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ABSTRACT

Reports of morphological differences between European anchovy (*Engraulis* cf. *encrasicolus*) from coastal and marine habitats have long existed in the ichthyologic literature, and have given rise to a long-standing debate on their taxonomic status. More recently, molecular studies have confirmed the existence of genetic differentiation between the two anchovy ecotypes. Using ancestry-informative markers, we show that coastal anchovies throughout the Mediterranean share a common ancestry, and that substantial genetic differentiation persists in different pairs of coastal/marine populations despite the presence of limited gene flow. On the basis of genetic and ecological arguments, we propose that coastal anchovies deserve a species status of their own (*E. maeoticus*) and argue that a unified taxonomical framework is critical for future research and management.

KEYWORDS

Anchovy, ecotypes, genetic divergence, partial reproductive barrier, ancestry-informative markers, taxonomy

MAIN TEXT

The European anchovy (commonly referred to as *Engraulis encrasicolus* L. – Clupeiform, Engraulidae) is a small pelagic fish with a large geographic distribution, spanning the north-eastern Atlantic and Mediterranean regions from the Baltic to the Black Sea. It is now recognised that this polytypic taxon consists of several genetically differentiated populations with contrasting abilities to occupy and forage in coastal environments (Borsa 2002, Oueslati et al. 2014, Le Moan et al. 2016, Montes et al. 2016, Catanese et al. 2017, Catanese et al. 2020, Huret et al. 2020). Some populations thrive preferentially in shallow coastal lagoons with highly variable salinity, while others are predominantly pelagic, with nevertheless a large overlap in their respective habitats (Le Moan et al. 2016, Catanese et al. 2017, Zuev 2019, Catanese et al. 2020, Huret et al. 2020). In the abundant literature on this species, the former are sometimes referred to as coastal, lagoonal or inshore populations, while the adjectives marine, pelagic or offshore are used for the latter. For the sake of simplicity, we will hereafter use the terms “coastal” vs. “marine” and relate these forms to morphology-based descriptions from the ichthyological literature. While the question of their taxonomical status as local races, subspecies or species has been pending for over a century (reviewed below), it is now well established by genetic evidence (Borsa 2002, Oueslati et al. 2014, Le Moan et al. 2016) that the coastal form constitutes one (or several) separate evolutionarily significant units (ESU) having received several specific Latin binomens in the past. In the present paper, we address the question of the possible unicity of the coastal form and its taxonomic consequences. We argue that coastal anchovy populations, despite being genetically differentiated from each other, share a common genetic ancestry can be genetically recognised throughout their range as a single ESU. We further show that despite ample opportunities for gene flow, the coastal form remains genetically distinct from the marine form, implying the existence of (partial) reproductive isolation barriers that justify taxonomical recognition.

Early works on the Atlantic / Mediterranean / Black Sea anchovies went in parallel with very few cross-comparisons. Since the very beginning, it has been suggested that ecological differences between anchovy morphs could point to the existence of separate entities, sometimes referred to as "races" (Grassi 1903, Zernov 1904, Zernov 1913, Maximov 1913). However, the nature of the underlying differences, either being inherited or reflecting plastic growth trajectories in contrasted environments, remained controversial (Grassi 1903, Lo Giudice 1911a, Lo Giudice 1911b) or were dismissed (Fage 1911, Tichy 1914). A clear report of this ecological differentiation dates back to a hundred years with Lo Giudice (1922), who was the first to use the terms of “coastal” and “pelagic” races for anchovy populations occurring in close proximity off the Italian coasts. Shortly thereafter, related work by Pusanov (1923) and Alexandrov (1925) differentiated the anchovies of the Azov Sea from those of the open waters of the Black Sea, and a subspecific status was proposed for the Azov Sea anchovies by Pusanov & Tseeb (1926) and Alexandrov (1927). Two decades later, in a study of anchovies from the Ionian Sea and Lake Ganzirri, Sicily, Dulzetto (1947) proposed a specific status for the latter population. Subsequent morphological studies confirmed the existence of ecophenotypic differentiation between coastal and marine anchovies in several other locations across the Mediterranean (see for instance Quignard et al. 1973), while there was still debate as to their eventual taxonomic status.

