



Population genetics meets phylogenetics: new insights into the relationships among members of the genus *Euthynnus* (family Scombridae)

Euthynnus population genetics and phylogeny

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Abstract *Euthynnus* (family Scombridae) is a genus of marine pelagic fish species with a worldwide distribution that comprises three allopatric species: *E. alletteratus*, *E. affinis* and *E. lineatus*. All of them targeted by artisanal and commercial fisheries. We analyzed 263 individuals from Atlantic and Pacific Oceans using two genetic markers, the mtDNA Control Region (350 bp) and nuclear calmodulin

(341 bp). The results obtained challenge the phylogeny of this group. We found a deep genetic divergence, probably at species level, within *E. alletteratus*, between the North Atlantic-Mediterranean and the Tropical East Atlantic. This deep genetic divergence was tested with several species delimitation methods. This complete phylogeographic association between the North Atlantic and the Tropical East Atlantic support the hypothesis of two cryptic species. In addition, population genetic heterogeneity was detected between the North East Atlantic–Mediterranean and North West Atlantic regions. Our results indicate two scales of differentiation in what is

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currently considered a single population. Accordingly, for management purposes, the populations of *E. alletteratus*, should be divided into a minimum of three management units. On the other hand, the high level of differentiation found in *E. alletteratus* contrasts with the shallow genetic divergence of *E. affinis* and *E. lineatus*.

Keywords Cryptic species · Evolutionary significant units · *Euthynnus* · Phylogenetics · Population genetics · Atlantic Ocean

Introduction

In the marine realm, and specifically for the pelagic species, the apparent absence of marine barriers, together with high migratory potential, large population sizes and pelagic planktonic stages, contribute to mitigate the genetic differentiation among regions (Palumbi, 1994). However, there is an increasing number of examples of genetic structuring in pelagic marine fishes (Waples, 1998; Smith et al., 2015; Pascual et al., 2017).

Some of these species of pelagic fishes are heavily targeted by artisanal and commercial fisheries as well as for recreational activities. Thus, the accurate assessment of population genetic structure is essential for the management and conservation of both the species and their populations. Because of their meat quality and abundance, the species of the family Scombridae, which include tunas, mackerels and bonitos, are one of the most valuable commercial species (Ward, 1995). The majority of scombrids are pelagic fishes capable of long migrations (Collette & Nauen, 1983) with extremely large populations (Collette et al., 2011; Juan-Jordá et al., 2011, 2013; Laconcha et al., 2015; Waples et al., 2018) and large effective population sizes (N_e) (Ely et al., 2005; Qiu

et al., 2013; Laconcha et al., 2015). All these factors contribute to make the analysis of their genetic population structure challenging. For instance, there is a considerable amount of literature about the biology and stock identification on major pelagic species such as the Atlantic Bluefin tuna [*Thunnus thynnus* (Linnaeus, 1758)] (Carlsson et al., 2004; Rooker et al., 2007; Díaz-Arce & Rodríguez-Ezpeleta, 2019). However, for other scombrids, particularly the small bodied size species, even basic aspects of their biology and genetic population structure are poorly understood, as is the case for the little tunny, *Euthynnus alletteratus* (Rafinesque, 1810). In spite of its relevance as a commercial resource for traditional and local fisheries along its distribution no management recommendation has been approved by ICCAT (International Commission for the Conservation of Atlantic Tunas) (ICCAT, 2020).

Members of genus *Euthynnus* are distributed worldwide in tropical and subtropical marine waters, and include three species distributed allopatrically: *E. alletteratus* displays an amphi-Atlantic and Mediterranean distribution, *E. lineatus* Kishinouye, 1920 is found primarily in the eastern Pacific Ocean, and *E. affinis* (Cantor, 1849) is found in the Indian and the central and western Pacific Oceans (Fig. 1) (Collette & Nauen, 1983). Distinction of these species is based on meristic characters and genetic markers. For example, *E. alletteratus* has no vomerine teeth but presents a higher gill raker count in the first arch than *E. affinis* and *E. lineatus*. The latter two, in turn, differ from each other on the total number of gill rakers, the total number of vertebrae and the presence or absence of vertebral protuberances (Collette & Nauen, 1983). Only two studies have used the mitochondrial marker COI to differentiate these three species (Kumar & Kunal, 2013; Santini et al., 2013). From a phylogenetic perspective, the genus *Euthynnus* was one of the latest groups that diversified within the scombrid family (Santini et al., 2013), thought to have appeared around 10 mya. Phylogenetic reconstructions and previous taxonomic studies agree that *E. alletteratus* would have diversified earlier from the common ancestor and that *E. lineatus* and *E. affinis* would be sister species that diverged more recently (Kumar & Kunal, 2013; Santini et al., 2013). Furthermore, although the genus *Euthynnus* has a worldwide distribution and occupies large areas where its members are subject to fisheries, there is scarce literature on

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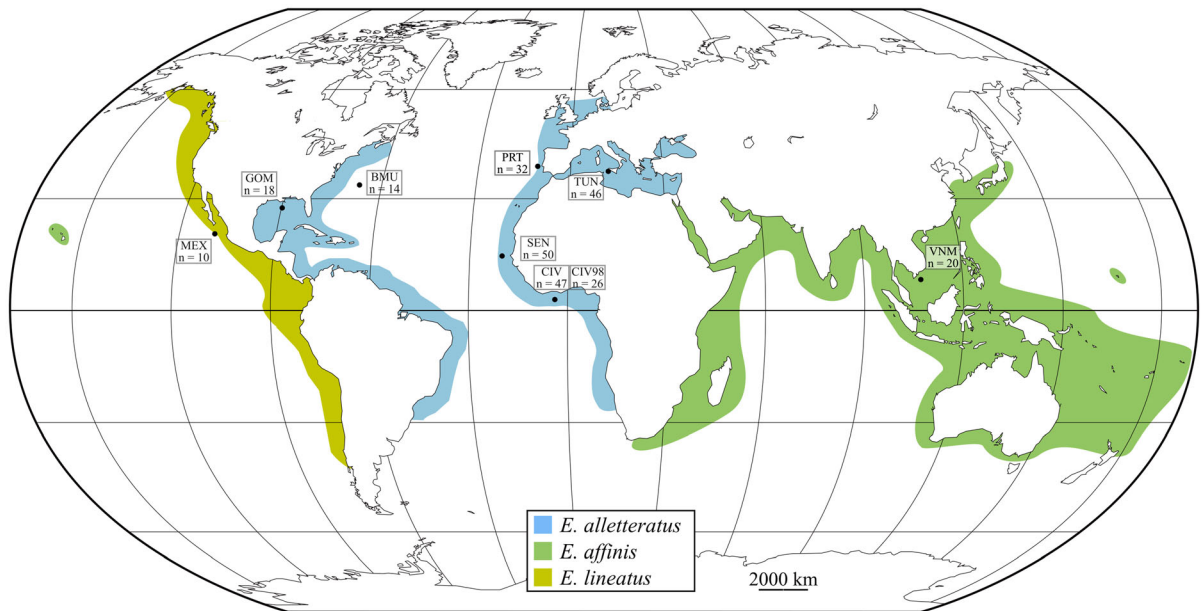


