrow line was directly proportional to the strength of the correlation with the morphometric parameters. The angle of the arrow line and the axes stands for the correlation between an environmental factor and the axes, i.e. the smaller the angle, the stronger the correlation. Permutation test (Legendre et al., 2011; Legendre and Legendre, 2012) with 999 permutations was applied in order to evaluate the significance of the ordination axes as well as the significance of constraining variables (i.e. the environmental factors). Furthermore, observations originating from each sub-area as well as their relative centroids and confidence ellipses (95%) were included in

the diagram in order to evaluate the differences in terms of morphological features and environmental conditions occurring in the sampling sites.

Statistical analyses on multivariate morphometric datasets were implemented in R environment, version 3.6.2 (R Core Team, 2019). In particular, the packages “UBL” (Branco et al., 2016) and “randomForest” (Liaw and Wiener, 2002) were used respectively for the pre-processing and the implementation of the classification models. The package “vegan” (Oksanen et al., 2019) was used for the performance of the CCA.

2.4. – The genetic analysis

Total genomic DNA was extracted from 121 larvae of *C. braueri* (70 larvae from the Strait of Sicily and 51 larvae from the Central-Eastern Tyrrhenian Sea) using the Dneasy tissue kit (Qiagen; www.qiagen.com) following the manufacturer's instructions. A 367 bp fragment representing the 12S ribosomal RNA (rRNA) gene was amplified by PCR using the primers 12S_CYC_F: 5'- TAAACCAGGATTAGATACCC-3' and 12S_CYC_R: 5'- GAGAGTGACGGGCGATGTGT-3' (Miya and Nishida, 1996). PCR amplification was performed in 50 µl total reaction volume. Each reaction contained 0.5 µM of each primer, 0.2 µM dNTPs, 1.5 µM MgCl₂, 1X PCR buffer, 1 U of Taq Polymerase (Invitrogen), and 50–100 ng of genomic DNA. The PCR conditions for amplification of *C. braueri* 12S were as follows: initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C (30s), annealing at 50 °C (45s) and the extension at 72 °C (60s) repeated for 30 cycles, and by a final extension at 72 °C for 10 min. Negative controls were included in all PCR runs to ascertain that no cross-contamination occurred. Double stranded products were checked with agarose gel electrophoresis and purified with the QIAquick PCR purification kit (Qiagen). Subsequently, PCR products were sequenced in the forward and reverse direction using an ABI Prism 3100 automated sequencer (Applied Biosystems). Sequences were carefully checked and deposited in GenBank under accession numbers reported in Table 4.

Sequences were aligned by MAFFT v.7 (Katoh et al., 2019) using the default values for gap opening and extension penalties. Genetic variability was estimated using two parameters: nucleotide diversity (π) described as the mean number of pairwise differences, and gene diversity (h) (Nei, 1978).

The ARLEQUIN software package v.3.5 (Excoffier and Lischer, 2010) was used to estimate the genetic diversity within each sample to analyze the molecular variance (AMOVA; Excoffier et al., 1992) and to estimate F_{st} values and to test for their significance using 16000 permutations. A hierarchical series of likelihood ratio tests (Huelsenbeck and Rannala, 1997), implemented by the 0.1.1 jModelTest (Posada, 2008) and using the Akaike Information Criterion, was applied to identify the appropriate nucleotide substitution model, with which to perform the Maximum Likelihood (ML) tree in MEGA X (Stecher et al., 2020). The HKY + I + G model best fitted the 12S dataset. The ML tree was then generated to provide a graphical representation of the relationship between *Cyclothone* populations. A haplotype network was constructed with Network v.10 (Bandelt et al., 1999) using the median-joining algorithm. Finally, the Tajima's D (Tajima, 1989) and Fu's F (Fu, 1997) were assessed in ARLEQUIN to test deviations from neutral molecular evolution. The parameter significance levels were calculated by generating 1000 random samples. Significant negative Tajima's D and Fu's F can be interpreted as signatures of population expansion.

3. Results

3.1. The larval spatial distribution

Larval stages of *C. braueri* are widely distributed over both study areas (Fig. 2).

Differences in terms of abundance emerged between regions. The Strait of Sicily showed higher larval densities compared with the

northern site (Table 1) and, in spite of the mesopelagic nature of the adults, larvae were abundant on the continental shelf as well as the deeper “offshore” zone, and mostly concentrated in the “East” sub-area. Differently, in the Central-Eastern Tyrrhenian Sea there was a generally lower larval density, with relatively higher values in the “South-East” sub-area (Fig. 2; Table 1). Most of the positive stations were distributed on the edge of the continental shelf and in the offshore stations, in agreement with the spawning behavior of this species (McKelvie, 1989; Yoon et al., 2007).

3.2. The environmental conditions in the study areas

The analysis of the mixed layer temperature (MLT), mixed layer salinity (MLS), mixed layer chlorophyll-a concentration (CHLa), and geostrophic current velocity (GCV) data showed marked difference within and between regions (Table 1 and Fig. 3). In the Strait of Sicily, temperature distribution evidenced an increasing thermal gradient from northwest to southeast, ranging from 18 °C to 26 °C. In the northwest sector, coastal colder waters were characterized by higher values of MLS and CHLa (Fig. 3a,c,e), suggesting the presence of coastal upwelling that allows the deeper waters (typically colder, saltier and richer in nutrient concentration) to reach the surface layers (Lafuente et al., 2002; Patti et al., 2010). The shape of the isotherm of 24 °C suggested the presence of a filament structure characterized by relatively colder and chlorophyll-enriched waters extending from the Sicilian coastal area to the archipelago of Malta in the south-eastern part of the study area. The distribution of the salinity was quite uniform in the study area, ranging from 37.5 to 37.75, with the exception of the coastal zone, where higher values were recorded. In particular, the presence of an intense saline front, characterized by a rapid increase from 37.75 to 38.5, was detected in the “East” zone (Fig. 3c). The mean geostrophic current fields confirm the presence of a very strong surface hydrodynamic system in this area, dominated by the Modified Atlantic Water (MAW) first approaching the Sicilian coast in the north-west zone and then splitting into two main branches: the first one flowing southward, towards the Tunisian coast (i.e., Atlantic Tunisian Current, ATC) and the other that flows south-eastward in a meandering pathway (Atlantic Ionian Stream, AIS) (Lafuente et al., 2002) (Fig. 3g). Moreover, an anticyclonic gyre is also observed in the south-east, identifying this zone as a potential retention area for the planktonic stage of fish larvae. In this hydrodynamic context, the saline front was spatially related to these oceanographic structure characterized by the fresher Atlantic waters that flow and interact with the saltier waters of the Mediterranean Sea (Bonanno et al., 2014).