Before the advent of genetic studies, several questions relative to the evolutionary origin and status of anchovy forms remained unanswered:

-1) Is phenotypic differentiation between coastal and marine anchovies a purely plastic response to living in different environmental conditions, or does it have a heritable genetic basis? In other words, are the coastal and marine forms freely interbreeding or are they partially or entirely reproductively isolated?

-2) In the latter case, are the various geographical populations of the coastal form closely related to one another (and likewise for the marine form), or do they constitute independent entities in each marine basin?

-3) What is the phylogeographic history behind this situation?

-4) Finally, what should their taxonomical status be?

These questions have now been partly solved by molecular population genetic studies, although the subject has been animated by intense debate. From the late 70s onwards, many studies had targeted a number of exploited fish species, including anchovies. Multiple papers reported either electrophoretic, mitochondrial, microsatellite or single nuclear polymorphism (SNP) variation patterns in anchovies at various geographical scales. However, most studies, surprisingly, seem to have stemmed from a *tabula rasa* with regards to the old morphological literature. It is further interesting to note that the first reports on mitochondrial DNA already evidenced two deeply divergent anchovy lineages. These two mitotypes were found to coexist in the same sampling locations, albeit in variable proportions, and therefore were not interpreted as reflecting the existence of two parapatric or quasi-sympatric entities (e.g. Bembo et al. 1995, Magoulas et al. 1996, Grant 2006, Silva et al. 2014, Vodyasova & Abramson 2017). Nevertheless, on the basis of a coupled morphometric and allozymic analysis, Bembo et al. (1996) concluded that there were necessarily two "stocks" among the Adriatic anchovies, primarily separated according to water depth. Hence, the question of the existence of two ecotypic forms has largely been overlooked, even in relatively recent studies (e.g. Borell et al. 2012, Zarraonaindia et al. 2012, Viñas et al. 2014, Silva et al. 2014). Most of these studies pointed toward the existence of a relatively strong genetic structure as compared to other highly dispersing broadcast spawners like sardines (e.g. Grant et al. 1998). This observation was not easy to account for without invoking unrealistic limitation on individual movement or strong environmentally induced selection occurring at each generation (see for instance Ruggeri et al. 2016 for the Adriatic).

By reanalysing published allozymic data, Borsa (2002) proposed that Mediterranean anchovies present a species complex with at least two forms, one of them corresponding to a coastal form that was later proposed to deserve a species rank on its own (*Engraulis albidus*, Borsa et al. 2004). After these first genetic clues, several studies have addressed the extent and evolutionary origin of divergence between anchovy forms with molecular markers (Bouchenak-Khelladi et al. 2008, Karahan et al. 2014, Oueslati et al. 2014, Le Moan et al. 2016, Montes et al. 2016, Catanese et al. 2017). These studies showed that coastal anchovies could be genetically characterised in areas as distant as the Bay of Biscay, Alboran Sea and the near Atlantic, Gulf of Lions, Siculo-Tunisian Strait, Tyrrhenian Sea, Adriatic Sea, and Levantine Basin, and that these were genetically more similar to each other than to geographically closer marine anchovies. As for anchovies in the Black and Azov seas and the related literature in Russian, see the review of Zuev (2019) that deals with all points above but point 2.

For the Atlantic and Mediterranean, Le Moan et al. (2016) more specifically addressed the question of the unique *versus* repeated evolutionary origin of the marine - coastal ecotype pairs. This genome-wide investigation revealed that coastal populations from the Bay of Biscay and the Gulf of Lions share a common ancestry that distinguishes them from the marine populations. The current existence of multiple ecotype pairs was thus not attributed to independent, *in situ* differentiation in response to parallel divergent selection, but to a secondary contact that probably took place about 300 kyrs ago between two pre-existing evolutionary lineages followed by their spatial redistribution. Since both ecotypes are highly mobile and often hybridise, historical gene flow following secondary contact has been sufficient to partially erode the genetic differences that existed between the two anciently diverged lineages. Some regions of the genome, such as those involved in eco-phenotypic differentiation, have however retained their divergence as a result of selection against unfit hybrid combinations and ecological selection. The use of ancestry-informative markers located in those genome regions that resist gene flow is thus crucial to be able to characterise the spatial and ecological structure of the present European anchovy populations, possibly explaining why the genetic distinction between marine and coastal anchovies was not evident in all molecular studies.

