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Temporal and spatial genetic variation of *Engraulis encrasicolus* in the Adriatic Sea

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Abstract

Small pelagic fish play a key role in the marine ecosystem, controlling predator abundance and regulating primary production rates by foraging on plankton. Alterations induced at different ecological levels by fishing activities and/or environmental changes are affecting the reproductive success of several small pelagics, including European anchovy (*Engraulis encrasicolus*), which is a major target of Adriatic mid-water trawl and purse-seine fisheries. In this study, we evaluated short-term genetic changes of the species in the Adriatic Sea by applying molecular markers in samples of three generations of European anchovy. Thirteen polymorphic microsatellite markers and a mitochondrial gene were used in cohorts of adults and larvae, collected at three sites in the north-east, central, and southern Adriatic Sea in 2015. Furthermore, temporal, and spatial genetic variation was assessed by comparing the above dataset with a sample of adult anchovy collected in 2012 at three sites close to those sampled in 2015. Expected heterozygosity was higher in adults than in larvae, suggesting a loss of genetic diversity and uneven reproduction. In addition, comparison of the two datasets demonstrated a change in the anchovy population structure from 2012 to 2015. In the reproductive event of 2015, this change led the two main genetic stocks described in the Adriatic Sea to merge into one. We suggest that the population structure of European anchovy in the north-eastern Adriatic may be influenced by changes in environmental parameters and by periodic alterations in the temporal pattern of population connectivity.

Keywords: *Engraulis encrasicolus;* genetic diversity; population structure; Adriatic Sea.

Introduction

Small pelagic fish (Clupeiformes) are a valuable food resource worth several hundred million dollars a year (FAO, 2020). Most Clupeiformes species are harvested from wild populations (Christensen, 2014). European anchovy (*Engraulis encrasicolus*) and European sardine (*Sardina pilchardus*) account for more than 40% of fish captures in the Mediterranean Sea; of these, more than half are caught in the Adriatic Sea (Tičina, 2003; FAO, 2018). Notably, anchovy are targeted by Italian northern Adriatic mid-water trawl fleets based predominantly in ports on the western coast (Tičina & Giovanardi, 1997).

Small pelagic fish are ecological key species due to their large biomass and complex dynamics and play a significant role in the marine food web, where they occupy an intermediate trophic level (Cury *et al.*, 2000). Their contribution is especially important in ecosystems, like the Adriatic Sea, which are dominated by the pelagic compartment, where European anchovy is one of the predominant species (Coll *et al.*, 2007). Physical, biological and behavioural mechanisms affect the population dynamics of small pelagic fish, which is characterized by wide biomass fluctuations over time and space (Lluch-Belda *et al.*, 1989; Alheit & Peck, 2019).

The life cycle of European anchovy is characterized by i) an r-selected life strategy (multiple pelagic spawner), ii) a short lifespan (~ 3 years), iii) high fecundity (~ 50,000 eggs *per* season) over a long spawning period (April-November) (Regner, 1985), iv) high early mortali-

ty and v) seasonal migrations between spawning and feeding grounds (Tičina, 2003; Morello & Arneri, 2009). Sexual maturity is reached by the end of the first year, when body length ranges from 7.5 to 11 cm (Kolitari, 2006). Depending on temperature, pelagic eggs hatch in 24/65 hours and post-hatching larval development takes from 1 to 4 days (Regner, 1985; Zorica *et al.,* 2018). Their fast growth rate makes anchovy larvae highly susceptible to variations in climate, food supply and physical processes (Bakun, 1996; Giannoulaki *et al.*, 2013). Combined with fishing, these factors have the potential to reduce local stocks to the point of collapse (Pitcher, 2001). Insufficient knowledge of these ecological processes affects our ability to make management predictions, prevent overexploitation (Alheit & Peck, 2019) and understand how evolutionary trajectories, determined by reproductive success and environmental pressure, can shape populations at different time and spatial scales (Burgess *et al.*, 2013; Somarakis *et al.*, 2018).

The life cycle features explain the high skewness of the individual's reproductive success (Hedgecock & Pudovkin, 2011; Ruggeri *et al.*, 2012). This stochastic phenomenon can exert severe effects on the genetic pool of local populations and, in combination with genetic drift, it may reduce genetic diversity if this is not offset by other mechanisms such as a stable population connectivity. Genetic diversity is a key evolutionary feature, enabling populations to respond promptly to environmental changes and to preserve sufficient evolutionary potential over time (Cuveliers *et al*., 2011). A sufficient genetic diversity makes it more likely that some individuals will possess genetic variants that are appropriate for novel conditions, enhancing the survival chances of the population. However, the high mortality of the early life stages can reduce genetic diversity among juveniles compared with the parental generation, impairing reproductive success (Gilbert-Horvath *et al.*, 2006). In particular, pelagic eggs and larvae face "survival windows" related to food availability, environmental conditions and predation; failure to overcome them affects the genetic contribution of a generation to the next reproductive event (Bakun, 1996; Fréon *et al.*, 2005). It has been suggested that reproduction resembles a "sweepstake process" (Sweepstake Reproductive Success, SRS) (Hedgecock, 1994), namely a chance-driven event where an individual's reproductive success does not depend only on its fitness. According to evidence obtained in other pelagic fish, SRS would exert several evolutionary effects on the population over time, reducing larval genetic diversity compared with adults and reducing the effective population size (N_e) compared with earlier generations while increasing offspring relatedness in each cohort (Hedgecock *et al.*, 2007).

Effective population size (N_e) is closely related to population abundance and reproductive strategy (Wright, 1931). In an ideal population N_e is equal to the census size (N_c), i.e., the total number of individuals in the population; however, this is rarely encountered in reality, especially due to differences in the genetic contribution of each individual to the next generation and to temporal fluctuations of N_c (Frankham, 1995; Hare *et al.*, 2011). The usual N_e/N_c ratio of pelagic fish species is in the order of 10^{-5}. A decline in N_e can severely reduce this ratio, determining a failure of the breeding event and resulting in the temporary disappearance of the local population (Hedrick, 2005). Another key index of genetic diversity is the effective number of breeders (N_b) (Waples & Teel, 1990), i.e., the number of adults contributing offspring to the next generation. In age-structured species like anchovy, N_b is easier to estimate and monitor than N_e, because it is calculated from a single cohort, not from the entire lifespan of the species (Waples & Antao, 2014). Studies of small pelagic fishes have documented temporal variations in genetic diversity suggesting that N_e and N_b may be lower than previously believed and that the interaction of fishing with other ecological factors can affect the genetic pool (Pinsky & Palumbi, 2014; Ruggeri *et al.*, 2016a).

Despite the marked dispersal ability of larval and adult stages, anchovy have been reported to exhibit an uneven genetic distribution even in the relative absence of physical barriers to connectivity (DeWoody & Avise, 2000; Hedgecock & Pudovkin, 2011). Molecular markers, like microsatellite DNA (or simple sequence repeats, SSRs or short tandem repeats, STRs) and mitochondrial DNA (mtDNA) fragments, have recently helped to unravel the genetic structure of anchovy. In the Adriatic Sea the stock was found to segregate in two main populations, one in the north-eastern and one in the western basin (Viñas *et al.*, 2013; Ruggeri *et al.*, 2016b). This genetic structure may be related to two main factors: topographical features, such as the islands and coves dotting the north-eastern Adriatic coast, which may hamper dispersion (Andrello *et al.*, 2013), and adaptive selection to local environmental conditions (Giannoulaki *et al.*, 2013; Ruggeri *et al.*, 2016b). On the other hand, a single anchovy stock for the entire Adriatic basin has been described (Fiorentino *et al.*, 2014). Given the high value of anchovy in the Adriatic Sea, this study employed polymorphic genetic markers to: i) investigate the genetic diversity of specimens from three areas of the basin during a reproductive event; ii) compare the results against the predictions of the SRS scenario, which forecasts a loss of genetic variation between adults and the offspring generated in a single reproductive event; and iii) evaluate the temporal stability of the genetic structure of the population by comparing three anchovy generations.