Fig. 1 Map depicting the distribution of the three recognized species of *Euthynnus* (Collette & Nauen, 1983). Sampling localities and the sample sizes at each location identified as

follows: Portugal (PRT), Tunisia (TUN), Bermuda (BMU), Gulf of Mexico (GOM), Senegal (SEN), Côte d'Ivoire (CIV), Viet Nam (VNM), Mexico (MEX)

the population genetics of its member species. No genetic evidences of population structure have been found in *E. affinis* among Indian locations (Kumar et al., 2012). Similarly, for *E. alletteratus* no genetic differentiation was detected within the Mediterranean (Allaya et al., 2015), nor between east and west Atlantic (Ward, 1995). However, preliminary results based on mtDNA (Alvarado Bremer & Ely, 1999) and on plastic and meristic characters (Gaykov & Bokhanov, 2008) suggests the presence of two stocks within the Atlantic Ocean.

The aim of the present study is to analyse the population genetic structure of *E. alletteratus* and provide new information on phylogenetic relationships within genus *Euthynnus*. Since genetic markers have been proved to be an effective tool for species and stock identification, the results presented here can help in the elaboration of plans for stock assignment and improve the current management strategies for this species.

Material and methods

Dataset assembly

Samples of individuals representing the three recognized species of *Euthynnus* individuals were analysed for two unlinked genetic markers, the mitochondrial DNA control region (mtDNA CR) and the nuclear calmodulin intron 4 (CaM), based on previous protocols (Alvarado Bremer et al., 1996; Chow & Takeyama, 2000). The dataset for the mtDNA CR consisted of sequences of *E. alletteratus* ($n = 175$), *E. affinis* ($n = 20$) and *E. lineatus* ($n = 10$), collected by international observers and researchers on scientific surveys or in commercial fisheries from five different locations (see Table 1 and Fig. 1 for details). Specimens were initially assigned to species based on their geographical sampling localities, since they have allopatric and non-overlapping distribution (Collette & Nauen, 1983). No meristic counts nor morphological descriptions were recorded. Additionally, mtDNA CR dataset was complemented with 58 sequences of *E. alletteratus* obtained in previous studies (unpublished data, unless noted), from three locations, (see Table 1), totalling 263 sequences for this marker. CaM marker dataset was created with a selection of individuals of

Table 1 Sampling data and variability indices, Sample size (n), number of haplotypes (M), haplotype diversity ($h \pm SD$), nucleotide diversity ($\pi \pm SD$) for mtDNA CR and number of individuals (n), number of alleles (A), heterozygosity ($H_S \pm SD$), nucleotide diversity ($\pi \pm SD$) for CaM marker

Species, regions and locations	Code	Year	mtDNA CR			CaM				
			n	M	$h \pm SD$	$\pi \pm SD$	n	A	$H_S \pm SD$	$\pi \pm SD$
<i>Euthynnus alletteratus</i>										
North East Atlantic + Mediterranean	NEA		78	16	0.641 ± 0.061	0.004 ± 0.003	16	4	0.704 ± 0.054	0.003 ± 0.002
Portugal	PRT	2018	32	9	0.643 ± 0.094	0.003 ± 0.002				
Tunisia	TUN	2018	46	12	0.644 ± 0.077	0.005 ± 0.003				
North West Atlantic	NWA		32	30	0.996 ± 0.009	0.022 ± 0.011				
Bermuda	BMU †	1993, 1995	14	14	1.000 ± 0.027	0.023 ± 0.013				
Gulf of Mexico (USA)	GOM	2002	18	18	1.000 ± 0.018	0.020 ± 0.011				
Tropical East Atlantic	TEA		123	68	0.977 ± 0.005	0.011 ± 0.006	14	13	0.934 ± 0.022	0.008 ± 0.005
Senegal	SEN	2018	50	31	0.958 ± 0.018	0.010 ± 0.006				
Côte d'Ivoire	CIV	1997–98, 2018	73	52	0.985 ± 0.006	0.011 ± 0.006				
<i>Euthynnus affinis</i>										
Viet Nam	VNM	2019	20	12	0.879 ± 0.065	0.006 ± 0.004	16	7	0.827 ± 0.036	0.005 ± 0.004
<i>Euthynnus lineatus</i>										
Mexico	MEX	2019	10	10	1.000 ± 0.045	0.023 ± 0.013	10	6	0.826 ± 0.046	0.004 ± 0.003

†Samples from the study of Alvarado Bremer & Ely (1999)

E. alletteratus ($n = 30$), *E. affinis* ($n = 16$) and *E. lineatus* ($n = 10$). Multiple sequence alignments were performed using ClustalW (Thompson et al., 1994) implemented in Geneious v7.1.9 (Kearse et al., 2012) and examined visually. CaM Sequences were phased using PHASE (Stephens et al., 2001) as implemented in DNAsp 6.12.03 (Rozas et al., 2017) in order to estimate the putative genotypes of the marker.

These sequence data have been submitted to the GenBank (www.ncbi.nlm.nih.gov/genbank) databases under accession number MN703152 - MN703207 and MN703208 - MN703342.