Now that the existence of two ecotypes has been widely recognised by several molecular studies, the way is paved for further investigations on the genetic bases of their physiological, behavioural and reproductive characteristics. Anchovies, being polytypic, have been able to occupy a wider range of habitats compared to a monotypic species (Zuev 2019, Catanese et al. 2020, Huret et al. 2020). As a first step, which is the very aim of this short paper, it remains however to adopt a common vocabulary and to clarify the present-day taxonomical situation. To this end, we produced genome-wide polymorphism data using a similar methodology as in Le Moan et al. (2016) and complemented their sampling design with more individuals throughout the Mediterranean and Black seas. Since reduced-representation genome sequencing generates large numbers of SNPs, we considered that a limited number of individuals per location was sufficient to adequately represent the genomic variability of any given location. Given the precise objectives of the current study, our analysis was limited to taxonomic assignment based on genotypic combinations at ancestry-informative markers.

To briefly summarize the methodology, individual genomic DNA of 30 samples collected from various sampling expeditions and local fisheries were used to generate RAD-sequencing libraries following a similar protocol to Baird et al. (2008). Sequencing was performed on an Illumina HiSeq2500 sequencer in single-read mode. Demultiplexed reads were matched to the same catalogue of loci as in Le Moan et al. (2016), after applying the same quality filters. We then merged the genotypes of the 30 newly sequenced individuals with those of 28 individuals from Le Moan et al. (2016), which were used as reference samples. Our final dataset was composed of 58 individuals representing five pairs of coastal/marine anchovy populations from the north-eastern Atlantic, the western Mediterranean, and the Black Sea (Fig. 1A; Supplementary Table S1). The filtered VCF file contained 2952 polymorphic loci genotyped in 58 individuals with a maximum rate of 30% of missing data and a minimum allelic frequency (MAF) of 4%. Genetic structure was visualised by Principal Component Analysis (PCA), performed using the R package SNPRelate (Zheng et al. 2012). A dendrogram based on an uncorrected nucleotidic similarity (identity by state IBS) matrix was constructed using the same software. Individual assignment to *K* ancestral populations were inferred with FastSTRUCTURE (Raj et al. 2014).

The genomic differentiation of the 58 individuals depicted in the first two PC axes (Fig. 1B) and the FastSTRUCTURE diagram (Fig. 1E) mainly distinguishes two groups of samples. PC 1 (7.56 % of total variance) clearly separates the individuals sampled in coastal waters on the right side from those sampled in marine conditions on the left (Fig. 1B). The second component (3.38 % of total variance) separates coastal individuals from the Gulf of Biscay from their western Mediterranean (Tunisia, Sicily, Gulf of Lions) and Black Sea (Kerch Strait) counterparts. This differentiation along PC 2 indicates that the Atlantic and Mediterranean coastal anchovies underwent significant differentiation, while their marine counterparts are less differentiated from each other (see also discussion in Catanese et al. 2017). Noticeably, some individuals appear in intermediate positions along PC 1, consistent with the identification of early-generation hybrids (e.g. F1, F2 and backcrosses) in Le Moan et al. (2016), as well as later-generation backcrosses between marine and coastal anchovies both in the Atlantic and Mediterranean. Such hybrids were also evidenced from anchovy eggs along the Thyrrenian coast (Catanese et al. 2020). Here we observe a similar pattern for some individuals from Crimea (Kerch Strait) which could potentially represent hybrids or admixed genotypes (Fig. 1B and 1E). The FastSTRUCTURE analysis also strongly captured the coastal/marine dichotomy at *K*=2 (Fig. 1E) without any significant changes for higher values of *K*. Individuals with mixed ancestry could correspond to different classes of hybrids as discussed above, an observation also reflected by their intermediate position in a dendrogram based on IBS distances (sup. Figure S2,).