Different conditions were found in the Central-Eastern Tyrrhenian sea, where warmer, saltier and more chlorophyll-enriched waters have been detected (Table 1 and Fig. 3b,d,f). In particular, MLT were higher than 25 °C in the entire study area, excluding a south-eastern coastal area where the values ranged between 23 °C and 24 °C. MLS ranged

between 37.5 and 38.2 and differences emerged among the three gulfs, with the “Middle” zone characterized by fresher waters (Fig. 3d). Very high CHLa values (>0.1 µg/l) were detected on the “North-West” and “Middle” zones and, more limitedly, in the coastal zone of the “South-East” sub-area (Fig. 3f). The analysis of the geostrophic current dataset highlighted a lower dynamics compared to the Strait of Sicily, with a lack of noticeable mesoscale oceanographic structures. (Fig. 3h).

GAMs allowed to define the relationship between the larval abundance and the environmental factors occurring in the surface layers. The general GAM model for the Strait of Sicily and including all environmental variables explained the 43.9% of the deviance. The environmental smooth terms that significantly affected the response variable were bottom depth ($F = 1.43$, $p < 0.05$), MLT ($F = 1.74$, $p < 0.01$), MLS ($F = 4.43$, $p < 0.01$) and GCV ($F = 1.07$, $p < 0.01$), while the $\log(\text{CHLa})$ resulted as a non-significant factor. Cubic regression splines allowed to determine the relationship between the response variable and the significant smooth terms (Fig. 4). In particular, a non-linear effect emerged with the bottom depth, which resulted positively correlated for depths <200 m (i.e. the continental shelf) and for depths >600 m (Fig. 4a). A positive approximately-linear relationship emerged for MLT >20 °C (Fig. 4b), which mainly occurred in the eastern sector of the study area. MLS positively affected the response variable for values around 37.7 and for values higher than 38.1 (Fig. 4c), i.e. the conditions on both sides of the saline front occurring in the “East” sub-area. A linear direct relationship emerged with the velocity of the geostrophic currents (Fig. 4d). Regarding the predictor importance, MLS ($\Sigma w = 1$) resulted as the most important predictor, followed by MLT ($\Sigma w = 0.99$) and GCV ($\Sigma w = 0.98$), while bottom depth ($\Sigma w = 0.86$) and $\log(\text{CHLa})$ ($\Sigma w = 0.5$) emerged as less important factor in explaining the larval abundance variability in the Strait of Sicily.

The general GAM model for the Central-Eastern Tyrrhenian Sea and including all environmental variables explained the 57.6% of the deviance and highlighted different relationships with the environmental covariates. Model selection outcomes identified the bottom depth ($F = 6.5$, $p < 0.01$) and the GCV ($F = 1.47$, $p < 0.01$) as the only significant smooth terms in the model. The cubic regression spline describing the effect of the bottom depth showed higher values of larval density for a bottom depth >200 m, i.e. in correspondence of the offshore zone, with a higher estimation around the 300 m isobath and for bottom depth >800 m (Fig. 4e). In contrast to the Sicilian region, where actually the dynamics of surface currents is quite high (max observed velocity is about 0.35 m/s), in Central-Eastern Tyrrhenian Sea larval density is inversely correlated with current velocity (Fig. 4f). Regarding the predictor importance, bottom depth ($\Sigma w = 1$) resulted as the most important predictor, followed by GCV ($\Sigma w = 0.94$), while MLS ($\Sigma w = 0.68$), $\log(\text{CHLa})$ ($\Sigma w = 0.51$) and MLT ($\Sigma w = 0.38$) emerged as less important factor in explaining the larval abundance variability.

Table 1

Summary of the spatial distribution of *Cyclothone braueri* at larval stage expressed as mean values (\pm SD) and frequency of occurrence in study two regions (in bold) and in their relative sub-areas. The mean values (\pm SD) of the collected environmental parameters are also reported for the same zones.

	Larval distribution		MLT [°C] mean \pm SD	MLS [PSU] mean \pm SD	CHLa [$\mu\text{g/l}$] mean \pm SD	GCV [m/s] mean \pm SD
	Density [$\text{n}/10\text{m}^3$] mean \pm SD	Freq. of occurrence [%]				
Strait of Sicily	0.65 \pm 1.22	64.6	22.37 \pm 2.41	37.85 \pm 0.32	0.037 \pm 0.019	0.121 \pm 0.074
West	0.50 \pm 0.84	57.7	19.08 \pm 1.59	37.79 \pm 0.07	0.057 \pm 0.037	0.184 \pm 0.088
Middle	0.19 \pm 0.24	51.7	18.94 \pm 2.10	37.80 \pm 0.06	0.053 \pm 0.022	0.139 \pm 0.058
East	0.86 \pm 1.42	64.4	24.11 \pm 0.77	37.96 \pm 0.40	0.034 \pm 0.010	0.172 \pm 0.083
Offshore	0.60 \pm 1.21	72.6	22.38 \pm 1.43	37.70 \pm 0.20	0.028 \pm 0.008	0.109 \pm 0.066
C-E Tyrrhenian Sea	0.17 \pm 0.38	47.3	25.67 \pm 0.74	37.92 \pm 0.13	0.149 \pm 0.293	0.057 \pm 0.026
North-West	0.11 \pm 0.17	43.5	25.79 \pm 0.49	37.94 \pm 0.15	0.123 \pm 0.146	0.032 \pm 0.018
Middle	0.13 \pm 0.22	42.1	26.02 \pm 0.40	37.84 \pm 0.12	0.278 \pm 0.452	0.038 \pm 0.021
South-East	0.24 \pm 0.53	53.1	25.23 \pm 0.91	37.98 \pm 0.09	0.046 \pm 0.024	0.072 \pm 0.025

