

Systematics of European coastal anchovies (genus *Engraulis* Cuvier)

François Bonhomme¹  | Laura Meyer¹ | Christine Arbiol¹ | Daniela Bănară² | Lilia Bahri-Sfar³ | Karima Fadhlouï-Zid⁴ | Petr Strelkov⁵ | Marco Arculeo⁶ | Laurent Soulier⁷ | Jean-Pierre Quignard⁸  | Pierre-Alexandre Gagnaire¹

¹ISEM, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France

²Aix-Marseille Université, Université de Toulon, CNRS, IRD, MIO UM 110, Mediterranean Institute of Oceanography, Marseille, France

³Biodiversité, Parasitologie et Ecologie des Écosystèmes Aquatiques, LR18ES05, 2092, Faculté des Sciences de Tunis, Université de Tunis El Manar, Tunis, Tunisia

⁴Institut National des Sciences et Technologie de la Mer, INSTM, La Goulette, Tunisia

⁵Department of Ichthyology & Hydrobiology, St Petersburg State University, St Petersburg, Russia

⁶Department STEBICEF, University of Palermo, Palermo, Italy

⁷Centre pour l'Aquaculture, la Pêche et l'Environnement en Nouvelle-Aquitaine, Bayonne, France

⁸Académie des Sciences et Lettres de Montpellier, Montpellier, France

Correspondence

Laura Meyer, Institut des Sciences de l'Évolution (UMR 5554), CNRS-UM2-IRD, Place Eugène Bataillon, Montpellier, F-34095, France.
Email: laura.meyer@umontpellier.fr

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

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

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 recognized 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, 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, 2020; Zuev, 2019; 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 and can be genetically recognized 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; Maximov, 1913; Zernov, 1904, 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,b) or were dismissed (Fage, 1911; Tichy, 1914). A clear report of this ecological differentiation dates back 100 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 and Tzeeb (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 1970s onwards, many studies had targeted a number of exploited fish species, including anchovies. Multiple papers reported 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.*, 1996a; Magoulas *et al.*, 1996; Grant, 2005; Silva *et al.*, 2014; Vodyasova & Abramson, 2017). Nevertheless, on the basis of a coupled morphometric and allozymic analysis, Bembo *et al.* (1996b) 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 limitations 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 characterized 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 300,000 years ago between two pre-existing evolutionary lineages followed by their spatial redistribution. Since both ecotypes are highly mobile and often hybridize, 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/or ecological selection. The use of ancestry-informative markers located in those genome regions that resist gene flow is thus crucial to be able to characterize 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 recognized 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 (Catanese *et al.*, 2020; Huret *et al.*, 2020; Zuev, 2019). As a first step, which is the aim of this short paper, it is necessary 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 was used to generate restriction-site associated DNA (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 (Figure 1a; Supporting Information Table S1). The filtered variant call format (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 visualized by principal component analysis (PCA), performed using the R package SNPrelate (Zheng *et al.*, 2012). A dendrogram based on an uncorrected nucleotide similarity (identity by state, IBS) matrix was constructed using the same software. Individual assignment to K ancestral populations was inferred with FastSTRUCTURE (Raj *et al.*, 2014).

The genomic differentiation of the 58 individuals depicted in the first two PC axes (Figure 1b) and the FastSTRUCTURE diagram (Figure 1e) mainly distinguishes two groups of samples. PC1 (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 (Figure 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 PC2 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 PC1, 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 (Figure 1b,e). The FastSTRUCTURE analysis also strongly captured the coastal/marine dichotomy at $K = 2$ (Figure 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 (Supporting Information Figure S2).

The present analysis allowed various geographical populations of coastal anchovy to be related 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 among 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