Genetic population analyses

The mtDNA CR haplotypes and CaM alleles were collapsed with DNAsp 6.12.03. Arlequin 3.5 (Excoffier & Lischer, 2010) was used to estimate the nucleotide (π) (Nei, 1987), haplotype diversity (h) (Nei & Tajima, 1981) indexes and expected heterozygosity (H_S) as gene diversity, as well to perform hierarchical analyses of molecular variance (AMOVA) and the ϕ_{ST} and F_{ST} pairwise comparisons (Wright, 1965; Excoffier et al., 1992). The comparison of samples from Côte d'Ivoire collected two decades apart (1998 and 2018) indicated temporal genetic homogeneity ($\phi_{ST} = 0.006$; $P = 0.251 \pm 0.004$) in this region. Based on these results, haplotypes from these two samples were pooled together and used as a single location in the subsequent analysis.

mtDNA CR data was used to reconstruct demographic history of each genetically differentiated pool. Demographic histories were inferred with Bayesian Skyline Plots (BSP) performed in BEAST v1.10.4 (Drummond et al., 2012). Each model was elaborated using BEAUTi v1.10.4 (Drummond et al., 2012), to avoid misrepresentation of the data and overestimate the population size, the whole alignments instead of collapsed haplotypes were used for the analysis. HKY + G was selected as the best nucleotide substitution model (see below) and the mutation rate was fixed at 4.9×10^{-8} substitutions/site/year (Alvarado Bremer et al., 2005; Allaya et al., 2015). Chain length of 50×10^6 , sampling every 1.000 generations, when needed, two runs were combined. Tracer v1.7.1 (Rambaut et al., 2018) was used to assess convergence with all ESS reaching the minimum values. Effective population size (N_e) was corrected by the generation time (τ) following the BEAST manual

recommendation. Generation time was estimated using the approach of Theisen et al. (2008) adjusting to the life-history traits of *E. alletteratus*. Thus, considering age at first maturity and a life-span of seven years (Saber et al., 2018; Lucena-Frédou et al., 2021), we estimated the generation time in 3.25 years. Haplotype networks for the mtDNA CR were constructed with the Minimum Spanning method implemented in PopART (Leigh & Bryant, 2015).

Phylogenetic analyses and species delimitation

Due to the different size of the two datasets, the alignments were analysed separately without concatenation of the sequences. Two different approaches were used for the phylogenetic reconstructions of the respective datasets, maximum likelihood and Bayesian inference. The best nucleotide substitution model for each marker was inferred with MEGA 7 on the basis of the Bayesian Information Criterion (BIC) (Kumar et al., 2016). For mtDNA CR maximum likelihood reconstruction was performed in MEGA 7 (Kumar et al., 2016), using Tamura 3-parameter + Gamma ($\alpha = 0.208$) as the best model with bootstrap 1,000 pseudoreplicates of node support. The sequences of three individuals belonging to the sister genus *Auxis* [*Auxis rochei* (Risso, 1810)] were used as outgroup. For CaM data maximum likelihood was performed using Jukes Cantor + Gamma + Invariable sites (I) ($\alpha = 0.05$, $I = 0.485$) with 1,000 pseudoreplicates of bootstrap support.

Bayesian inference was also tested for both mtDNA CR and CaM data using BEAST v1.10.4 (Drummond et al., 2012) with two tree priors, namely Speciation Yule Process and Coalescent Constant Size (Yule, 1924; Gernhard, 2008). BEAUTi v1.10.4 (Drummond et al., 2012) was used to elaborate the different models for running BEAST. For mtDNA CR Yule and Coalescent tests were generated with the second-best nucleotide substitution model (HKY + G) since T92 was not available. The two tests were constructed under the assumption of strict clock and base frequencies estimated. The analysis was run with a MCMC (Markov Chain Monte Carlo) chain length of 10 million generations, sampling every 1.000 generations. For the CaM marker, Yule and Coalescent prior tests were constructed with Jukes Cantor + Gamma + Invariable sites, strict clock, base frequencies estimated and MCMC of 20 million

generations sampling each 1.000 generations in both runs. In all runs, transition kernel states were checked to reach the minimum value and ESS were assessed to converge with Tracer v1.7.1 (Rambaut et al., 2018). Trees summary reconstructions were performed with TreeAnnotator v1.10.4 with a burn in of 10%, to avoid negative branches common ancestor heights option was selected. Final trees were represented graphically and edited with FigTree v1.4.3 (Rambaud, 2012). D_A Genetic distances (Nei et al., 1983) among different phylogenetically differentiated groups were estimated in MEGA 7.

Different methods were used to identify putative species. For the ABGD (Automatic Barcode Gap Discovery) (Kekkonen & Hebert, 2014) three distances were tested, Jukes-Cantor (JC69), Kimura (K80) (TS/TV = 2) and Simple Distance. For mtDNA CR alignment parameters were set as follows Pmin: 0.001, Pmax: 0.1, Steps 10 and Relative Gap With: 1.5. while for CaM alignment settings were Pmin: 0.000001, Pmax 0.1, Steps 10 and Relative gap with 0.5. ABGD was run under the web server version available from <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>. MtDNA CR and CaM ultrametric trees generated under the two priors -Coalescence and Speciation- were used as input trees for GMYC (Generalized Mixed Yule Coalescent) (Pons et al., 2006), and single and multiple-threshold versions were tested <https://species.h-its.org/gmyc/> (Zhang et al., 2013). Maximum likelihood trees were used for PTP (Poison Tree Process) and mPTP (multiPTP). These methods were run with the default options as implemented in the web server <http://www.exelixis-lab.org/> (Kapli et al., 2017). For PTP and mPTP Likelihood Ratio Test (LRT) were calculated as $-2\ln\lambda$, being λ the relation between the null model likelihood and the likelihood of the model itself (Huelsenbeck & Bull, 1996). Finally, putative species were tested with bPTP (bayesian PTP) analyses (Zhang et al., 2013), for the two markers MCMC converge after 500.000 generations, thinning was set as 100 and a burn-in of 0.1, under the online version available from <https://species.h-its.org/>.

Results

The mtDNA CR sequence comparison included 103 polymorphic sites out of 350 bp, which defined 135

haplotypes (Table 1). CaM alignment revealed 10 polymorphic sites out of 341 bp, after allele phasing, the alignment resulted in 27 putative alleles.

Population genetics analyses

The highest values of haplotype diversity for the mtDNA CR were obtained in *E. alletteratus* from Bermuda and Gulf of Mexico, and in *E. lineatus* from Mexico's East Pacific Coast. In each of those locations no haplotype was shared (Table 1). Conversely, the pooled samples of *E. alletteratus* from Portugal and Tunisia had the lowest haplotype diversity ($h = 0.641 \pm 0.061$), with one haplotype shared in 46 out of 78 individuals (58.9%), while also displaying the lowest value of nucleotide diversity ($\pi = 0.004 \pm 0.003$). Based on ϕ_{ST} values of *E. alletteratus* in the North East Atlantic and Mediterranean area (NEA), no significant genetic divergence was found between the Portugal and Tunisia locations ($\phi_{ST} = 0.007$; $P = 0.217 \pm 0.004$). A similar situation occurs in the North West Atlantic area (NWA) with no significant genetic differences between Bermuda and the Gulf of Mexico ($\phi_{ST} = 0.054$; $P = 0.049 \pm 0.002$). Differentiation was also lacking $< (\phi_{ST} = 0.011$; $P = 0.071 \pm 0.007)$ among the two Tropical East Atlantic locations (TEA) Senegal and Côte d'Ivoire.