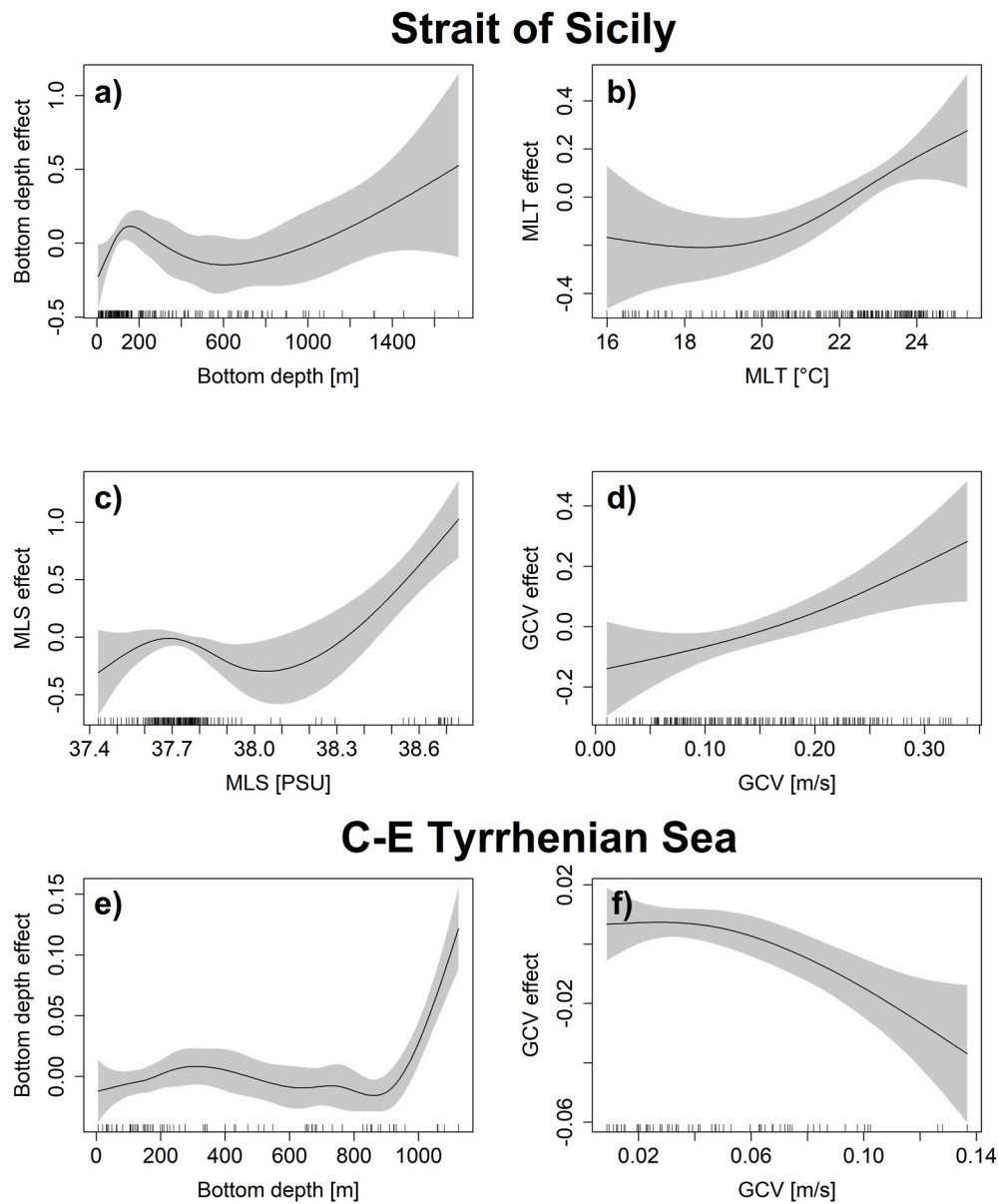


Fig. 4. Significant cubic regression splines estimated in GAMs performed on the log-transformed larval density [larvae/m³] (response variable) and environmental factors (explanatory variables) recorded in the Strait of Sicily (a–d) and Central-Eastern Tyrrhenian Sea (e–f). In each panel, gray region defines the confidence interval of the estimated smooth term, while black lines in the foot represents the distribution of the covariate expressed as a 1-d rug plot.

3.3. – The morphometric analysis

Univariate testing (Kruskal-Wallis rank sum test) was performed singularly on the size-normalized morphometric parameters among the different regions (i.e., the Strait of Sicily and the Central-Eastern Tyrrhenian Sea). All morphometric parameters showed a significant difference between regions (p -values < 0.05). PERMANOVA testing performed on the multivariate morphometric dataset confirmed the evidence obtained through the univariate approach, denoting a significance difference between the two regions ($F = 4.1843$, p -value < 0.01). The Random Forest (RF) model based on size-corrected morphometric parameters implemented for the discrimination of the all identified sub-areas in the Central Mediterranean Sea correctly classified 56.2% of the observations (Table 2, model A). However, the distribution of the classification error is heterogeneous among the sub-areas. In particular, four sub-areas were better discriminated (total error lower than 44%): the Sicilian “West” and “Middle” and the Tyrrhenian “North-West” and “Middle” sub-areas. The Sicilian “East” has been mostly confused with

the Sicilian “Offshore” areas, indicating more similar morphological features in larvae collected in the two zones. Similarly, the Tyrrhenian “South-East” area has been mostly confused with the other sub-areas located in the same region. The pattern of a higher classification error in sub-areas located in the same region is also highlighted by the values of two components of the total error: the “error within region” and the “error between regions” (Table 2, model A). Indeed, excluding the best identified area, i.e., the Tyrrhenian “Middle” area, the former error is always higher than the latter. Variable importance was estimated by the model and identified BD (body depth) as the most important parameter for the correct classification of the model, followed by ED (eye diameter), ML (mouth length), PL (pre-anal length) and HL (head length) (Suppl. 1, model A). The mean values of the morphometric parameters in all sub-areas are reported in Fig. 5 and showed different patterns among sub-areas that have been focused through the analysis specifically dedicated to the two regions.

Regarding the Strait of Sicily, a univariate test (Kruskal-Wallis rank sum test) was performed singularly on the size-normalized morpho-

Table 2

Confusion matrices and relative classification errors of the Random Forest models based on the morphometric dataset. For each sub-area, (S) and (T) indicate the reference region, i.e., Strait of Sicily and Central-Eastern Tyrrhenian Sea respectively. The Model A was performed taking into account the dataset collected in both regions of the Central Mediterranean Sea. “Error within region” reports the misclassification (%) with the other sub-areas of the same region. “Error between regions” reports the misclassification (%) with the sub-areas of the other region. “Total error” report the sum of the two previous reported errors, i.e. the general misclassification error (%) for each sub-area. Models B and C were performed considering the two regions independently (Strait of Sicily and Central-eastern Tyrrhenian Sea respectively). “Total error” regarding each sub-area is also reported for the model B and C. The % of correct classification is reported on top of the matrices. In each matrix the number of the observations correctly classified for each sub-area is shown in bold.