Material and Methods

Study area

The Adriatic Sea is a semi-enclosed basin in the central-eastern Mediterranean Sea characterized by the widest continental shelf in the Mediterranean Sea and by a distinctive habitat (Artegiani *et al.,* 1997a, b). It is divided into two geographical sub-areas (GSAs): GSA 17 (Central and Northern Adriatic Sea) and GSA 18 (Southern Adriatic basin) (GFCM, 2001). Its high productivity, the

spatial and temporal heterogeneity of the ecological parameters and the broad continental shelf provide unique conditions for the reproduction and persistence of numerous species, especially small pelagic fish (Coll *et al.*, 2009; Giannoulaki *et al.*, 2013). The Adriatic Sea is characterized by a cyclonic thermohaline circulation, the East Adriatic Current (EAC), that flows northward along the eastern coast (Marini *et al.*, 2010), the Western Adriatic Current (WAC), that flows southward along the Italian coast (Artegiani *et al.*, 1997a) and a bottom current, the Dense Water Outflow Current (DWOC) (Cushman-Roisin *et al.*, 2001). Local gyres like the South Adriatic Gyre (SAG) have the potential to enhance water mixing and promote its enrichment by concentrating nutrients (Artegiani *et al.*, 1997b) (Fig. 1).

Sampling method and data collection

Anchovy samples were collected in 2015 during acoustic surveys of small pelagic fish conducted in the framework of the MEDIAS Project (http://www.medias-project.eu/medias/website/ MEDIAS, 2019). In GSA 18 and western GSA 17, sampling was conducted by Italian researchers onboard r/v G. Dallaporta (Leonori *et al.*, 2012, 2017), whereas in eastern GSA 17 it was performed by Croatian researchers onboard r/v BIOS DVA (Tičina *et al.*, 2006). The sample included 144 adult (A) individuals and 144 larvae (L) collected during the spawning season (June and September) at three sites: i) south-western Adriatic Sea (respectively ASA and LSA specimens), ii) central Adriatic Sea (ACA and LCA) and iii) north-eastern Adriatic Sea (ACR and LCR) (Fig. 2, Table 1). This dataset was combined and compared to a dataset (Ruggeri *et al.*, 2016b) composed of three samples (35 specimens each) of adult anchovies, which were collected in July and September 2012 in areas close to the collection sites of the present sample, namely BAA, south-western Adriatic, PEB, central Adriatic and JAB, north-eastern Adriatic (Fig. 2). The latter specimens had been analysed using the same microsatellite markers as in the present study and were included to gain insight into genetic diversity variation in three anchovy generations, from 2012 to 2015 (Fig. 2, Table 1).

Larval specimens were sampled using a WP2 plankton net (200 µm mesh size) whereas adults individuals were caught with a pelagic trawl net. All specimens were stored in 70% ethanol solution at room temperature. Larvae were sorted in the laboratory under a stereomicroscope, stored individually and preserved in absolute ethanol at -20 °C.

Satellite measurements of water temperature, salinity, and primary production for 2012 and 2015 were obtained for each collection site. Mean monthly satellite data with a horizontal resolution of ~ 6.5 km and 72 unevenly spaced vertical levels were obtained from the EU Copernicus Marine Service Information (http://marine.copernicus.eu/) and extracted using SeaDAS software (v.7.4) (Simoncelli *et al.*, 2019; Teruzzi *et al.*, 2019).

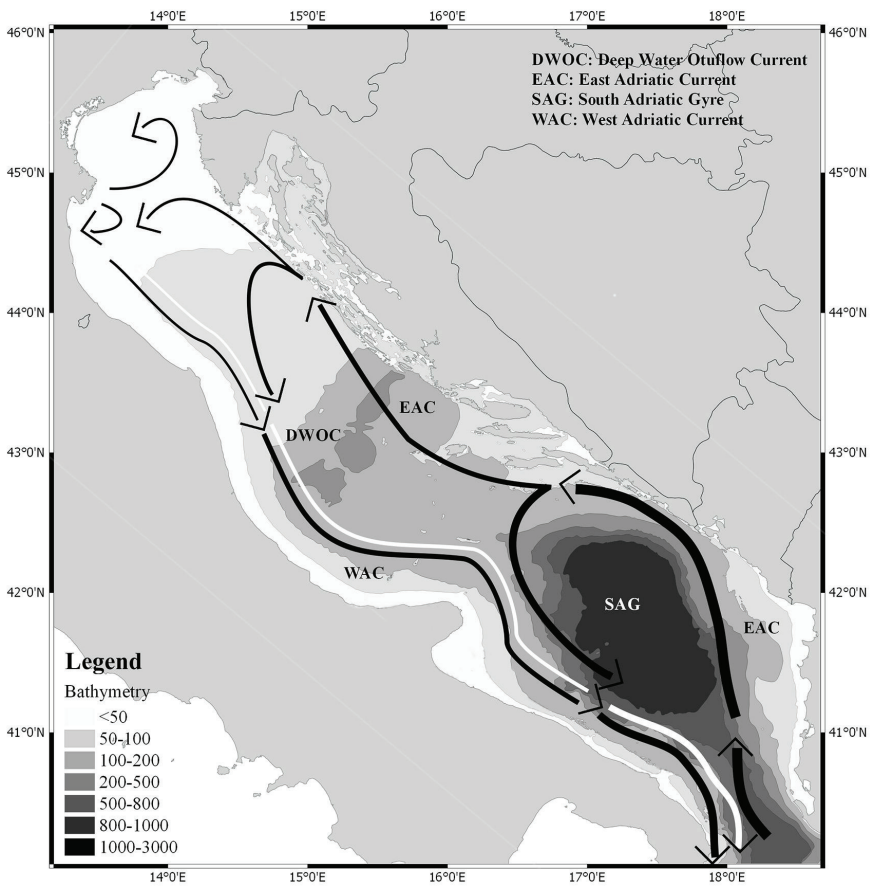

Fig. 1: Adriatic Sea surface circulation (redrawn from https://commons.wikimedia.org/wiki/File:Adriatic_Sea_Currents_2.svg).