By contrast, a high degree of genetic heterogeneity between regions was observed (Table 2). High heterogeneity was detected between the NEA region and NWA region ($\phi_{ST} = 0.405$; $P = 0.000 \pm 0.000$) (Table S1 in Supporting Information), although these regions shared some haplotypes. AMOVA showed that 37% of the variation corresponds to differences between regions ($d.f$ 1; $P = 0.000$). In addition, significant high genetic differences were found between the North East Atlantic-Mediterranean region and Tropical East Atlantic region (TEA) ($\phi_{ST} = 0.918$; $P = 0.000 \pm 0.000$) and also between the NWA and the TEA regions ($\phi_{ST} = 0.866$; $P = 0.000 \pm 0.000$). Moreover, within the *E. alletteratus* none of the haplotypes was shared between the individuals from North Atlantic-Mediterranean (West and East) and the TEA area. Similarly, no haplotype was shared between the three *Euthynnus* species. The comparison between *E. affinis* (Viet Nam) versus *E. lineatus* (Mexico) showed a lower ϕ_{ST} value ($\phi_{ST} = 0.302$; $P = 0.000 \pm 0.000$) (see Table 2). In an

Table 2 Pairwise genetic differentiation between samples. *E. alletteratus* North East Atlantic and Mediterranean (NEA), North West Atlantic (NWA), Tropical East Atlantic (TEA), *E. affinis* Viet Nam (VNM) and *E. lineatus* Mexico (MEX)

	NEA <i>E. alletteratus</i>	NWA <i>E. alletteratus</i>	TEA <i>E. alletteratus</i>	VNM <i>E. affinis</i>	MEX <i>E. lineatus</i>
NEA <i>E. alletteratus</i>		0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
NWA <i>E. alletteratus</i>	0.388		0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
TEA <i>E. alletteratus</i>	0.918	0.866		0.000 ± 0.000	0.000 ± 0.000
VNM <i>E. affinis</i>	0.964	0.873	0.906		0.000 ± 0.000
MEX <i>E. lineatus</i>	0.949	0.822	0.890	0.302	

Below diagonal ϕ_{ST} values, above diagonal p values based on data from mtDNA CR

AMOVA comparing the three species and regions 89.21% of the variation was explained by differences between species/regions ($d.f$ 4; $P = 0.000$) (see Table S2 in Supporting Information).

Haplotype network reconstruction for *E. alletteratus* resulted in two separate clusters with a complete segregation of the North Atlantic-Mediterranean individuals from the ones from the Tropical East Atlantic (TEA). The north Atlantic cluster showed a star-like pattern with a central haplotype being present in 46 out of 78 individuals, the TEA haplotypes drew a scattered pattern (Fig. 2A). Accordingly, Bayesian Skyline Plots indicates different population dynamics for each location (Fig. 2B, C and D). BSP in NEA haplotypes suggest a recent demographic history with a gradual increase of population size in the last 25,000 years while BSP for NWA individuals indicates a population growth that started around 75,000 years ago. The BSP in the Tropical East Atlantic area reflects a moderate episode of population expansion that started around 125,000 years ago.

As with the mitochondrial marker, CaM showed the lowest gene diversity and nucleotide diversity in the NEA *E. alletteratus* individuals, while the TEA individuals presented larger genetic diversity (Table 1). Pairwise F_{ST} showed significant differentiation between the NEA and the TEA with all alleles private to the respective region. Surprisingly, although significant differences were found between *E. affinis* and *E. lineatus*, the F_{ST} between them was lower than the expected (Table 3) with several shared alleles. Finally, although the nuclear CaM marker showed a lower value of differentiation than the mtDNA CR, it was still highly significant ($F_{ST} = 0.654$; $d.f = 3$; $P = 0.000$).

Phylogenetic analyses and species delimitation

The mtDNA CR phylogenies reconstructed with Bayesian Inference and maximum likelihood clearly segregates the lineages of *E. alletteratus* into two reciprocally monophyletic clusters that correspond to the entire North Atlantic + Mediterranean and to the Tropical East Atlantic, respectively (Fig. 3). The nodes of these two groups are well supported by bootstrap values and posterior probabilities. These two clusters of *E. alletteratus* are the sister group to a single well-supported cluster composed by *E. affinis* + *E. lineatus*. In this group some of the CR lineages of the two species are lumped together but it can be identified a lineage consisting only of *E. lineatus* (see Fig. 3 inset). Of the two Bayesian priors, Speciation Yule Process recovered better supported nodes than Coalescence, but in both instances the tree topology for the most internal branches was the same. CaM phylogenetic reconstruction also separates *E. alletteratus* from the entire North Atlantic + Mediterranean and TEA into two clusters (Fig. 4). For this marker, *E. alletteratus* from North Atlantic + Mediterranean emerges as the sister taxon of *E. affinis* + *E. lineatus*. The *E. affinis* + *E. lineatus* alleles are gathered into one cluster with no internal partitioning, with three CaM alleles shared between these two species (but no mtDNA CR haplotype was shared between *E. affinis* and *E. lineatus*). This topology is well supported for the two priors tested through Bayesian Inference, although in this case Coalescent constant size recovered well supported nodes (Fig. 4). Unfortunately, maximum likelihood phylogenetic reconstruction for CaM could not recover a congruent topology showing low bootstrap