A - Central Mediterranean Sea % of correct classification: 56.2%	West (S)	Middle (S)	East (S)	Offshore (S)	North-West (T)	Middle (T)	South-East (T)	Error within region	Error between regions	Total error
West (S)	50	4	10	9	3	3	10	0.258	0.180	0.438
Middle (S)	3	73	3	4	1	3	1	0.114	0.057	0.170
East (S)	13	4	32	27	4	3	6	0.494	0.146	0.640
Offshore (S)	10	10	22	27	6	9	5	0.472	0.225	0.697
North-West (T)	1	0	6	4	61	6	11	0.191	0.124	0.315
Middle (T)	5	1	5	3	3	66	5	0.091	0.159	0.250
South-East (T)	1	3	8	9	18	10	40	0.315	0.236	0.551
B - Strait of Sicily % of correct classification: 60.32%					C - Central-eastern Tyrrhenian Sea % of correct classification: 69.11%				Total error (B)	Total error (C)
West (S)	99	5	10	10	29	2	10	North-West (T)	0.202	0.293
Middle (S)	6	107	6	6	3	32	6	Middle (T)	0.144	0.220
East (S)	16	4	63	42	8	9	24	South-East (T)	0.496	0.415
Offshore (S)	27	13	54	31				(T)	0.752	

metric parameters among the four identified sub-areas (Fig. 2), highlighting a significant difference of HL (head length; $\chi^2 = 25.377$; $p < 0.01$), BD (body depth, $\chi^2 = 31.563$; $p < 0.01$), and ED (eye diameter; $\chi^2 = 43.659$; $p < 0.01$) among groups, with higher values in the “East” and the “Offshore” zones (Fig. 5a). The multivariate pairwise test (PERMANOVA) performed among zones identified significant differences between the “East” and all other zones, while no differences were detected among the other groups (Table 3). The Random Forest classification model correctly classified 60.32% of the observations. The confusion matrix (Table 2, model B) shows that the model is able to perform a good classification of the observations collected in the “West” (~20% of classification error) and the “Middle” (~14% of classification error) sub-areas. On the other hand, according to the model A, the higher classification error of the “East” (~50%) and “Offshore” (~75% of error rate) is mostly due to a mix-up between these two sub-areas, characterized by similar morphometric values in the collected larvae. In agreement with the univariate test, the variable importance of the Random Forest analysis identified firstly BD and secondarily ED and HL as the most important parameters for the correct classification of the groups (Suppl. 1, model B). The findings of the morphometric analysis allowed us to identify two different morphotypes relative to the Sicilian region: one characterized by size-corrected higher values of BD, ED, and HL (hereafter called “thick morphotype”) spatially distributed in the “East” and “Offshore” waters; the other one concentrated in the “West” and in the “Middle” sub-areas dominated by larvae showing lower values of these parameters (hereafter called “thin morphotype”).

Considering the Central-Eastern Tyrrhenian Sea, the univariate Kruskal-Wallis rank sum test shows that BD (body depth) was the only morphometric parameter that showed significant differences ($\chi^2 = 6.5077$; $p < 0.01$) among the sub-areas “North-West,” “Middle,” “South-East,” as a consequence of lower normalized BD values in the “South-East” zone (Fig. 5b). PERMANOVA test confirmed the “South-East” zone as significantly different to the other two sub-areas (Table 3). The Random Forest classification model correctly identified 69.11% of the observation based on the morphometric parameters. The classification error ranged between 29 and 42% among sub-areas (Table 2, model C). BD was the most important parameter for the correct discrimination of these groups (Suppl. 1, model C). As this parameter assumed lower values in the “South-East” compared with the other two sub-areas (Fig. 5), based on the results of the morphometric analysis, we identified a “thick morphotype” in the “North-West” and “Middle” sub-areas

and a “thin morphotype” in the “South-East” zone.

The canonical correspondence analysis (CCA) allowed to highlight the relationship between the larval morphology and the environmental conditions occurring in the sampling areas. Permutation test indicated the significance of both axes (CCA1, $F = 15.42$, $p < 0.01$; CCA2, $F = 12.47$, $p < 0.01$). Among the constrained variables, only MLT ($F = 9.79$, $p < 0.01$) and CHL ($F = 10.44$, $p < 0.01$) have been identified as parameters significantly correlated to the axes describing the two-dimensional space of the diagram (Fig. 6). MLT was inversely correlated with the first axis (CCA1), which also accounted for the BD morphometric variability, while CHL is inversely correlated with the second axis (CCA2). The other morphometric parameters exhibited a weaker (HL, PL and ML) or absent (ED) relationship with the canonical axes. The distribution of the observations based on the sub-area of origin substantially confirmed the results of the RF classification models. Indeed, larvae identified as “thick”, i.e. collected in the “East” and “Offshore” sub-area of the Sicilian region and in the “North-West” and “Middle” sub-areas of the Tyrrhenian Sea, mostly occupied the left side of the diagram, characterized by higher values of BD and MLT. Among them, the last two Tyrrhenian sub-areas were also discriminated for higher values of CHL, as indicated by their centroids in the third quadrant, while Sicilian sub-areas were mostly located in correspondence of the positive part of the CCA2, i.e. lower values of CHL. Consistently with these patterns, the “thin morphotype” (i.e. larvae collected in the “West” and “Middle” sub-areas of the Strait of Sicily and in the “South-East” sub-area of the Tyrrhenian region) was mostly located in the right part of the diagram, which is mainly described by lower values of BD and MLT, although a limited overlapping of observations emerged between the “South-East” and “East”-“Offshore” sub-areas.

3.4. The genetic results

A 367-bp portion of the 12S ribosomal RNA (rRNA) gene was sequenced in 121 individuals of *C. braueri* from the Strait of Sicily (sub-areas “West,” “Middle,” “East,” and “Offshore”) and Central-Eastern Tyrrhenian Sea (sub-areas “North-West,” “Middle,” and “South-East”). Of these 367 bases, 91 were variable and 59 were parsimony informative. The analysis of the investigated sequences led to the identification of a total of 24 distinct mtDNA haplotypes, of which 8 haplotypes were shared among samples. In particular, the haplotype H5, shared among