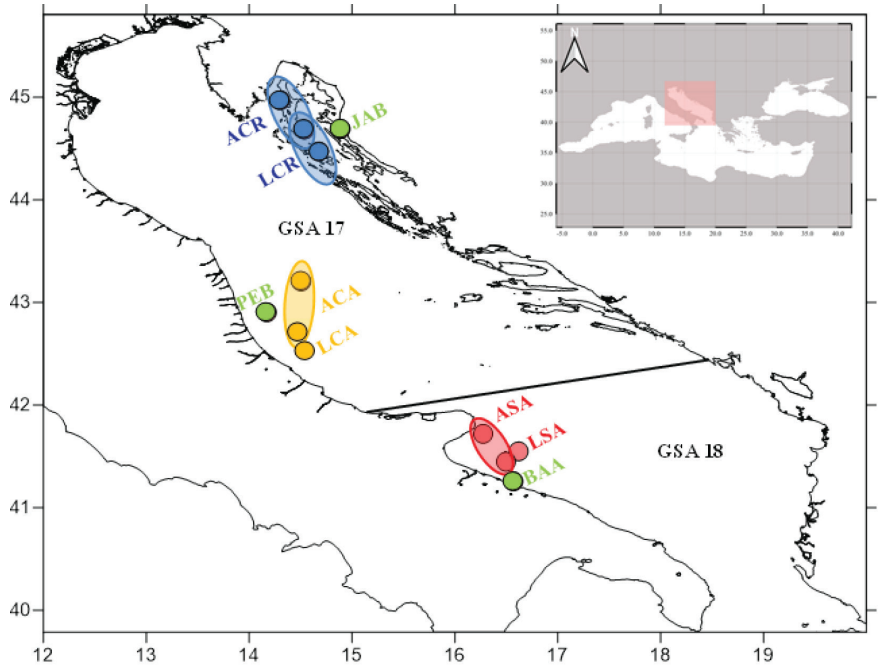

Fig. 2: Map of sampling locations. Adults (A) and larvae (L) sampled in 2015: in blue north-eastern Adriatic Sea (ACR and LCR); in yellow central Adriatic Sea (ACA and LCA); in red southern Adriatic Sea (ASA and LSA). In green adults sampled in 2012: BAA; PEB; JAB from Ruggeri *et al*. (2016b).

Table 1. Summary of sampling sites and samples; SA = southern Adriatic Sea; CA = central Adriatic Sea; CR = north-eastern Adriatic Sea; A= adults; L= larvae.

	Station	Sample name	Stage	N	Sampling coordinates	Sampling depth (m)	Sampling date (month/ year)
SA	LIT 3	**LSA**	L	48	41°32.910'N 16°37.152'E	71	Jun-2015
CA	LIT 12	**LCA**	L	48	42°37.482'N 14°28.698'E	100	Jun-2015
CR	LEM 3	**LCR**	L	48	44°28.302'N 14°40.608'E	59	Sep-2015
	LEM 4				44°41.148'N 14°32.268'E	83	
SA	AIT 3	**ASA**	A	48	41°26.850'N 16°29.808'E	9	Jun-2015
	AIT 4				41°43.128'N 16°16.470'E	14	
CA	AIT 11	**ACA**	A	48	43°12.252'N 14°30.348'E	9	Jun-2015
	AIT 12				42°35.796'N 14°30.198'E	13	
CR	AEM 4	**ACR**	A	48	44°41.148'N; 14°32.268'E	83	Sep-2015
	AEM 8				44°58.218'N 14°18.150'E	51	
SA	BAA	**BAA**	A	35	41°15.32'N 16°34.13'E	23	Jul-2012
CA	PEB	**PEB**	A	35	42°54.19'N 14°10.31'E	58	Sep-2012
CR	JAB	**JAB**	A	35	44°41.47'N 14°53.32'E	83	Sep-2012

DNA extraction, amplification and genotyping

DNA was extracted from the whole body of larval specimens or from a caudal fin fragment of adults using a MagCore® HF16 automated nucleic acid extractor (Genomic DNA Tissue Kit, code 401). Quantity and quality of DNA were evaluated by means of a spectrophotometer assay. All samples were screened at 13 polymorphic microsatellite loci described previously (Landi *et al.*, 2005; Pakaki *et al.*, 2009; Lin *et al.*, 2011); 11 loci were labelled with fluorescent dyes and multiplexed in three separate reactions, the remaining 2 loci were amplified individually. Polymerase chain reaction (PCR) products were separated on a 2% agarose gel and stained with GelRed™ (Biotium, Inc.) to check for size and PCR specificity. PCR products in multiplex were genotyped by automatic sequencing using an ABI-PRISM 3130xl genetic analyzer (Applied Biosystems) and the program Peak Scanner (http://www.appliedbiosystems.com/peakscanner.html) was used for peak identification. PCR products in simplex were genotyped by vertical electrophoresis on 5% denaturing polyacrylamide gel and visualized by a silver staining protocol (Benbouza *et al.*, 2006). PCR amplification conditions and microsatellite genotyping were optimized as described in Ruggeri *et al.* (2016b).

A PCR-based assay of the cytochrome b gene of mtDNA with three primer pairs was used to detect two mitochondrial lineages, clade A and clade B, which have been

described in the Adriatic (Magoulas *et al.*, 2006; Viñas *et al.*, 2013; Ruggeri *et al.*, 2016b). The visualization of the resulting amplicons on 2% agarose gel produced two band patterns: a single amplicon of ~ 580 bp for clade A individuals and 2 amplicons of 180 bp and 580 bp, respectively, for clade B individuals (Kristoffersen & Magoulas, 2008).

Raw data quality

The overall quality of raw data (i.e., allele misidentification), the occurrence of null alleles and the incidence of other genotyping errors (allele dropout and stutter peaks) were assessed with MICROCHECKER 2.2.1 (Van Oosterhout *et al.*, 2004). The excess of homozygous genotypes predicted by the Brookfield algorithm (Brookfield, 1996) was removed. Since null alleles mostly affect parameters associated with genotypic frequencies, the fixation index (F_{ST}) was calculated by comparing uncorrected and corrected datasets. F_{ST} values were estimated with FSTAT 2.9.3.2 software (Goudet, 2001).

Statistical analysis

Microsatellite toolkit (Park, 2001) and FSTAT 2.9.3.2. were used to calculate three key genetic diversity indices: the mean number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosity and inbreeding coefficient (F_{IS}). Departure from Hardy-Weinberg equilibrium (HWE) was tested with Genepop 4.0.11 (http://genepop.curtin.edu.au) (Rousset, 2008) using a Markov Chain Monte Carlo (MCMC) method with 100 batches of 10,000 iterations each and a burn-in period of 1000 iterations; the first 1000 iterations were discarded before sampling. An MCMC chain was used to determine whether any locus pair was in linkage disequilibrium. A sequential Bonferroni adjustment of p-values was used to account for a possible increase in type-I error (false positive) for multiple comparisons (0.05/α; α = pairwise test) (Rice, 1989).

The reduction in genetic diversity between adults and larvae cohorts, predicted by the SRS scenario, was investigated with two tests. The first compared H_E variation in adults and larvae collected in 2015. H_E was preferred to other genetic diversity indices because it is more stable over generations and is independent of the level of polymorphism characterizing the microsatellite markers; this might pose problems for some parameters (*e.g.* allele richness and mean N_A), because it increases the variance (Dalongeville *et al.,* 2016). Pairwise comparisons of H_E of adults and larvae from each of the three sites and then of all adults (2015 + 2012) against larvae were conducted in PAST 4.02 (Hammer *et al.*, 2001). Next, a two-paired test was applied to assess the significance of each pairwise comparison of the average H_E values of adults and larvae. The second test, performed to investigate the predictions of the SRS scenario, involved estimating N_e and N_b by two different approaches. Since all larvae had been collected in June and September of the same year, they were assumed to belong to the same cohort. When samples include specimens of the same age group generated by a single breeding cycle and/or spawning season, estimates of N_b can be translated into estimates of N_e *per* generation (Waples *et al.*, 2014). This was achieved using a maximum likelihood sibship assignment method (Wang, 2009; Wang & Santure, 2009) implemented in COLONY 2.0.2.2 software (Jones & Wang, 2010) assuming random mating. Parent-offspring relationships were identified by comparing the genotype of each larval specimen to the genotype of all putative parents. If more than one parent was identified, the categorical assignments were examined to measure the likelihood of each parent–offspring pair being true based on the genotype and allele frequencies in the population. For comparison purposes, N_e and 95% confidence intervals (CI95) were also estimated with *N*e-Estimator 2.0 software (Do *et al.*, 2014) using the linkage disequilibrium method (Waples & Do, 2008) and the molecular coancestry method (Nomura, 2008). The N_b/N_c ratio was compared in specimens from each collection site. The N_c estimates for the northern, middle and southern Adriatic Sea were obtained with acoustic data from the MEDIAS dataset, assessed with the standard echo-integration method (Simmonds & MacLennan, 2005). Acoustic data were logged and processed according to the MEDIAS protocol (http://www.medias-project.eu/medias/website/handbooks). Biomass calculation was performed at 38 kHz. The echo-integration method requires a relation linking the echo strength to the length-weight equation, to convert an acoustic signal into fish biomass/density, known as Target Strength (TS), modelled as follow: TS=20 logL+b20 where L refers to the fish length in cm and b20 is a constant for a given species.