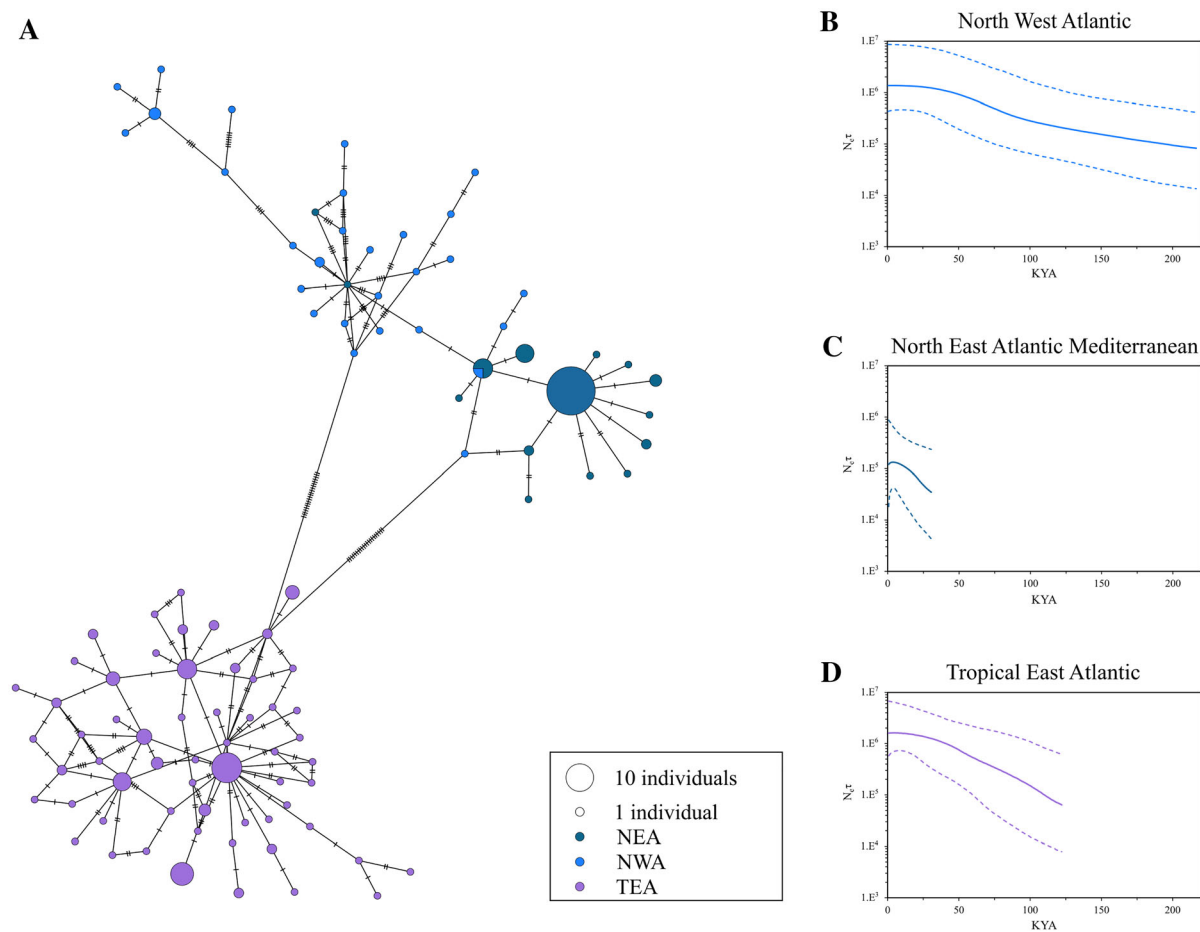


Fig. 2 Haplotype network of *E. alletteratus* haplotypes (A). Bayesian Skyline Plots for the North East Atlantic and Mediterranean lineage, NEA (B); North West Atlantic, NWA (C); and the Tropical East Atlantic clade, TEA (D). In the y-axis solid lines represent the median $N_e \tau$ (τ , generation time) of each

population as the product of the effective population size and the generation time for this species, dotted lines indicate the 95% highest probability density limits. X-axes correspond to the time expressed in thousands of years ago (KYA)

Table 3 CaM pairwise differentiation between samples. Below diagonal F_{ST} between samples, above diagonal p -values. *E. alletteratus* North East Atlantic + Mediterranean (NEA), *E.*

alletteratus Tropical East Atlantic (TEA), *E. affinis* Viet Nam (VNM), *E. lineatus* Mexico (MEX)

	NEA	TEA	VNM	MEX
NEA		0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
TEA	0.687		0.000 ± 0.000	0.000 ± 0.000
VNM	0.751	0.576		0.000 ± 0.000
MEX	0.783	0.601	0.147	

values and several inconsistencies were detected in the allele clustering (data not shown). In accordance with the Bayesian approach, estimation of D_A distances for the CR between *E. alletteratus* from the North

Atlantic + Mediterranean vs. TEA ($D_A = 0.117$) was higher than the distance ($D_A = 0.006$) between *E. affinis* and *E. lineatus* (Table S3 in Supporting Information).

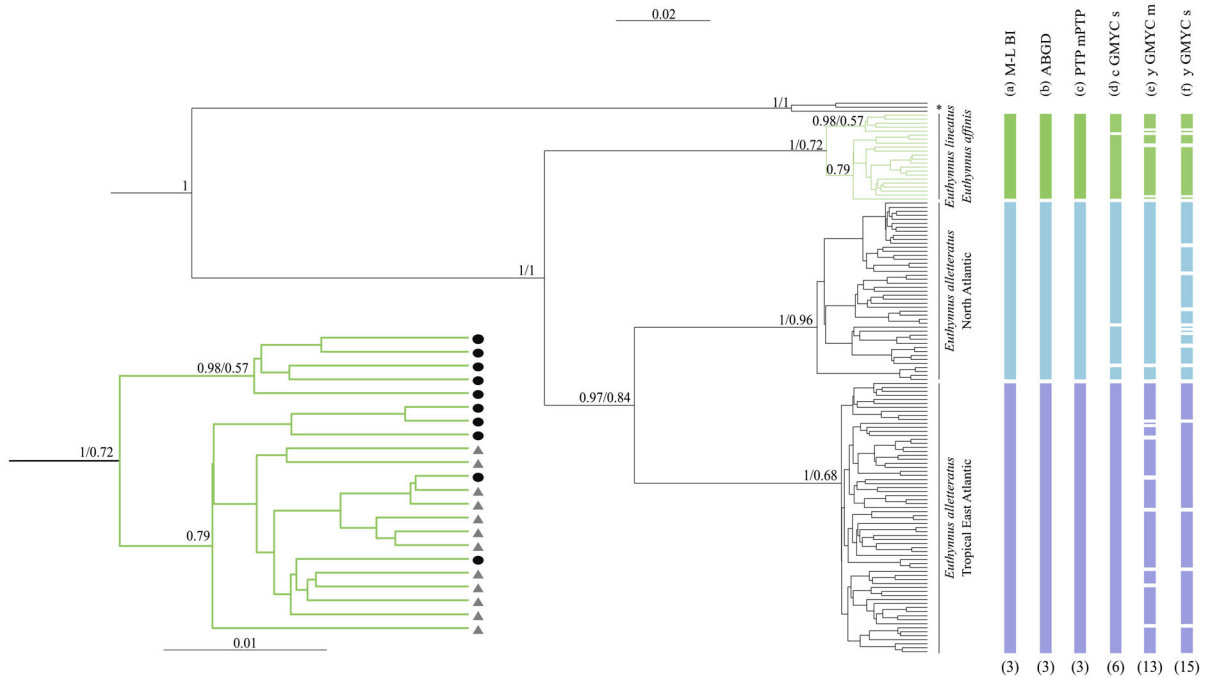


Fig. 3 Bayesian Inference tree under Speciation Yule Process hypothesis for the mtDNA CR marker, nodes support from posterior probabilities (left) and bootstrap values (right) are shown, † indicates three *Auxis rochei* sequences that were used as outgroup. Green branches correspond to the *E. lineatus* and *E. affinis* clade; in the inset black circumferences indicate *E. lineatus* haplotypes, and grey triangles *E. affinis* haplotypes. Bars summarize the results for the species delimitation methods tested, with the number of putative species/clusters depicted at