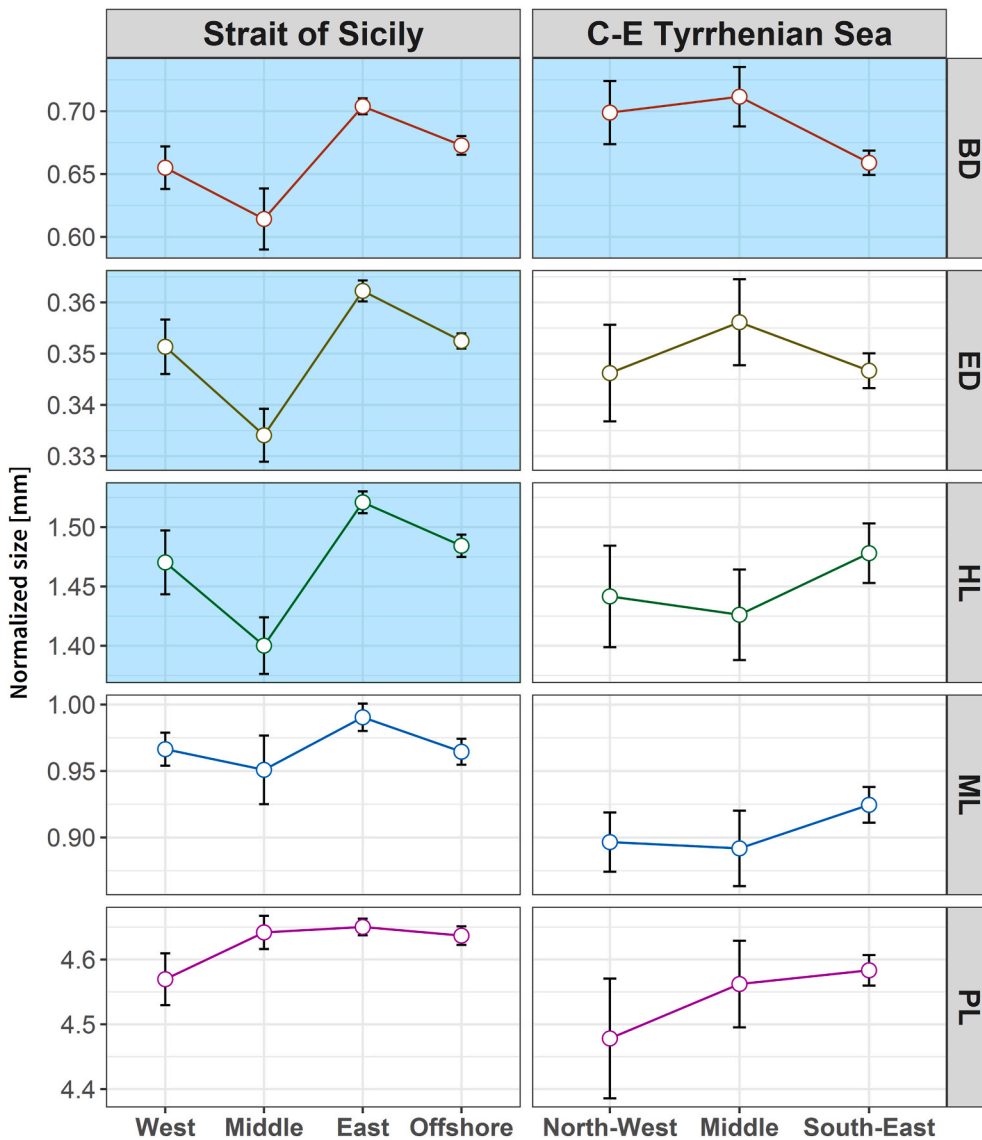


Fig. 5. Morphometric parameters (mean ± std error) of *Cyclothone braueri* larvae collected in (left) Strait of Sicily and (right) Central-Eastern Tyrrhenian Sea, and their corresponding sub-areas. In light blue background the variables exhibiting significant difference in univariate Kruskal-Wallis rank sum test are evidenced; they are also the most important parameters for the correct classification of the sub-areas within each region as emerged by the Random Forest models. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Pairwise permutational analysis of variance (PERMANOVA) statistical test based on 999 permutations. P-values are Bonferroni corrected. Significant p-values are evidenced in bold.

A - Sub-areas of the Strait of Sicily				B - Sub-areas of the Central-Eastern Tyrrhenian Sea		
F = 2.9718, p-value < 0.01				F = 2.2792, p-value < 0.034		
	West	Middle	East	North-West	Middle	South-East
Middle	0.211			0.981		
East	0.034	0.02		0.039	0.014	
Offshore	0.34	0.072	0.039			

30% of individuals of all sub-areas, could be considered ancestral (Table 4). The 66.6% of haplotypes were private (present in 1 or 2 individuals). Concerning the genetic variation within each group, the population of the East sub-area in the Strait of Sicily had the highest value of haplotype diversity (0.797), while in the Tyrrhenian Sea, the population of the South-East sub-area had the lowest (0.57) (Table 5). The AMOVA analysis computed revealed structure along the studied distribution area of *C. braueri* (Fst = 0.26 p < 0.0001) (Table 6).

The Maximum Likelihood tree was built using our data and 4 additional partial 12S sequences of *C. braueri* from Balearic Sea samples

reported in GenBank database (KC616353-KC616356) and the sequence of *C. pygmaea* as an outgroup (Suppl. 2). All haplotypes formed two clusters (named haplogroup A and haplogroup B) supported by bootstrap values that were not very high and were without any geographical pattern (Suppl. 3). The sequence analysis of the haplotypes belonging to the two clusters showed that they differ for a nucleotide substitution at position 230. In this position, all sequences of the haplogroup A have a thymine (T), while those of the haplogroup B have a cytosine (C) (Suppl. 4). The median joining network showed that from the haplotype H5 descend several private haplotypes (starlike topology), while the remaining haplotypes show a reticulate topology probably due to the high gene flow between samples (Fig. 7). The two haplogroups A and B are not well differentiated in the network.

The selected neutrality test applied to the entire sample of *C. braueri* (Strait of Sicily and Tyrrhenian Sea) to detect expansion of populations provided different results. In particular, the Tajima's D tested on the entire sample of *C. braueri* regardless of the sub-areas was statistically not significant (D = -0.37; p = 0.38); the Fu's F, which is more sensible to detect population growth, was negative and statistically significant (F = -13.64; p = 0.0001).



Fig. 6. Diagram of the CCA implemented on the morphometric and environmental datasets. Blue arrows indicate the relationship between the constrained variables and the two canonical axes identified by the analyses. The spatial location of the morphometric parameters in the diagram is represented by the red crosses. Single observations were projected using different colors and shape points based on the sampling site. Moreover, the centroids and the relative confidence ellipses (95%) were also calculated and added to the diagram in order to provide a better description of the relationships with the canonical axes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4

List of 12S haplotypes from *Cyclothone braueri* samples collected in Strait of Sicily (S) and Central-Eastern Tyrrhenian Sea (T). Sub-areas within each region are also indicated in the appropriate column as follows. Regarding the Strait of Sicily: West (W); Middle (MS); East (E); Offshore (Off). Regarding the Central-Eastern Tyrrhenian Sea: North-West (NW); Middle (MT); South-East (SE). The number of specimens belonging to each sub-area is reported in parentheses.