The degree of admixture of the larvae collected at the three sites was evaluated by assignment tests performed in Geneclass 2 (Piry *et al.*, 2004) based on the allele frequency method (Paetkau *et al.*, 1995). The tests compared larval against adult specimens collected in 2015 and then against the pooled adult samples (2015 + 2012), assuming a lifespan of ~ 3 years, to examine the effect of overlapping generations. Individuals assigned to a given site with a confidence greater than 70% were retained and used to calculate the percentage of larvae that were recruited from the local stock and of those that had drifted from elsewhere.

The population structure was assessed using a multivariate ordination method performed with Discriminant Analysis of Principal Components (DAPC) (Jombart *et al.*, 2010) available in the adegenet R package (Jombart, 2008). DAPC implements a test of principal components of genetic variation (PCA) to find the largest difference among groups of samples while minimizing differences within each group. The population structure was also assessed using the cytochrome b gene of mtDNA, which allowed identifying clades A and B (Kristoffersen & Magoulas, 2008).

The effect of the environmental parameters on the genetic structure of the population was investigated by analysis of variance (ANOVA) and Tukey's *post-hoc* test

performed with R (R Core team, 2013), to seek differences in environmental variables in different years, GSAs (GSA 18_West, GSA 17_West, GSA 17_East) and depth layers, which were considered as fixed effects.

Results

Raw data quality

In the 2015 sample, PCR amplification of the 13 microsatellite loci was successful in 287/288 specimens, the exception being an LSA individual. Since all the larvae from each batch had been collected at the same time and preserved with the same standardized protocol, either the DNA quality of the individual that failed to amplify decayed immediately or else it was not an anchovy larva (e.g., *Sardinella aurita*), considering that the PCR primers were designed for *Engraulis* spp. The missing data consisted of 12/3731 (0.32%) genotypes over the 13 loci. Although the data showed no allele dropout and minimal stuttering, signal of null alleles was detected in 53/117 (45.3%) global tests. Altogether, null alleles were found at all loci except Ee2-165b. Their incidence was highest at Ej-41.1, Ej-27.1 and Enja-83, where null alleles were detected at all three sites. Null alleles are common in microsatellites; since they can bias genetic statistics, occasionally even increasing F_{ST} values, we applied the correction for null alleles to infer F_{ST} values (Chapuis & Estoup, 2007) and removed the excess of homozygous genotypes according to the Brookfield algorithm (1996). In the corrected dataset, null alleles were removed from 22/53 significant tests. In the raw dataset, pairwise F_{ST} values ranged from -0.0033 to 0.0248 and identified 10 significant pairwise tests out of 36 tests (Table 2A), whereas in the corrected dataset they ranged from -0.0024 to 0.0267 and identified 4 significant pairwise tests (Table 2B). This clearly suggests that null alleles markedly affected the dataset, and that the Brookfield correction greatly improved the quality of the raw data. The corrected dataset was thus used for all further tests.

Genetic diversity in the adult and larval samples, N_b and N_e

The mean number of alleles (N_A) ranged from 9 at Ee2-165b to 42 at Ee2-407 (Table S1). In larvae, N_A *per* sample over all loci ranged from 14.15 alleles in LCA specimens to 15.62 alleles in LSA specimens. Expected (H_E) and observed (H_O) heterozygosity were lowest in LCA (H_E = 0.8080 and H_O = 0.7251), whereas the highest values were found respectively in LSA (H_E = 0.8135) and LCR (H_O = 0.7370) specimens (Table 3). In adults, N_A *per* sample over all loci ranged from 14.46 alleles in ACA to 14.62 alleles in ASA. Expected and observed heterozygosity were lowest in ACA (H_E = 0.8266) and ACR (H_O = 0.7101), respectively, and highest in ACR (H_E = 0.8335) and ACA (H_O = 0.7406) specimens (Table 3). Significant departure from Hardy-Weinberg proportions over all loci was found at 10 loci (Ee2-91b, Ej-41.1, Ee10, Ej-27.1, Ej35, Enja-83, Ee2-507, Eja17, Ej-2, Ee2-508). In 78 pairwise tests, significant linkage disequilibrium was detected only between Ej35 x Ee2-135.

Table 2. A) Raw dataset of pairwise F_{ST} values; B) Dataset corrected for null alleles (values in bold: significant after Bonferroni correction). Larvae (L) and adults (A) sampled in 2015 in the southern (LSA and ASA), central (LCA and ACA) and north-eastern Adriatic Sea (LCR and ACR). Adults sampled in 2012: BAA, PEB, JAB.

A	LSA	LCA	LCR	ASA	ACA	ACR	JAB	BAA	PEB
LSA	0	-0.0005	0.0014	0.0001	0.0019	0.0016	**0.0201**	0.0024	0.003
LCA	-0.0005	0	-0.0022	0.0011	0.0019	**0.0045**	**0.0267**	0.0012	0.0042
LCR	0.0014	-0.0022	0	0.0005	-0.0001	**0.005**	**0.0196**	-0.0002	0.002
ASA	0.0001	0.0011	0.0005	0	-0.0015	-0.0008	**0.022**	-0.0005	0.0007
ACA	0.0019	0.0019	-0.0001	-0.0015	0	-0.0003	**0.0222**	-0.0002	0.0011
ACR	0.0016	**0.0045**	**0.005**	-0.0008	-0.0003	0	**0.0245**	0.0021	0.0013
JAB	**0.0201**	**0.0267**	**0.0196**	**0.022**	**0.0222**	**0.0245**	0	**0.0163**	**0.0226**
BAA	0.0024	0.0012	-0.0002	-0.0005	-0.0002	0.0021	**0.0163**	0	-0.0024
PEB	0.003	0.0042	0.002	0.0007	0.0011	0.0013	**0.0226**	-0.0024	0

B	LSA	LCA	LCR	ASA	ACA	ACR	JAB	BAA	PEB
LSA	0	-0.0014	0.0003	-0.0007	0.0017	0.0001	**0.0186**	0.0009	0.0025
LCA	-0.0014	0	-0.0027	0.0011	0.0017	0.0032	**0.0248**	0.0004	0.0036
LCR	0.0003	-0.0027	0	0.0003	-0.0006	0.0035	0.0179	-0.002	0.0016
ASA	-0.0007	0.0011	0.0003	0	-0.0013	-0.0015	0.0211	-0.0018	0.0004
ACA	0.0017	0.0017	-0.0006	-0.0013	0	-0.0014	**0.0214**	-0.0019	0.0001
ACR	0.0001	0.0032	0.0035	-0.0015	-0.0014	0	**0.0218**	-0.0005	-0.0004
JAB	**0.0186**	**0.0248**	0.0179	0.0211	**0.0214**	**0.0218**	0	0.015	0.0232
BAA	0.0009	0.0004	-0.002	-0.0018	-0.0019	-0.0005	0.015	0	-0.0033
PEB	0.0025	0.0036	0.0016	0.0004	0.0001	-0.0004	0.0232	-0.0033	0

Table 3. Summary of the genetic variability observed at 13 microsatellite loci. Larvae (L) and adults (A) sampled in 2015 in the southern (LSA and ASA), central (LCA and ACA) and north-eastern Adriatic Sea (LCR and ACR). Adults sampled in 2012 (BAA, PEB, JAB). N = number of individuals correctly genotyped; N_A = mean number of alleles observed; H_E = expected heterozygosity; H_O = observed heterozygosity; SD = standard deviation; F_{IS} = inbreeding coefficient.