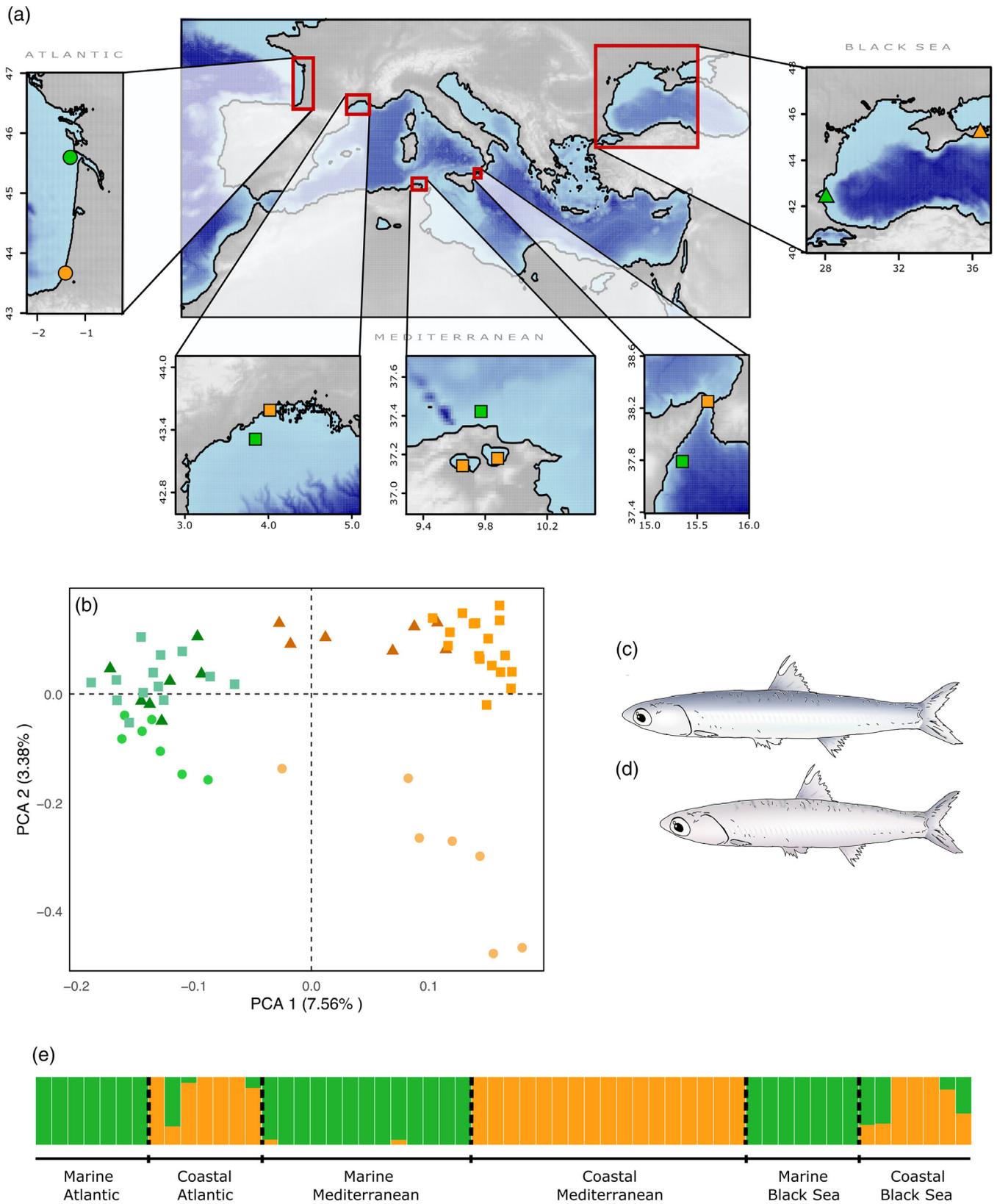


FIGURE 1 (a) Sampling locations of *Engraulis cf. encrasicolus*. Symbols represent locations (●, Atlantic; ■, Mediterranean; ▲, Black Sea) while colours represent habitat type (green, marine; orange, coastal). (b) Principal component analysis based on 2952 SNPs in 58 individuals (symbols correspond to 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

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 (Figure 1c,d). 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) and Karahan *et al.* (2014) as well as earlier works. A conspicuous difference in otolith shape has also been reported and used to identify putative 'stocks' (Messaoud *et al.*, 2011; 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. maoticus* (Pusanov & Tzeeb, 1926, with a diagnosis in Latin; Supporting Information 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' near 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. maoticus* (Pusanov & Tzeeb, 1926), which applies to all coastal populations that have been found to share a common ancestry. Pusanov and Tzeeb (1926) published a comparative 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. maoticus*.

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 recognized the shared molecular bases associated with the two eco-phenotypically divergent forms, a harmonized nomenclature is critically lacking. We propose that it is time to take this step to make better sense of the future generation of whole-genome sequence data on anchovies. This will aid characterization 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. maoticus*) 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 to ultimately better preserve these hidden layers of biodiversity.

ACKNOWLEDGEMENTS

We are grateful to Dr Paola Rinelli (CNR Messina) for the samples from Lake Ganzirri and the Ionian Sea and to Dr M.L. Molino, Director of the Riserva Naturale Orientata Capo Peloro, for authorization PROT. INT. N.7140/VI DEL to collect from Lake Ganzirri (91/12/2017). We are also grateful to Dr Aurelio Mazzè of the University of Palermo and Christine Bibal from ISEM for helping us to find some old papers. Lastly, we would like to thank the Montpellier Bioinformatics Biodiversity platform as well as the GenSeq sequencing platform. This work was financed through the basal annual funding of ISEM.

ORCID

François Bonhomme  <https://orcid.org/0000-0002-8792-9239>

Jean-Pierre Quignard  <https://orcid.org/0000-0002-1908-3235>

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How to cite this article: Bonhomme, F., Meyer, L., Arbiol, C., Bănar, D., Bahri-Sfar, L., Fadhlaoui-Zid, K., Strelkov, P., Arculeo, M., Soulier, L., Quignard, J.-P., & Gagnaire, P.-A. (2022). Systematics of European coastal anchovies (genus *Engraulis* Cuvier). *Journal of Fish Biology*, 100(2), 594–600. <https://doi.org/10.1111/jfb.14964>