Haplotype Code	N	Region	Sub-areas	Haplogroup	GenBank Acc. N°
HAP1	1	S	W	B	MT892772
HAP2	1	S	W	A	MT892773
HAP3	16	S/T	W(7)/E(3)/SE(3)/MT(3)	B	MT892774
HAP4	2	S	W	A	MT892775
HAP5	37	S/T	W(7)/MS(1)/E(2)/Off(6)/SE(11)/MT(6)/NW(4)	A	MT892776
HAP6	1	S	W	A	MT892777
HAP7	1	S	W	A	MT892778
HAP8	1	S	MS	A	MT892779
HAP9	3	S	MS	A	MT892780
HAP10	7	S	MS	A	MT892781
HAP11	7	S/T	MS (5)/Off (1)/NE (1)	A	MT892782
HAP12	2	S	MS	A	MT892783
HAP13	1	S	MS	A	MT892784
HAP14	1	T	NW	B	MT892785
HAP15	8	T	NW	A	MT892786
HAP16	2	T	NW	A	MT892787
HAP17	6	S/T	E(2)/Off(1)/SE(2)/MT(1)	A	MT892788
HAP18	4	T	SE(1)/MT(3)	A	MT892789
HAP19	4	T	SE(2)/MT(2)	B	MT892790
HAP20	1	T	SE	A	MT892791
HAP21	9	S	E(7)/Off(2)	A	MT892792
HAP22	1	S	E	A	MT892793
HAP23	4	S	E(2)/Off(2)	B	MT892794
HAP24	1	S	E	B	MT892795

Table 5

Diversity measures for *Cyclothone braueri* collected in the Strait of Sicily (S) and Central-Eastern Tyrrhenian Sea (T) and the relative sub-areas for 12S ribosomal gene: number of sequences (n), number of haplotypes (Nh), haplotype diversity (h), nucleotide diversity (π) and relative standard deviation (SD).

Region	Location	Date of collection	n	N _h	h ± SD	π ± SD
S	West	June 2013	20	7	0.774 ± 0.065	0.004 ± 0.0007
S	Middle	June 2013	20	7	0.595 ± 0.073	0.002 ± 0.0003
S	East	July 2013	18	7	0.797 ± 0.066	0.005 ± 0.0005
S	Offshore	July 2013	12	5	0.652 ± 0.133	0.004 ± 0.0009
T	North-West	July 2013	16	5	0.708 ± 0.094	0.004 ± 0.0005
T	Middle	July 2013	15	5	0.733 ± 0.084	0.005 ± 0.0010
T	South-East	July 2013	20	6	0.568 ± 0.119	0.003 ± 0.0010

4. Discussion

The present study is the first attempt to study the ecology of the early life stage of *C. braueri* in the Central Mediterranean Sea considering a regional scale. We took advantage of almost simultaneous surveys conducted in the southern (Strait of Sicily) and in the northern part (Central-Eastern Tyrrhenian Sea) of the Central Mediterranean Sea to assure the sampling of early life stages spawned by potentially unrelated populations in different heterogeneous environments. In this context, we found a high density and a wide dispersion of *C. braueri* larvae in both regions, confirming the cosmopolitan features of this mesopelagic species. In the Strait of Sicily, in spite of the mesopelagic nature of the adults, *C. braueri* larvae were found both on the continental shelf and in the deeper zone, highlighting the importance of a sampling design that

Table 6
Summary statistics of the Analysis of Molecular Variance (AMOVA).

Structured tested	Source of variation	Variance component	Percentage of variation	F-statistic	P
All populations on a unique group	Among populations	0.29498 Va	26.20	$F_{ST} = 0.26$	0.00001
	Within populations	0.83088 Vb	73.80		

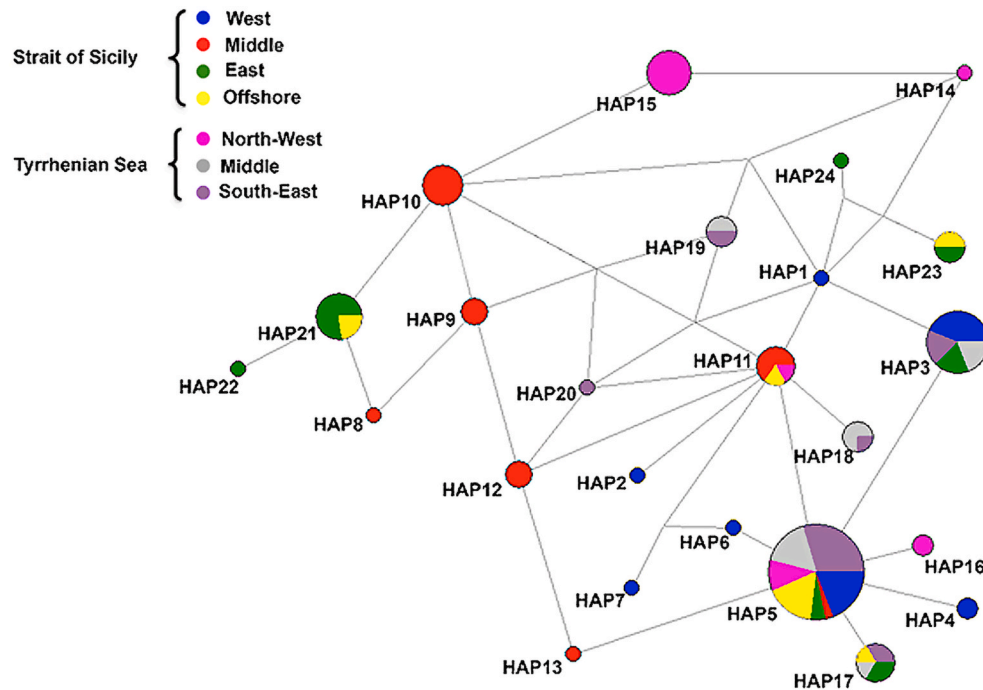


Fig. 7. Median-joining network (star-like topology) of the haplotypes identified by sequencing the 12S ribosomal gene from *Cyclothone braueri* larvae collected in the regions of the Strait of Sicily, the Central-Eastern Tyrrhenian Sea and in their respective sub-areas.