Results of species delimitation based on mtDNA CR data are summarized in Fig. 3. Excluding the outgroup, the ABGD analysis recovered three clusters (putative species) irrespective of the distance model used. In agreement with the populations analyses presented above, ABGD separated the *E. alletteratus* individuals from North Atlantic-Mediterranean from those from TEA region as belonging to two different species while grouping *E. affinis* and *E. lineatus* into one species. For simple distance some partitions (prior maximal distances [P] ranging from 0.001668 to 0.004642) grouped the dataset into four putative species, with a single haplotype as representative of the fourth species. In addition, PTP and mPTP recovered three species, with one cluster formed by *E. affinis* and *E. lineatus*, and two different clusters for individuals from the North Atlantic + Mediterranean region and another for those from the TEA region ($P < 0.001$, and $P < 0.001$). Conversely, bPTP

the bottom: **a** Maximum likelihood (M-L) and Bayesian Inference (BI) phylogenetic reconstruction, **b** Automatic Bar Gap Discovery, **c** Poisson tree process and multi-rate Poisson tree process, **d** coalescent reconstruction and Generalized Mixed Yule Coalescent single threshold (cGMYC), **e** Yule speciation process and Generalized Mixed Yule Coalescent multiple threshold (yGMYCm), **f** Yule speciation process and Generalized Mixed Yule Coalescent single threshold (yGMYCs)

retrieved an unrealistic number of species ranging from 39 to 112 and being almost every individual haplotype is identified as a putative species. With GMYC, the most realistic delimitation was obtained under the coalescent prior, in which single threshold performed better than multiple threshold and recovered six clusters as putative species (Confidence Interval [CI] 6–15), with the number of entities increasing to nine if the outgroup is included (CI 9–18). Multiple threshold recovered 14 clusters (CI 13–16) and 18 entities (CI 16–20) (Figure S4 in Supporting Information). On the other hand, GMYC under Yule Speciation Process multiple threshold, while not prove to be better, recovered 13 clusters (CI 13–14) and 18 entities (CI 17–18) (Fig. 1), whereas with single threshold recovered 15 clusters (CI 7–21) and 22 entities (CI 8–28), including the outgroup. Although all the results clearly overestimated the

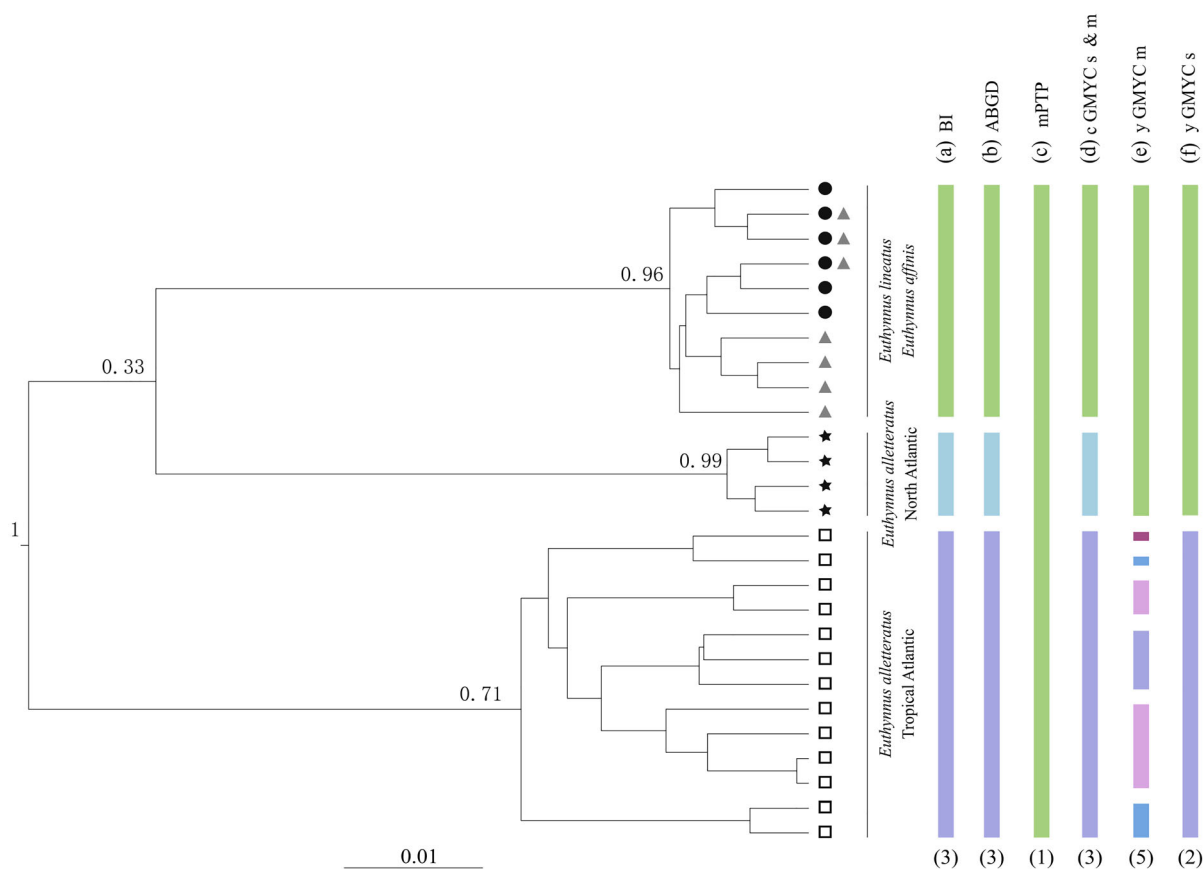


Fig. 4 Bayesian Inference tree with Coalescent constant size prior for the CaM marker, values on nodes depict support of posterior probabilities. Black circumferences indicate *E. lineatus* alleles, grey triangles *E. affinis* alleles, black stars North Atlantic *E. alletteratus* and white squares South Atlantic *E. alletteratus* alleles. Shared alleles between *E. lineatus* and *E. affinis* are represented with the two symbols (black circumferences and grey triangles). Bars summarize the results of species