The present analysis allowed to relate various geographical populations of coastal anchovy to each other through the identification of common genetic bases that distinguish them from their marine counterparts. Le Moan et al. (2016) showed that genetic divergence between coastal and marine ecotypes was restricted to about 20-25% of the genome. These genomic regions contain ancestry-informative markers that are useful for ecotype assignment and for identifying hybrid genotypes. Although hybrids are relatively common, heterogeneous genome divergence between ecotypes indicates that the barrier to gene flow is sufficiently strong for the two ecotypes to persist in a parapatric/quasi-sympatric (although not entirely syntopic) situation without a complete re-mixing of their genomes. This contrasts with the relative genetic homogeneity amongst populations of the same ecotype throughout their geographical range. Hence, it can be considered that marine and coastal anchovies fulfil the conditions to be treated as separate species. In line with one of the most fundamental components of the biological species concept, the two anchovy forms are maintained as distinct genotypic clusters despite their spatial overlap (Mallet 2020). This situation thus calls for a re-examination of the taxonomic status of anchovy ecotypes. According to the rule of anteriority, we discuss in what follows the correct naming of each ecotype.

Concerning the marine or offshore ecotype, we shall follow Borsa et al. (2004) who state: "*No type is known for this species and Linnaeus' original description is too vague to allow the distinction between the two species.../... . For the sake of stability, we propose to arbitrarily maintain the specific name* encrasicolus *to the apparently most common and widespread anchovy species in the seas of Europe. .../...also referred to as "oceanic" or "open-sea" anchovy*". This fish is often referred to as "blue anchovy" or "green anchovy", depending on location. The genetic homogeneity of marine anchovies has now been confirmed throughout a large part of its range, and hence the numerous subspecific trinomens that were given to local populations should be considered invalid. It should also be noted that, despite being described as marine/offshore/oceanic/pelagic, individual identification by multilocus genotyping has shown that these fish are able to enter continental systems such as estuaries (see for instance the individuals of the marine taxon identified in the Adour estuary, Gulf of Biscay, in Le Moan et al. 2016). Borsa et al. (2004) have deposited a neotype and voucher specimens for this species at the Musée National d'Histoire Naturelle (MNHN), Paris (neotype MNHN 2002-1775; vouchers MNHN 2002-1776 to MNHN 2002-1844).

As for the coastal ecotype, which is the focus of the present study, our results point to the genetic homogeneity of this taxon throughout most of its range, although subtle genetic differentiation may exist among coastal populations due to limited genetic connectivity between them (Oueslati et al. 2014, Le Moan et al. 2016, Catanese et al. 2017). Coastal anchovies also display common morphological features that separate them from marine anchovies (Fig. 1C and 1D). Generally, this includes paler dorsal colouration (they are often locally referred to as "white", "yellow" "grey" or "silver anchovy" in different regional languages), smaller size at maturity, fewer vertebrae, a dorsal fin implanted closer to the tail and a proportionally bigger eye. For more details, see the morphological descriptions in Borsa et al. (2004), Quignard et al. (1973), Tortonese (1967), Karahan (2014) as well as earlier works. A conspicuous difference in otoliths shape has also been reported and used to identify putative "stocks" (Messaoud et al. 2012, Vodyasova & Soldatov, 2017).

Until now, there have been, to our knowledge, three attempts at providing a morphological diagnosis and attributing a Latin binomen or trinomen to coastal anchovy populations. These are *E. e. maeoticus* (Pusanov & Tzeeb 1926 – with a diagnosis in Latin, sup. Figure S3) from the Sea of Azov, *E. russoi* (Dulzetto 1947) from Sicilian lagoons, and lastly *E. albidus* (Borsa et al. 2004 – with diagnostic features in English) from the Gulf of Lions. A mention should also be made for *E. e. symaetensis* (Dulzetto 1940) which was collected from the "beach" nearby a small estuary near Catania (Sicily). Interestingly, the morphological analysis performed by this last author indicates morphometric characteristics that are apparently intermediate to those of *E. russoi* and those of the marine Ionian Sea *E. encrasicolus* (data reanalysed in Tortonese 1967, who dismissed *symaetensis* as a valid name). Since these samples have disappeared, it will not be possible to confirm whether they were *bona fide* coastal anchovies that were locally introgressed, or a mixed stock containing hybrids.