	Stage	Year	N	N_a	SD(N_a)	H_e	SD(H_e)	H_o	SD(H_o)	F_{is}
LSA	L	2015	47	15.62	7.98	0.8135	0.0368	0.7326	0.0186	0.099
LCA	L	2015	48	14.15	6.80	0.8080	0.0385	0.7251	0.0184	0.101
LCR	L	2015	48	15.08	7.17	0.8115	0.0320	0.7370	0.0185	0.092
ASA	A	2015	48	14.62	7.94	0.8274	0.0285	0.7386	0.0182	0.104
ACA	A	2015	48	14.46	7.98	0.8266	0.0323	0.7406	0.0185	0.098
ACR	A	2015	48	14.54	7.47	0.8335	0.0293	0.7101	0.0192	0.144
BAA	A	2012	35	12.62	6.73	0.8213	0.0309	0.7279	0.0220	0.116
PEB	A	2012	35	13.69	6.86	0.8284	0.0298	0.7171	0.0220	0.137
JAB	A	2012	35	11.77	4.82	0.7649	0.0294	0.7275	0.0219	0.043

In the sample from the north-eastern Adriatic, the two-paired test comparing $H_{E\text{-}ACR}$ = 0.833±0.029 and $H_{E\text{-}LCR}$ = 0.811±0.032 did not yield significant differences (p = 0.1447). Despite the higher H_E values of adults; non-significant test results were also obtained when comparing the central ($H_{E\text{-}ACA}$ = 0.827±0.032 *vs* $H_{E\text{-}LCA}$ = 0.808±0.038; p = 0.1162) to the southern Adriatic ($H_{E\text{-}ASA}$ = 0.827±0.029 *vs* $H_{E\text{-}LSA}$ = 0.814±0.037; p = 0.2024) specimens. Again, comparison of the H_E of all adults to all larval specimens showed higher though not significantly different values in the former ($H_{E\text{-}Adults}$ = 0.829±0.030 *vs* $H_{E\text{-}Larvae}$ = 0.811±0.036; p = 0.0627) (Fig. 3).

The effective number of breeders (N_b), obtained with the maximum-likelihood method in COLONY, ranged from 98 (LCA and LCR) to 114 (LSA). The census size (N_c) obtained from the MEDIAS dataset was 2.41E+10 for GSA 17_West, 3.48E+09 for GSA 18_West and 1.59E+09 for GSA 17_East, resulting in a low N_b/N_c ratio with an order of magnitude of $10^{-8/-9}$ (Table 4A).

The effective population size (N_e), estimated with N_e-Estimator software according to the linkage disequilibrium method, ranged from 169.3 (CI95: 44.2-inf) in JAB to infinite values in LCA (CI95: 552.9-inf), ACA (CI95: 127.1-inf) and BAA (CI95: 159.8-inf) specimens (Table 4B), whereas the N_e estimates calculated by the molecular coancestry method ranged from 49.9 (CI95: 49.9-inf) in JAB to infinite values in most of the other samples (Table 4B). Difficulties in obtaining specific estimated and finite CI95 values were met with both approaches, due to the high SSR variability in Engraulidae (Zarraonaindia *et al*., 2009; Borrell *et al*., 2012). Furthermore, N_e values of adults and larvae at each site did not display significantly different mean or min-CI95 values.

Assignment test

Evaluation of adults and larvae by the assignment test in Geneclass identified 66.6% LCR, 60.4% LCA and 55.3% LSA individuals with a confidence greater than 70%. As regards the LCR specimens, only 6% were attributed to local ACR parents, whereas most were assigned to ACA (47%) or ASA (47%) parents (Fig. 4A). Of the LCA specimens, 34% were assigned to local ACA parents, whereas 45% were attributed to ASA parents and 21% to ACR parents (Fig. 4B). The LSA individuals were assigned to local ASA (38%), ACA (31%) or ACR (31%) parents (Fig. 4C). When the test was performed

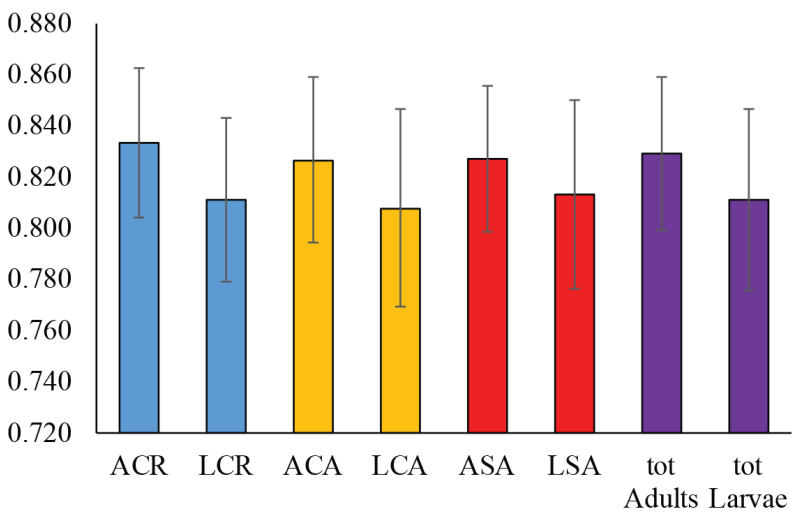

Fig. 3: Mean expected heterozygosity (H_E) of the adult (A) and larval (L) specimens sampled in 2015 in the north-eastern (ACR and LCR), central (ACA and LCA) and southern Adriatic (ASA and LSA), tot Adults (ACR+ACA+ASA) and tot Larvae (LCR+LCA+LSA).

Table 4. Estimated N_b and N_e and 95% confidence intervals (CI95). A) N_b estimated with the Maximum-likelihood sibship method; N_c obtained from the MEDIAS dataset. B) N_e estimated with the Linkage disequilibrium method and the Molecular coancestry method. Larvae (L) and adults (A) sampled in 2015 in the southern (LSA and ASA), central (LCA and ACA) and north-eastern Adriatic Sea (LCR and ACR). Adults sampled in 2012 (BAA, PEB, JAB).