delimitation methods, with the number of putative species/clusters depicted at the bottom: **a** Bayesian inference phylogenetic reconstruction, **b** Automatic Bar Gap Discovery, **c** multi-rate Poisson tree process, **d** coalescent reconstruction and Generalized Mixed Yule Coalescent single threshold, **e** Yule speciation process and Generalized Mixed Yule Coalescent multiple threshold, **f** Yule speciation process and Generalized Mixed Yule Coalescent single threshold

number of species, all tests were statistically significant ($P < 0.001$). Species delimitation results obtained with the CaM marker using ABGD tests performed with JC and K80 distances differentiate *E. alletteratus* from the North Atlantic-Mediterranean locations from the ones from TEA locations, again, one unique cluster was detected for the *E. affinis* and *E. lineatus* species ($P = 0.001$ – 0.002) (Fig. 4). However, with Jukes Cantor and Kimura models one partition ($P = 0.004$) clustered all alleles into a single species, and the same results were obtained using simple distance ($P = 0.001$). PTP reported all alleles except one to belong into a single species ($P < 0.001$), similarly

mPTP reported all alleles as a single species ($P < 0.001$), while bPTP reported an unrealistic number of species 19 (CI 3–25). In relation to GMYC results, Yule speciation single threshold recovered 2 clusters (CI 1–10) and 2 entities (CI 1–26) while multiple threshold recovered four clusters (CI 1–8) and five entities (CI 1–9), but in both cases without statistical support. On the other hand, GMYC for Coalescent reconstruction single and multiple thresholds recovered 3 clusters (CI 3–9 and CI 3–7, respectively) and 3 entities (CI 3–15 and CI 3–15) being both statistically significant ($P < 0.05$), GMYC grouped indistinctly individuals of *E. affinis* and *E. lineatus* into a single cluster, and split *E. alletteratus*

into two different species, individuals from the North Atlantic-Mediterranean area and individuals from the TEA area.

Discussion

Patterns of genetic differentiation within *Euthynnus* spp.

In this study, the distribution of genetic variation for *E. alletteratus* was characterized. To our knowledge this is the largest genetic study on this species in terms of the number of individuals characterized and in geographic coverage. Furthermore, the *E. alletteratus* variation was compared with the genetic variation of the other two species of the Genus: *E. lineatus* and *E. affinis*.

The most salient finding of this paper is the deep genetic divergence within *E. alletteratus*, which separates the samples from the North Atlantic from the TEA (see below). Previously, Alvarado Bremer & Ely (1999) identified this CR discontinuity when they compared two small samples sizes from NWA and the TEA region. These authors concluded that an amphiatlantic pattern of genetic differentiation existed and that the coastal habits of *E. alletteratus* could account for the absence of trans-Atlantic gene flow that gave rise to the genetic discontinuity. In here this interpretation is partially rejected. The comparison of amphiatlantic samples in the North Atlantic reveals that only about 40% of the variation is explained by differences between populations, and that occasional contact between the NWA and the NEA must exist as evidenced by shared haplotypes between the two regions. By contrast, the *E. alletteratus* from the TEA shows no evidence of gene flow with the NWA and the NEA populations, respectively, ($\phi_{ST} = 0.866$ and 0.918), which is further supported by the reciprocal monophyly of mtDNA and CaM clades. This level of differentiation contrasts dramatically with the shallow index of differentiation ($\phi_{ST} = 0.302$) separating *E. affinis* from Viet Nam from *E. lineatus* from Mexico, which is lower than that between the two populations of *E. alletteratus* at opposite sides of the North Atlantic ($\phi_{ST} = 0.388$). The deep genetic differentiation is corroborated by the different demographic histories suggested by the BSP and network analyses. The NEA population, which include samples from the

Mediterranean, seems to have the most recent population history, probably consequence of population expansion after the last glacial period. A similar pattern, population expansion after last glaciation, has been observed in the Mediterranean population of other pelagic species (eg, Atlantic bluefin tuna, *Thunnus thynnus*; and swordfish, *Xiphias gladius* Linnaeus, 1758) (Alvarado Bremer et al., 2005). Nevertheless, this assumption should be taken in caution since only one location from the Mediterranean Sea is analysed.

Phylogeny, phylogeographic association, and species delimitations

The phylogeographic association of genetic discontinuities at both the mtDNA and nDNA markers between the North Atlantic and the TEA suggest the presence of two cryptic species of *E. alletteratus* within the Atlantic Ocean. Both mtDNA CR and CaM data split *E. alletteratus* individuals into two reciprocally monophyletic clusters supported by bootstrap pseudoreplicates and posterior probabilities (see Figs. 2 and 3). The mtDNA CR divergence between the two groups of *E. alletteratus* ($D_A = 11.7\%$) is nearly 20 times larger than the CR divergence ($D_A = 0.6\%$) between *E. lineatus* (Mexico) and *E. affinis* (Viet Nam), and similar to the distance that separates *E. alletteratus* respectively from *E. affinis* and from *E. lineatus* (S. Table 3). Further, the level of divergence between the independent lineages of *E. alletteratus* falls within the range of D_A values between species-pairs reported for members of the also cosmopolitan genus *Sarda*, which range from 11.2 and 17.1% (Viñas et al., 2010). This division of *E. alletteratus* into two sister taxa was supported by CR data using M-L BI, ABGD and PTP mPTP, and by CAM data using BI, ABGD and cGMYCs&m (see Figs. 2 and 3). The analysis of CR with cGMYCs also identifies *E. alletteratus* from the TEA as a species separate from the representative samples from North Atlantic, although the latter are also divided into three separate species with no phylogeographic association within that region. By contrast, the results obtained with yGMYCm and yGMYCs using *E. alletteratus* CR data resulted in the identification of nine and five species, respectively, in the TEA, and two and nine species in the North Atlantic. The analysis of CaM using yGMYCm resulted in the partition of the TEA