includes both areas in studies aimed at exploring the distribution of mesopelagic species during the planktonic stage. This spatial distribution of the planktonic stages and the outcomes of the multivariate GAM suggested a significant effect of the hydrodynamic in advecting larvae from spawning to retention areas. Indeed, higher densities were found in the Eastern sector of the study area, where the waters were warmer and characterized by the presence of a saline front indicative of a retention area (Agostini and Bakun, 2002; Lafuente et al., 2002). In this framework, the shape of the cubic regression splines estimated from the GAM confirmed this relationship, describing higher larval densities for warmer waters and for the salinity values that characterized the saline front. In addition, higher densities were significantly related to higher current velocities, connoting an important effect of the larval advection. This evidence is in agreement with other studies that linked the spatial distribution of meso-bathypelagic species at the planktonic stage to the mesoscale oceanographic structures (e.g., geostrophic currents, eddies, fronts) occurring at the surface level in the same region (Cuttitta et al., 2004, 2016b, 2018). In this study, the analysis of the geostrophic currents and/or the lagrangian simulations performed in the area confirmed the occurrence of energetic and complex hydrodynamics system that hence can underlie the spatial heterogeneity of planktonic stages, advecting larvae from the spawning areas (i.e., the offshore zone) to the coastal zone and in particular in the South-East sector of the study area. Similar to other studies focused on different ichthyoplankton species in the same region, the advection was mainly controlled by the Atlantic Ionian Stream (AIS) that transported planktonic stage and concentrated them in the eastern retention area (Cuttitta et al., 2004; Patti et al., 2018, 2020; Torri et al., 2018). This interpretation would explain the presence of a high number of larvae on the eastern continental shelf, as also emerged from the model outcome, despite the

mesopelagic nature of the spawning in *C. braueri*. In addition, this study represents the first description of the spatial distribution of *C. braueri* early life stage in the Central-Eastern Tyrrhenian Sea where, in contrast to the southern region, the sampling and the multivariate model evidenced a pattern driven essentially by the bathymetry. Indeed, most of the larvae was found beyond the isobath of 200 m, in agreement to the spawning behavior of the adults (McKelvie, 1989; Yoon et al., 2007), suggesting the absence of noticeable coastward larval transport. Altimetry data supported this hypothesis as only a weak (or absent) physical forcing linked to the movement of the surface water masses has been detected in this area during the sampling period. This pattern is comfortably supported by the outcome of the GAM, that allowed to identify the bottom depth as the most important predictor explaining the spatial variability of the larval density. Moreover, the positive effect of high depths and low current velocity supported the hypothesis of spatial distribution substantially linked to the spawning behavior of the adults in the offshore zone.

Moreover, this study was aimed at determining the population structure of *C. braueri* taking into account for the first time both morphometric and genetic approaches. The first analysis highlighted the presence of two distinct morphotypes in both study areas, namely called “thick morphotype” and “thin morphotype”. On one side, the presence of different phenotypes in distinct areas could be the signal of different sub-populations characterized by a limited genetic flow. On the other, it could be the effect of different environmental factors affecting the state of fish larvae, e.g., in terms of nutritional conditions. In our study, morphometric and genetic analysis support the second hypothesis. Several studies showed that the body shape is a good proxy of the nutritional status of fish larvae (Battini et al., 1995; Ehrlich et al., 1976; Frank and McRuer, 1989; Powell and Chester, 1985; Theilacker, 1978),

which in turn is mainly affected by temperature and food availability conditions (Buckley, 1984; Ferron and Leggett, 1994; Heath, 1992). Similar to these results, in our study the “thin” shape has been found in sub-areas characterized by lower values of temperature and/or lower chlorophyll-a concentration, while the “thick morphotype” characterized the warmer and nutrient-enriched waters. In light of this evidence, the spatial distribution of the two morphotypes could be linked to the occurrence of more or less favorable temperature and trophic conditions that, influencing the metabolism rate and the food availability respectively, affect the nutritional conditions and hence the morphological features of the larval stages.

This interpretation is supported from the results emerged in our study, where both MLT and CHLa are related to the presence of the two morphotypes. In the Strait of Sicily, the “thin” morphotype has been found in the area affected by upwelling, i.e., in the colder waters of the “West” and “Middle” zones. In particular, among these two areas the lowest values in terms of size-corrected morphometric parameters have been found in the coastal “Middle” sub-area that was also characterized by lower food availability. Conversely, larvae exhibiting the “thick morphotype” have been collected in warmer sub-areas (“East” and “Offshore”) and the highest values of morphometric parameters emerged in the “East” sub-area, where, in addition to the warmer waters, relatively nutrient-enriched waters have been detected due to the presence of a filament mesoscale oceanographic structure (Bignami et al., 2008; Torri et al., 2018). These observational patterns have been comfortably confirmed by the multivariate analysis. On one side, Random Forest classification models allowed to identify the body depth (BD) as the most important morphometric parameter that allows for the discrimination of the sampling sites. On the other side, CCA confirmed this finding and highlighted a correspondence between higher values BD (i.e. the “thick morphotype”) and warmer and food-enriched waters. These two environmental factors seem to act synergistically in affecting the morphological features of the larval stage. Among regions, the highest and the lowest values of BD have been found in the waters characterized respectively by the highest (the Tyrrhenian sub-areas “North-West” and “Middle”) and the lowest (The Sicilian sub-area “Middle”) values of temperature and CHLa concentration. Accordingly, a less considerable, albeit existing, discrimination of the observations in the ordination diagram emerged between the “South-East” (“thin morphotype”) and the “East”-“Offshore” (“thick morphotype”) zones in correspondence of more similar environmental conditions.

In addition, this synergic action has been evidenced at the intra-regional scale. In the Strait of Sicily, it was interesting to note that the highest and the lowest values of morphometric parameters have been evidenced in correspondence of areas characterized by common patterns in terms of temperature and food availability, as in the “East” zone (where both parameters were relatively higher) and in the “Middle” zone (where both parameters were relatively lower) respectively, while limited morphological differences emerged where temperature and food availability showed an opposite patterns (i.e., low temperature and high food availability as in the “West” zone and vice versa in the “Offshore” sub-areas). Based on these findings, the “East” zone of the Strait of Sicily can be identified as favorable areas for the larval development in *C. braueri*. In addition to this, it is notable that also other studies supported the identification of the “East” area as one of the most favorable retention zones in the Strait of Sicily for the larval development of fish species that spawn during the summer season, as the case of European anchovy, *Engraulis encrasicolus* (Cuttritta et al., 2015; Falco et al., 2020; Patti et al., 2020). Similar patterns have been found in Central-Eastern Tyrrhenian Sea. Indeed, in this region the synergic effect of temperature and food availability matched coherently with the morphometric findings, as the “thick” morphotype was found in warmer and trophic waters (the two northern sub-areas) and the “thin” morphotype was found in the rest of the study area (the “South-East” zone). In this case, the two northern sub-areas (“North-West” and “Middle” in this study) could identified as more suitable areas for the larval development

compared to the South, although further studies focused on this topic could be needed.