A	Maximum-likelihood sibship method				
	N_b value	N_c value	CI95 min	CI95 max	N_b/N_c
LSA	114	3.48E+09	77	179	3.274E-08
LCA	98	2.41E+10	66	152	4.059E-09
LCR	98	1.59E+09	65	152	6.175E-08

	Linkage disequilibrium method			Molecular coancestry method		
B	N_e value	CI95 min	CI95 max	N_e value	CI95 min	CI95 max
LSA	1194.9	122.2	inf	inf	inf	inf
LCA	inf	552.9	inf	106.8	0.1	536.2
LCR	307.6	76.2	inf	inf	inf	inf
ASA	1065.8	136.7	inf	inf	inf	inf
ACA	inf	127.1	inf	inf	inf	inf
ACR	258.7	65.4	inf	71.3	8.6	198.5
BAA	inf	159.8	inf	inf	inf	inf
PEB	259.8	50.3	inf	79	0.1	396.4
JAB	169.3	44.2	inf	49.9	0	250.6

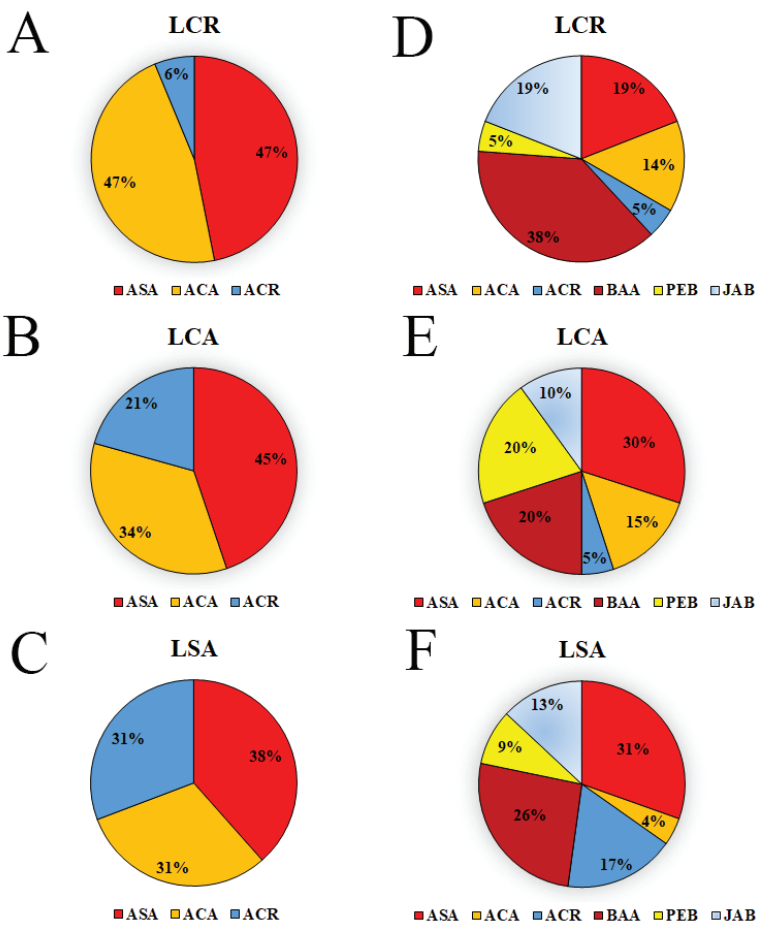

Fig. 4: Left: Assignment test of the larvae (L) to adult (A) specimens sampled in 2015 in the north-eastern (ACR and LCR), central (ACA and LCA) and southern Adriatic (ASA and LSA). A) LCR with ASA, ACA and ACR parents; B) LCA with ASA, ACA and ACR parents; C) LSA with ASA, ACA and ACR parents. Right: Assignment test of the larvae (L) to adult (A) specimens sampled in 2012 (JAB, PEB and BAA) and 2015. D) LCR with ASA, ACA, ACR, BAA, PEB and JAB parents; E) LCA with ASA, ACA, ACR, BAA, PEB and JAB parents; F) LSA with ASA, ACA, ACR, BAA, PEB and JAB parents.

on the all-adult 2015 + 2012 sample, it identified 47.9% LCR, 41.6% LCA and 44.6% LSA specimens with a confidence greater than 70%. Of the LCR specimens, only 5% were assigned to local ACR parents; the other larvae were assigned to BAA (38%), JAB or ASA parents (each 19%) (Fig. 4D). The LCA specimens were assigned to local ACA parents (15%) or to ASA (30%), PEB or BAA (20% each), JAB (10%) or ACR (5%) parents (Fig. 4E). The LSA specimens were assigned to local ASA parents (31%), or to BAA (26%), ACR (17%), JAB (13%), PEB (9%) or ACA (4%) parents (Fig. 4F).

Stock structure and environmental variables

The DAPC multivariate analysis retained 218 PCAs and 8 Discriminant Axes (DAs); DA1 and DA2 accounted for 78.52% of the explained variance (respectively 46.85% and 31.67%). The first axis, DA1, suggests the separation of JAB from one side and of PEB from the other side, whereas DA2 suggests the separation of LCR, LCA and LSA specimens from most adult individuals of the 2012 and 2015 samples (Fig. 5).

Mitochondrial DNA analysis confirmed earlier reports of a strong presence of clade B in the Adriatic Sea, with frequencies ranging from 51% to 87%. Interestingly, in the JAB sample, clade A showed a slightly higher frequency than in the other samples, with a value of ~ 35% (Fig. 6).

Testing with ANOVA highlighted significantly different chlorophyll-a concentrations among GSAs and significantly different salinity in the different GSAs, years, and depth layers, whereas temperature showed no significant differences (Table 5). Tukey's *post-hoc* test demonstrated that western GSA 17 was the most heterogeneous area in terms of salinity at the different depth layers, highlighting significant differences within the GSA itself and between the depth layers of GSA 18 and eastern GSA 17

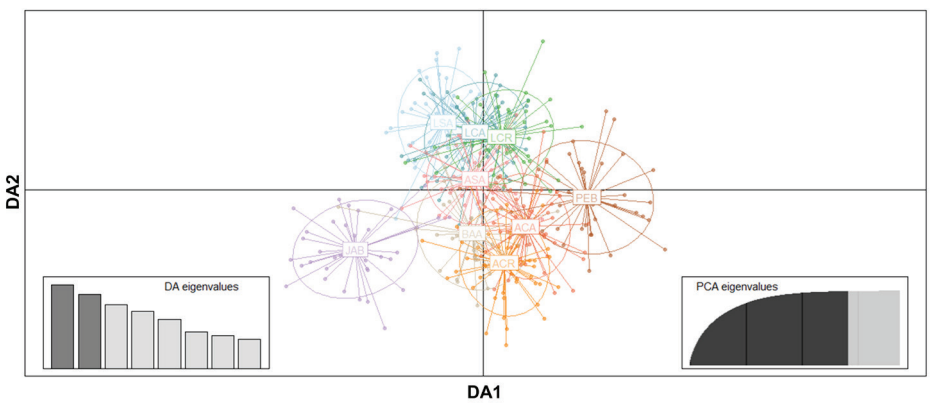

Fig. 5: Graphical outcome of DAPC multivariate analysis. Samples from the different sites are shown in different colours with 95% inertia ellipses. The X and Y axes of the scatterplot describe the first and second discriminant function and explain 78.5% (respectively 46.85% and 31.67%) of the genetic variance.

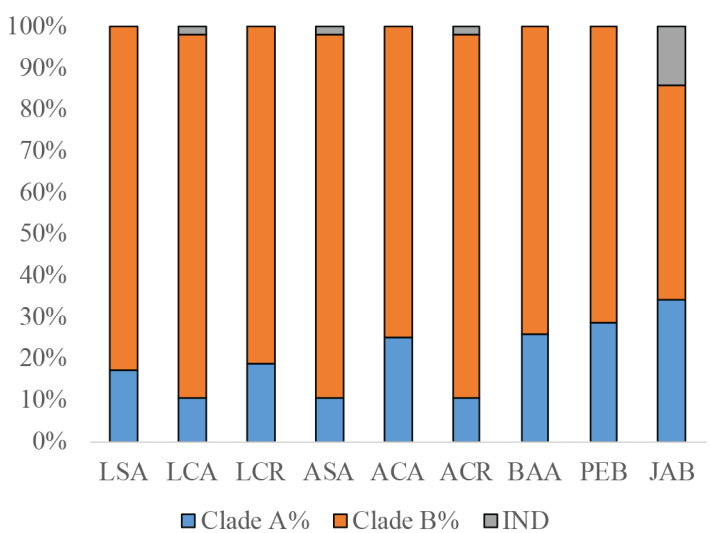

Fig. 6: Percentage of the two mitochondrial clades and of indeterminate (IND) individuals. Larvae (L) and adults (A) sampled in 2015 in the southern (LSA and ASA), central (LCA and ACA) and north-eastern Adriatic Sea (LCR and ACR). Adults sampled in 2012: BAA, PEB, JAB.