Given the nomenclatorial rule of antecedence, the only valid name for the coastal species is *E. maeoticus* (Pusanov & Tzeeb 1926) which applies to all coastal populations that have been found to share a common ancestry. Pusanov & Tzeeb (1926) published a comprative diagnosis for what they considered to be a subspecies and named it after the antique Meotian people that used to inhabit the banks of the Azov Sea. Since, to our knowledge, no type specimens were deposited by Pusanov nor Dulzetto , those secured by Borsa et al.(2004) at MNHN (type registered as MNHN 2002-1716, paratypes MNHN 2002-1717 to MNHN 2002-1774) under the name *E. albidus* should be considered as valid type specimens of *E. maeoticus*.

We believe that placing the biological diversity observed for anchovies within a clear and unified taxonomical framework will greatly benefit future research across a variety of disciplines. Although various recent studies have recognised the shared molecular bases associated with the two eco-phenotypically divergent forms, a harmonised nomenclature is critically lacking. We propose that it is time to take this step in order to make better sense of the future generation of whole-genome sequence data on anchovies. This will aid characterisation of the molecular bases and biological functions associated with the species' ecological divergence. Furthered by these molecular advances, eco-physiological studies will hopefully be able to shed some light on the biology of marine (*E.* cf. *encrasicolus*) and coastal (*E. maeoticus*) anchovies, investigating the genetic bases of behavioural, physiological and life history traits that explain the persistence of the two species despite their large co-occurrence. Such advances would also provide valuable tools to improve current fishery models and to move towards a management of stocks that takes the biological duality of anchovies into account. Last but not least, we hope that this taxonomic recognition in one of the most emblematic fishes in the Mediterranean ecosystem will encourage future consideration of cryptic subdivisions that also exist in other fish species, in order to ultimately better preserve these hidden layers of biodiversity.