The hypothesis of the nutritional larval condition underlying the morphological differences is also supported by the morphometric parameter that was found to be more effective for the multivariate discrimination of the study areas, i.e. the “Body Depth” (BD). From an ecological point of view, the BD parameter seems to be a good proxy for the larval nutritional status of this species. Although more focused studies should be addressed on *C. braueri* (e.g., mesocosm experiments), this evidence is in agreement with previous studies on other fish species at larval stage (Battini et al., 1995; Diaz et al., 2009; Gwak et al., 1999; Martin and Malloy, 1980). In these papers, morphometric parameters linked to the thickness of the body showed a better response to the environmental stressors compared with parameters describing length measures (e.g., head length or pre-anal length parameters used for this study), most likely because the latter are strictly related to the endoskeleton. On the other hand, BD-like parameters could be more related to the physiological status of the individuals because they could be more directly affected by the muscular tone and/or by the amount of stored lipids. Therefore, the choice of BD-like parameters could be appropriate for studies aimed at determining the larval condition using a morphology-based approach.

Although our analysis demonstrated the key role of environmental factors in affecting the larval morphotype, it also suggests the potential effect of other source of variability underlying the morphological differences emerged between regions. Indeed, the general Random Forest model (model A) evidenced higher morphological similarity in larvae collected in the same region in spite of the differences in terms of environmental conditions. It was indicative that the larvae collected in the “East” and the “Offshore” zones of the Strait of Sicily has been mostly confused with the other larvae sampled in the two colder zones of the same region, despite more similar environmental conditions with the Tyrrhenian “South-East” south-area. In light of these results, in recognizing the fundamental role of the pelagic environment, we cannot exclude the potential role of other local factors able to influence the larval condition but not examined in this study, such as the food quality and/or the parental effect (see Catalán et al., 2020 for recent researches on this topics), in affecting the larval morphology and diversifying larvae belonging to different subpopulations.

Our results, based on our investigation on the variability of the 12S rRNA gene, suggest that the observed morphometric interregional heterogeneity is not due to genetic factors. From the genetic point of view, analyses highlighted the presence of 24 mtDNA haplotypes that are possible to gather into two distinct haplogroups. However, in spite of the morphometric detection of two spatially separated morphotypes, molecular analysis evidenced the co-occurrence of these two phylogroups within regions and sub-areas. There was thus no geographical pattern driving the inter-relationship among the haplotypes. AMOVA analysis also corroborated this finding showing a higher variability within populations compared with the variability among populations, suggesting the co-presence of alternative genotypes in the same areas. It is also noteworthy that this study revealed an intraspecific genetic variability in this species and signatures of population expansion as suggested by the significant negative values of Fu's F_s test. Our results integrated the findings by Miya and Nishida (1996), who considering the 12S and 16S rRNA genes of three individuals of *C. braueri* taken from western South Pacific, western North Atlantic and Mediterranean Sea, showed the absence of an intraspecific variation (except for a single nucleotide substitution at position 93 in the 16S rRNA). The co-occurrence of two main clades in the Mediterranean Sea has already been evidenced in other fish species, such as swordfish (*Xiphias gladius*) (Alvarado Bremer et al., 2005; Pappalardo et al., 2011), the Atlantic bonito (*Sarda sarda*) (Viñas et al., 2004), the European anchovy (*Engraulis encrasicolus*) (Cuttritta et al., 2015), the greater amberjack (*Seriola dumerili*) (Šegvić-Bubić et al., 2016) and the common pandora (*Pagellus erythrinus*) (Angiulli et al., 2016). The common line among these studies is the lack

of geographical patterns within the Basin that can be attributed to a secondary contact between historical populations that diverged in allopatry after the removal of ecological or geological barrier. In particular, past geographical isolations due to climate cycles during the late Pliocene and throughout the Pleistocene seem to have triggered a deep genetic divergence of phylogroups that have successively had a secondary mixing. The non-geographic distribution pattern of the two *C. braueri* phylogroups could be due to their simultaneous arrival in the basin from the Atlantic where they diverged, as already hypothesized for other pelagic fish species (Šegvić-Bubić et al., 2016). An alternative hypothesis could take into consideration the secondary contact between the two phylogroups after an initial separation of a Mediterranean population from an Atlantic one, during glaciations, and subsequent return to the Mediterranean by the Atlantic population. This scenario was hypothesized to explain the population structure of small pelagic fishes such as *Engraulis encrasicolus* (Magoulas et al., 2006) and *Sarda sarda* (Viñas et al., 2004). However, it should be noted that i) we cannot test the above mentioned hypothesis because we had not Atlantic samples in our study, and ii) a deep structure among haplogroups has not been detected in this study. Indeed, we found not very high, but moderately robust, bootstrap values supporting the two divergent haplogroups in the ML tree and no clear separation between the two haplogroups in the network. Considering the circumglobal distribution of *C. braueri*, a wider number of populations and individuals than those examined herein should be studied to confirm the genetic structure and demographic history of this species.

5. Conclusions

The multidisciplinary approach to the study of the mesopelagic species *C. braueri* allowed us to obtain useful information on the ecological implications of environmental factors on the spatial distribution as well as on the condition of larval stages and on genetic structure of the populations of this species in the Central Mediterranean Sea. This study identified two larval morphotypes (namely called “thin” and “thick” morphotypes) not connected to two detected phylogroups but rather linked to the different nutritional conditions derived from the environmental variability in terms of temperature and food availability on a regional scale. In this context, from a methodological point of view, the body depth morphometric parameters emerged as an appropriate candidate in order to infer useful insights on the larval condition, although further analysis more specifically addressed on the biochemical and physiological larval condition could be also addressed for this scope.

The conclusions of this work encourage further analyses focused on the genetic structure of the fish population, which showed patterns similar to other Mediterranean fish species and suggest a common history in terms of separation, genetic divergence and secondary contact that could be further explored. Moreover, our ecological findings lay the groundwork for the further identification of the role of key environmental factors in controlling the population biomass. Water temperature and food availability could play an important role also in terms of larval growth rate and thus in modulating natural mortality of early life stages, that in turn could affect the recruitment and hence the biomass of this dominant species in the mesopelagic zone. Biomass fluctuation of one of the main constituents of the mesopelagic community could thus induce significant effect on the marine ecosystem, affecting the food web and the populations of the higher trophic layers that represent fundamental marine resources. Furthermore, increasing the knowledge of organisms residing in mesopelagic environments is an essential effort in order to evaluate mesopelagic fish species as further marine resources to be sustainably exploited by fisheries. In this framework, data collection efforts focusing on the deep environments should be increasingly encouraged. Moreover, this work emphasizes the key role of the oceanographic cruises in collecting information not only of the photic zone but also on the deeper layers of the ocean. Indeed, the occurrence

of early life stages of meso- and bathy-pelagic species in surface layers represents a valuable signal, able to provide insight into one of the most unknown systems of the Earth.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2021.105379>.

Author statement

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