Table 5. Mean monthly chlorophyll-a (Mean_ChlA), temperature (Mean_T) and salinity (Mean_S) in 2012 and 2015 as tested by analysis of variance. Df = degree of freedom; F = Fisher parameter. Significant values shown in bold ($p < 0.001$).

Mean_ChlA	Df	F	p	Mean_T	Df	F	p	Mean_S	Df	F	p
Year	1	1.8424	0.176	Year	1	0.0002	0.988	Year	1	29.4712	**1.29E-07**
GSA	2	75.0477	**<2.00E-16**	GSA	2	1.8607	0.158	GSA	2	510.5953	**<2.00E-16**
Depth	3	0.0239	0.995	Depth	3	2.5919	0.053	Depth	3	87.4668	**<2.00E-16**
Year: GSA	2	0.0395	0.961	Year: GSA	2	0.0569	0.945	Year: GSA	2	8.4991	**0.0003**
Year: Depth	3	0.0178	0.997	Year: Depth	3	0.0054	0.999	Year: Depth	3	0.007	0.999
GSA: Depth	6	0.1004	0.996	GSA: Depth	6	0.0077	1	GSA: Depth	6	52.5618	**<2.00E-16**
Year: GSA: Depth	6	0.0122	1	Year: GSA: Depth	6	0.0034	1	Year: GSA: Depth	6	0.0448	1.000
Residuals	264			Residuals	264			Residuals	264		

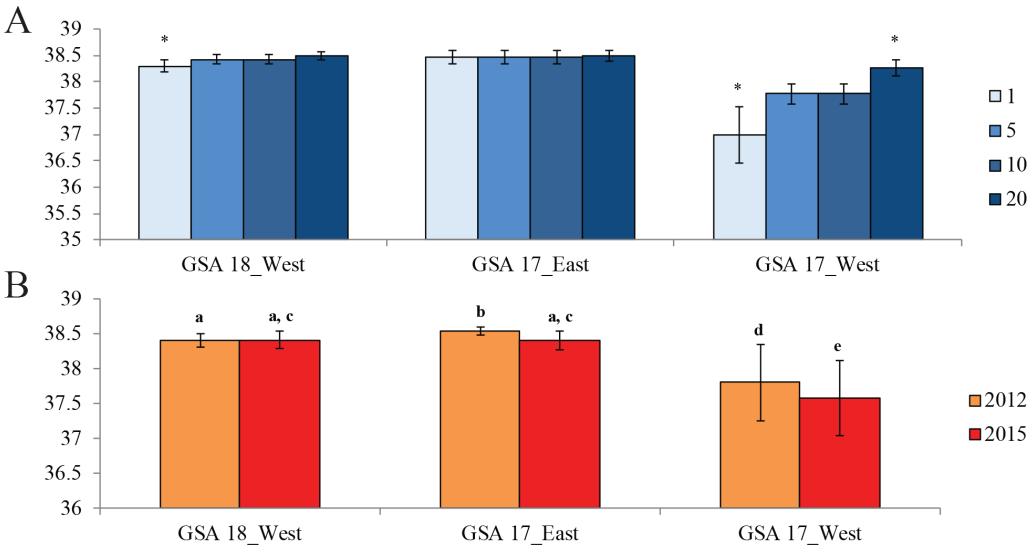

Fig. 7: A) Mean monthly salinity in GSAs 17 and 18 at different depth layers (1, 5, 10, 20 metres) in 2015. B) Monthly mean salinity in the different GSAs and years (2012 and 2015). Asterisks and low-case letters represent statistical significance and significant differences ($p < 0.001$; Tukey's *post-hoc* test).

(Fig. 7A). Moreover, in 2012 and 2015 mean salinity was significantly different in eastern compared with western GSA 17, but not with GSA 18. In 2012 mean salinity differed among the GSAs, whereas in 2015 differences between eastern GSA 17 and GSA 18 were not significant (Fig. 7B).

Discussion

Genetic diversity across anchovy generations

Small pelagics are characterized by large populations and high fecundity rates counterbalanced by high levels of mortality. Such loss of recruiters is usually determined by unpredictable events; notably, shrinking of the adult fraction can reduce the genetic diversity of offspring compared with the parental cohorts. This reproductive strategy, which privileges gamete production over survival of the early life stages, has been called "sweepstake reproductive success" (Hedgecock & Pudovkin, 2011). To explore its effects, we examined the variability of three key indices of genetic diversity – N_A, H_E and H_O – in cohorts of adults and larvae, collected in three different areas of the Adriatic Sea potentially, belonging to different populations. Furthermore comparison of N_A and H_O of adult and larval specimens demonstrated a similar level of genetic diversity at the three sites; this implies that such indices may be insufficiently sensitive to the genetic changes taking place in the single reproductive event investigated, especially due to the highly polymorphic nature of the microsatellite markers used and their proneness to mutation (Zarraonaindia *et al.*, 2009). In particular, N_A is deeply affected by marker polymorphism and the evaluation of multiple markers with different levels of polymorphism results in the accumulation

of a large evenness (Hoban *et al.*, 2013). Nonetheless, our estimated values of genetic diversity indices are consistent with those reported in other anchovy populations (Borrell *et al.*, 2012), and in other species (DeWoody & Avise, 2000), including heavily exploited fish like Atlantic herring (Larsson *et al.*, 2010), European sardine (Ruggeri *et al.*, 2013), sea urchin (Flowers *et al.*, 2002) and kelp rockfish (Gilbert-Horvath *et al.*, 2006). Instead, H_E is more sensitive to the loss of allelic variants associated with reproductive events. As an index based on HWE, and independent of the broad variation in each marker's allele polymorphism, it is a valuable estimator to assess variation in gene diversity across generations. Comparison of adult and larva specimens collected in 2015 highlighted lower though not significantly different H_E values ($p = 0.0627$) in larvae both within and among sites. Despite the lack of statistical support, our findings seem to suggest a loss of genetic diversity due to SRS. These data can be explained by an imbalance between the high level of SSR polymorphism and the size of the study sample: given the large anchovy population in the Adriatic Sea, such imbalance results in a statistical type-II error (false negative). In addition, detection of the possible reduction in genetic diversity between adults and offspring can be hampered by the presence of overlapping generations acting as a reservoir of diversity (Gilbert-Horvath *et al.*, 2006). The loss of genetic diversity across cohorts, a key element of SRS, depends on the amount of gene flow in post-juvenile stages (Hutchinson *et al.*, 2003). If gene flow is not hampered by physical barriers, successive mixing in nursery areas – due to passive larval transport by currents – enables preservation of the population. These considerations highlight the need to protect the main nursery areas of the Adriatic to avoid critical erosion of genetic diversity over time.