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FIGURE and CAPTION

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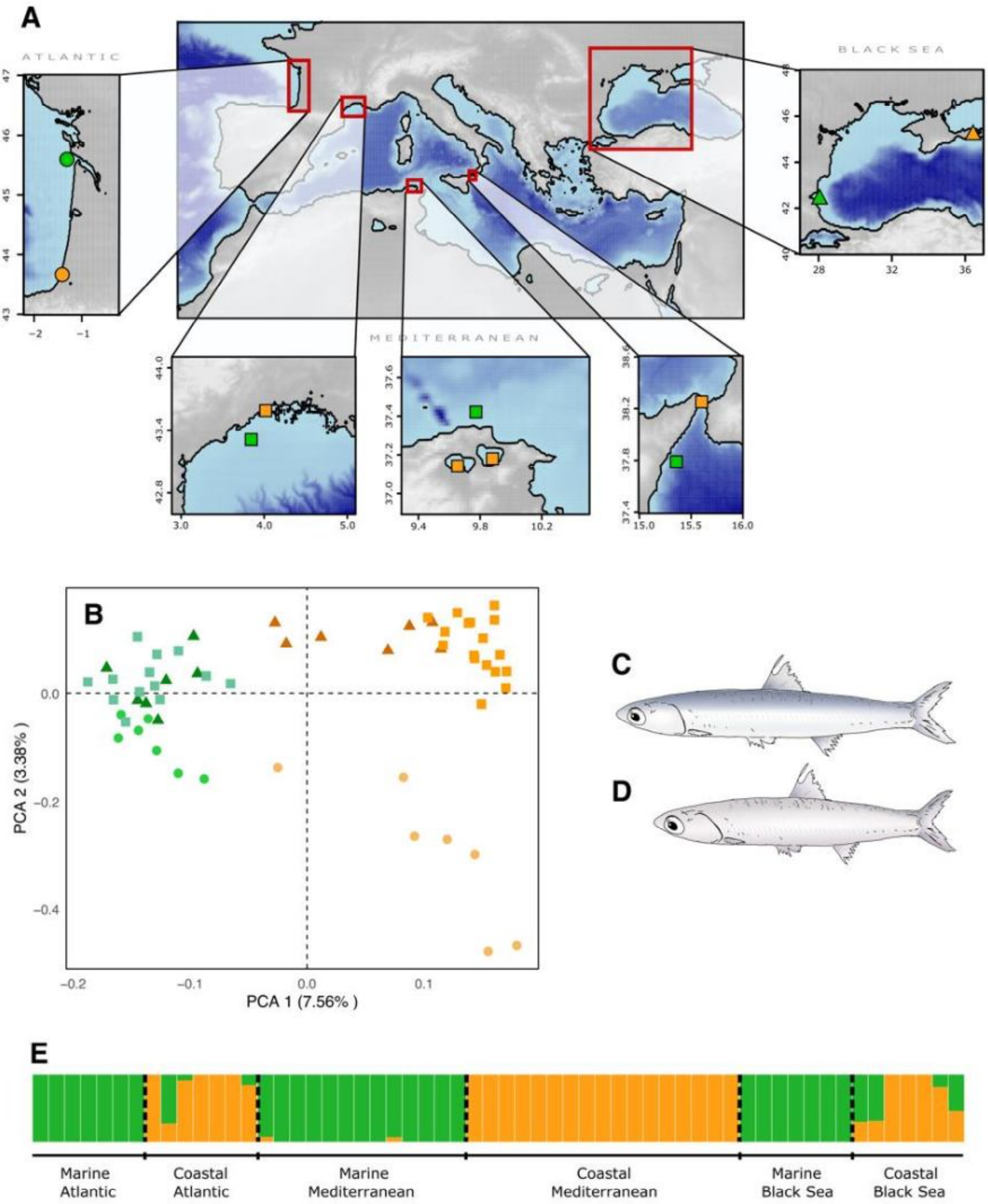
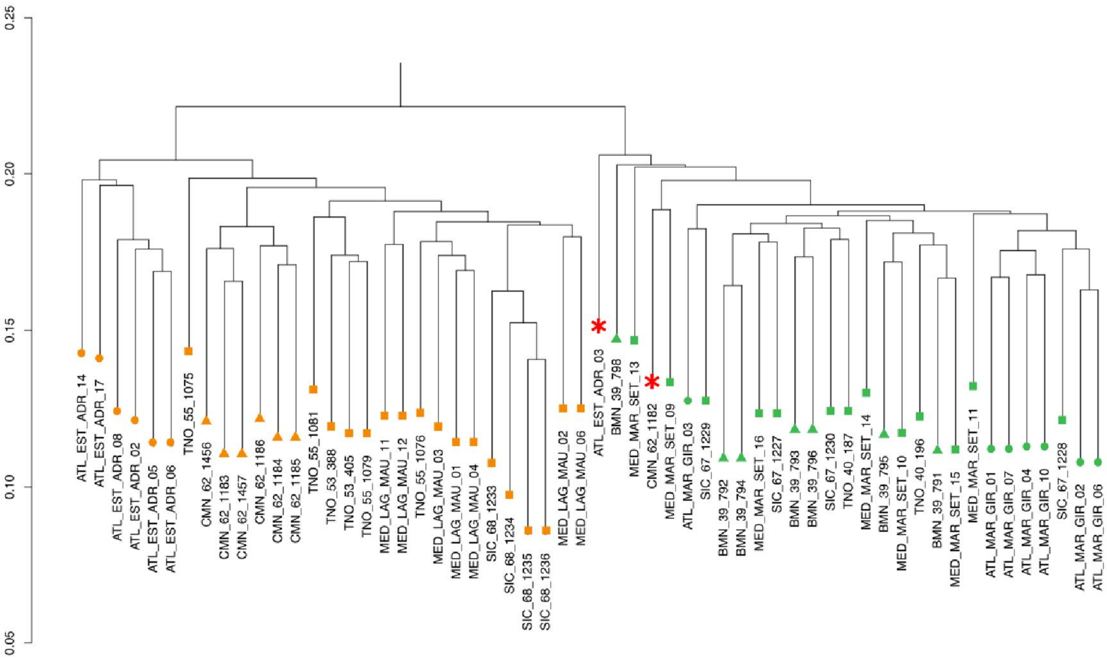


Figure 1. (A) Sampling locations of *E. cf. encrasicolus*. Symbols represent locations (●: Atlantic; ■: Mediterranean; ▲: Black Sea) while colours represent habitat type (green: marine; orange: coastal). (B) Principal component analysis based on 2952 SNPs in 58 individuals (symbols correspond to those used in *A*). Schematic representations are shown for anchovies from (C) marine and (D) coastal habitats. (E) Individual ancestry proportions as determined by FastSTRUCTURE with K=2 clusters identified

SUPPLEMENTARY MATERIAL

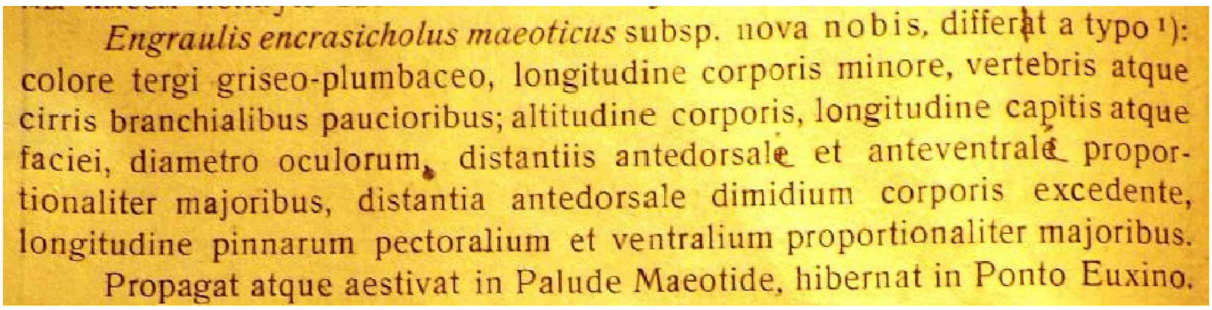
Supplementary Table S1. Anchovy samples used in the current study. Samples marked with an asterisk (*) are from Le Moan et al. (2016).

Sample ID	Numb	Habitat type	Location	Lat	Long	Year
ATL-ADR *	7	Estuary	Gulf of Biscay - Adour	43.51	-1.49	2006
ATL_GIR *	7	Open Sea	Gulf of Biscay - Offshore	45.59	-1.27	2002
BMN_39	7	Open Sea	Black Sea - Offshore	42.46	28.12	2010
CMN_62	7	Coastal	Black Sea - Kerch Strait	45.24	36.50	2017
MED_MAU *	7	Lagoon	Gulf of Lion – Mauguio lagoon	43.58	4.02	2013
MED_SET *	7	Open Sea	Gulf of Lion - Offshore	43.30	3.85	2005
SIC_67	4	Open Sea	Sicily – Ionian Sea	37.78	15.36	2018
SIC_68	4	Lagoon	Sicily – Ganzirri lagoon	38.26	15.61	2018
TNO_40	2	Open Sea	Tunisia NW - Offshore	37.42	9.78	2009
TNO_53	2	Lagoon	Tunisia NW – Bizerte lagoon	37.18	9.88	2009
TNO_55	4	Lagoon	Tunisia NW – Ichkeul lagoon	37.14	9.66	2011



Supplementary Figure S2. Dendrogram for 58 anchovy samples based on an IBS distance matrix. Symbols correspond to those used in Fig. 1. Red stars indicate samples that had been collected in coastal areas, but which cluster with marine samples.

Supplementary Figure S3: Original Latin diagnosis from Pusanov & Tseeb (1926)



Engraulis encrasicholus maeoticus subsp. nova nobis, differat a typo 1): colore tergi griseo-plumbaceo, longitudine corporis minore, vertebris atque cirris branchialibus paucioribus; altitudine corporis, longitudine capitis atque faciei, diametro oculorum, distantiis antedorsale et anteventrale proportionaliter majoribus, distantia antedorsale dimidium corporis excedente, longitudine pinnarum pectoralium et ventralium proportionaliter majoribus.

Propagat atque aestivat in Palude Maeotide, hibernat in Ponto Euxino.

Translation (from the present paper authors)*: Engraulis enchrasicholus maeoticus* new subspecies, different from the type[1] by : belly colour lead-grey, smaller body length, smaller number of vertebrae and branchial filaments; body height, head and face length, eye diameter, anterodorsal and anteroventral distances proportionally larger; anterodorsal distance superior than half the body length; length of the pectoral and ventral fin rays proportionally larger.

Migrates and aestivate in Maeotide Lagoon (*Azov Sea*), overwinters in Euxinus Pontus (*Black Sea*).

1) : individuis ponticis (*Black Sea individuals*)