Genetic diversity also depends on N_c and/or N_b (Waples *et al.*, 2008); for Adriatic anchovy the N_b estimates ranged from 98 to 114 and the N_b/N_c ratio was in the order of 10^{-8} - 10^{-9}, which is much smaller than the 10^{-5} found in several marine species (Hare *et al.*, 2011). The low N_b values suggest repeated SRS events, which together with genetic drift can lead to local adaptation triggered by environmental drivers (e.g., salinity and temperature), or to topographically distinctive habitats (e.g., coves and islands) (Diopere *et al.,* 2018; Ivanova *et al.,* 2021). The low N_b/N_c ratio supports the notion that anchovy are subject to SRS, which induces extreme variability in the individual's reproductive success and consequently demographic instability (Ruggeri *et al.*, 2016a). As regards N_e, it is difficult to estimate it in large populations characterized by high genetic variability and a weak population structure, since it requires very large sample sizes (Larsson *et al.*, 2010). For this reason, as in other studies of large marine populations, our N_e estimate has broad confidence intervals that affect resolution (Larsson *et al.*, 2010; Cuveliers *et al.*, 2011). Despite these limitations, comparison of our N_e estimates with the conservation guidelines of the revised 50/500 rule (Frankham *et al.*, 2002) yields interesting results. To maintain evolutionary potential and minimize inbreeding and loss of genetic diversity, N_e should be as large as possible (≥ 1000) (Frankham *et al.*, 2014). Whereas N_e was similar in adults and larvae from the same site, between-site comparisons showed higher N_e values in the samples from the central and southern Adriatic than in the north-eastern Adriatic; the latter finding agrees with the N_e reduction predicted by the SRS in small populations (Allendorf, 1986; Ruggeri *et al.*, 2016a,b). It is also worth noting that N_e values of the 2012 sample from the north-eastern Adriatic (JAB) were much lower than those of any other sample analysed in 2012. This reflects that this was a transient small population.

Assignment test and population structure

The assignment test, which provided information on the level of admixture of larval samples from the three different areas of the Adriatic Sea, indicated the probability that each of them originated from local adults. In the 2015 sample, the assignment of larvae to parents became increasingly heterogeneous passing from north to south. In fact, only 6% of the north-eastern larvae (LCR) were assigned to local adults (ACR); the remaining larvae were equally assigned to central (ACA) and southern (ASA) adults (Fig. 4A); in contrast, larvae from the southern Adriatic (LSA) were equally assigned to ASA, ACA and ACR parents (Fig. 4A, B, C). These data support the notion that larval nurseries play a significant role in the central and southern Adriatic, in line with the view that the western coast is the main spawning and nursery area in the basin, where the seasonal cyclonic gyre (SAG, Fig. 1) promotes self-recruitment and larval retention (Malavolti *et al.*, 2018). The assignment test conducted with the pooled adult individuals (2012 and 2015), suggested a reduction, from 19% in 2012 to 5% in 2015 (Fig. 4D), of the contribution of local spawners in the reproductive event in the north-eastern Adriatic. These data can be explained by ocean transport; in fact, although the larvae collected in the north-eastern Adriatic may indeed have been the offspring of local parents, the sampling area may serve as a nursery for early stages transported by currents from elsewhere. Sharing of nursery areas by multiple stocks has been described in several marine species with pelagic reproduction, which depend on sea surface currents to concentrate the larval stages in sheltered areas to complete development (Siegel *et al.*, 2008). Otherwise, a failure of the local breeders in 2015 can be postulated. The anchovy biomass inhabiting the north-eastern Adriatic was smaller than that of the western Adriatic (Table 4); a smaller biomass, combined with changes in abiotic factors, may have adversely influenced the spawners and/or larval stage development. A significant change in salinity, recorded between 2012 and 2015 in the north-eastern Adriatic, lends support to the notion (Mediterranean Sea Physics Reanalysis dataset, Simoncelli *et al.*, 2019). Moreover, a temporal instability of boundaries between the two genetic populations, previously identified by Ruggeri *et al.* (2016b), may have induced a migration from the south-eastern (GSA 18) to

the north-eastern Adriatic (eastern GSA 17), resulting in merging of the two populations in the 2015 spawning events (Fréon *et al.*, 2005). The marked difference in the biomass of the two populations may have involved strong competition during the reproduction event, resulting in a limited contribution by spawners from eastern GSA 17. Further support to the notion of a temporal break of population boundaries in the Adriatic is provided by the genetic structure sketched by mtDNA and nuclear SSR marker analysis in the three anchovy generations (2012-2015). Both mitochondrial lineages (clades A and B) have been described in the Adriatic (Magoulas *et al.*, 2006; Viñas *et al.*, 2013; Ruggeri *et al.*, 2016b). Their divergence has been attributed to isolation during the Pleistocene glaciations and to interaction of the factors that are shaping population genetic structure today. At present, the two clades show a mosaic pattern, possibly a result of secondary contact (Magoulas *et al.*, 2006). Clade B is commonly predominant in the Adriatic Sea; yet clade A was found at higher frequency in the 2012 sample from the north-eastern Adriatic (JAB) and decreased in 2015. This finding suggests the possible migration of spawners from the central and southern Adriatic Sea to the north-eastern Adriatic during the 2015 reproductive events, as also supported by the nuclear SSR marker test results. In fact, DAPC analysis demonstrated a change in the genetic structure of the population in the north-eastern Adriatic between 2012 and 2015, with only the JAB sample, collected in 2012, showing genetic differences from the other samples. The low F_{ST} values of all the samples, except JAB, combined with its significantly different composition, lend some support to our hypothesis of a low level of genetic differentiation with a single population in the whole Adriatic basin in 2015. Therefore, our findings are consistent with a temporal change in the genetic structure and population connectivity from 2012 to 2015. The larval retention due to the action of oceanic fronts and gyres is a well-known isolating mechanism in pelagic fish (Warner & Cowen, 2002). The oceanic front between the Istrian Peninsula and the Mid Adriatic Pit and the anticlockwise gyre off the Po River delta have the potential to hamper gene flow, contributing to explain the north-south genetic discontinuity highlighted by Ruggeri *et al.* (2016b). Yet, since both are ephemeral (Artegiani *et al.*, 1997a,b), they hamper the movement and mixing of larvae and juveniles only temporarily, which does not contrast with the finding of a single homogeneous population described in this work. In addition, larval connectivity is an intermittent and heterogeneous process characterized by an annual time scale and affected by a variety of biological and physical processes. This stochasticity is inherently unpredictable, due to the chaotic nature of the coastal circulation and the relatively short larval time scales (Siegel *et al.*, 2008). At the regional scale, environmental variables (i.e,. salinity and temperature) have been demonstrated to influence larval development, survival and growth (Morello & Arneri, 2009; Giannoulaki *et al.*, 2013) as well as adult maturation and reproductive success (Zorica *et al.*, 2013). Ruggeri *et al.* (2016b) have reported a significant association of four outlier loci with environmental variables and a correlation of higher salinity with the existence of a north-eastern anchovy stock; interestingly we found a significant change in salinity between 2012 and 2015 in the north-eastern Adriatic (Fig. 7B). Altogether, our data suggest that the genetic structure of *E. encrasicolus* populations in the Adriatic Sea may be affected by short-term events and that their genetic pool may reflect annual differences induced by interactions among biological factors affecting anchovy populations and the complex climatic and oceanographic characteristics of the Adriatic basin. Long-term monitoring is expected to enhance our knowledge of anchovy genetic diversity dynamics and genetic structure in the Adriatic Sea, identifying the spatial boundaries of stock units and supporting fishery management decisions and conservation efforts directed at sustainable harvesting.

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Supplementary Data

The following supplementary information is available online for the article:

Table S1. Summary of genetic variability observed at 13 microsatellite loci in the sampled populations. $N_{A\ TOT}$ = Number of alleles observed in a specific locus; N_A = number of alleles observed *per* location; N = number of individuals correctly genotyped; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient estimates; R_S = allelic richness estimates standardized at 22 individuals.