sample into four species with a fifth grouping the North Atlantic *E. alletteratus* + *E. affinis* + *E. lineatus* as one species. Several authors reported that GMYC tend to over split the individuals in many more clusters than the ones expected (Zhang et al., 2013; da Silva et al., 2018). Long branches and fast evolutionary rates can lead to over split clusters while they are not different species, thus, reflecting population structure rather than different species. The GMYC also present low accuracy in species with large effective population sizes (Esselstyn et al., 2012), consistent with the results obtained here. Thus, it appears that GMYC approaches fail to delimit the processes of speciation and coalescence revealed by CR data, perhaps due to the excessive variability characteristic of this mtDNA segment and attempts to improve the resolving power using mGMYC only increased the number of putative species identified. Nevertheless, this genetic marker has been proved to be marker of choice for delimitating species in the Scombridae family (Viñas et al., 2010; Ollé et al., 2019) with proved higher power of resolution than the traditional COI marker used for DNA barcoding (Viñas & Tudela, 2009). By contrast, the analysis of CaM data using mPTP failed to separate any of the *Euthynnus* spp. included in this study (Fig. 3). It has been reported before that mPTP and PTP tend to underestimate the number of species (da Silva et al., 2018), which is consistent with the results obtained here. No improvement of species identification is observed for the CaM marker under Yule speciation process ultrametric tree reconstructions. However, for CaM data both single and multiple GMYC approaches under coalescent prior of the phylogenetic reconstructions reported the same three clusters as ABGD and mPTP and PTP mentioned above. As can be seen the different methodologies lead to different conclusions, some of which are highly unrealistic. We advocate that species delimitation methods should include several unlinked markers and different methodologies, including phylogenetic analyses. Then, the most plausible hypothesis is the one shared by most markers and methodologies.

Considering all the results across methods and markers and rationalizing the number of species, we propose that *E. alletteratus* fishery targets two putative cryptic species that occupy different regions of the Atlantic basin. These results are consistent with the morphological differentiation of *E. alletteratus* along

the western coast of Africa. Gaykov & Bokhanov (2008) compared samples collected from Morocco to Liberia (Central Eastern) against samples collected from Nigeria to Namibia (South-eastern) and found significant differences ($P < 0.01$) in preanal distance, length of the second dorsal and anal fins base, distance between the caudal fin ends, maximum and minimum body depth, maximum and minimum body width, distance between caudal peduncle keels, and differences in meristic counts in the number of rays in the first dorsal and anal fins. It is very likely that the differences detected in here between the North Atlantic and the TEA samples at the molecular level correspond to the morphological differences identified by Gaykov & Bokhanov, 2008. The North-Tropical Atlantic phylogeographic pattern observed in *E. alletteratus* fits with the previously defined biogeographic realms (Spalding et al., 2012), that are associated with the North and South Atlantic gyres and the Atlantic Equatorial Current System. This association can be the result of oceanic forces that favoured the reproductive isolation between north and tropical individuals or driven by distinct habitat preferences between species. This association with the oceanic biogeographic realms can help to understand how individuals that are geographically more distant (Tunisia vs Gulf of Mexico) depict lower genetic differentiation than individuals that inhabit closer areas (Portugal vs Senegal). A similar pattern has been observed in other pelagic species such as *Delphinus* spp. (Amaral et al., 2012) and the swordfish with the use of both mtDNA CR and nuclear markers, including CaM (Alvarado Bremer et al., 1996; Chow et al., 2007). North–South differentiation is supported by the mtDNA CR marker with the split of two clusters of *E. alletteratus* well supported for bootstrap values and posterior probabilities. Moreover, amphi-Atlantic population differentiation have been detected in other small scombrid species such as *Sarda sarda* (Bloch, 1793) (Viñas et al., 2010) and *Scomber scombrus* Linnaeus, 1758 (Rodríguez-Ezpeleta et al., 2016). Additional information is needed to examine the patterns of genetic variation along the eastern coast of Africa, from Morocco to Liberia, which was not sampled in this study. Conversely, in the NWA, the southern portion of its range, from the Caribbean Sea to the northwest coast of South America, needs to be examined.

The high level of differentiation found in *E. alletteratus* within the Atlantic Ocean contrasts with the shallow genetic divergence that separates *E. affinis* and *E. lineatus* collected roughly 15,000 km apart. The D_A value (0.6%) and the absence of reciprocal monophyly for either of the two markers is more consistent with population differentiation than that expected for two separate sister species (Table 2 and Table 3; Figs. 2 and 3). Several hypotheses can be invoked to account for this surprising result. First, the range of *E. affinis* extends from Indian Ocean to Hawaii in the Pacific Ocean, while *E. lineatus* is primarily found from southern California to Peru in the East Pacific (Fig. 1), although two stray specimens have been collected in the Hawaiian Islands (Collette & Nauen, 1983). Conversely, there are reports of *E. affinis* captured in areas as far as Baja California and Ecuador, and authors and governmental authorities seems to acknowledge the presence of two different stocks of *E. affinis* in Western Pacific and Central-Eastern Pacific (Ward, 1995) probably related to a heterogeneous distribution of two highly divergent clades (Jackson et al., 2014). Thus, one possibility is that the sample of *E. lineatus* from the East Pacific (Mexico) analysed in here included transient *E. affinis* individuals originating in the Central-Eastern Pacific. A second hypothesis is the small sample sizes characterized in this study compared to those of *E. alletteratus*. Small sample sizes may result in false negatives, that is the failure to detect differences between two species (Björklund & Bergeck, 2009). This explanation, however, is unlikely given the high-resolving power of CR data to differentiate among tunas and billfishes (Alvarado Bremer et al., 1997; McKenzie & Alvarado Bremer, 2017). A third hypothesis is incomplete lineage sorting. While reciprocal monophyly is expected between species-pairs, there are several examples of paraphyly due to retention of ancestral polymorphisms or hybridisation in fishes (Campton & Utter, 1985; Smith, 1992; Scribner et al., 2000), and such events have also been reported for marine pelagic species (Chow & Kishino, 1995; Alvarado Bremer et al., 2005; Rooker et al., 2007; Viñas et al., 2010). Given that *E. affinis* and *E. lineatus* are one of the youngest lineages upon scombrid species (Santini et al., 2013), the possibility of incomplete lineage sorting needs to be considered. It is worth mentioning that the *E. lineatus* sample contains a strongly supported cluster of lineages that

are private to that sample, which may be support the mixed sample hypothesis, but it is also consistent with the potential of unidirectional gene flow from west to east in the Pacific. Accordingly, a fourth hypothesis needs to be advanced, where *E. affinis* and *E. lineatus* are populations of a single species. Traditionally they have been classified as separate species on the basis of differences in meristic counts and morphological characters (Collette & Nauen, 1983). Unfortunately, samples from Mexico were collected under the assumption of belonging to *E. lineatus* and no meristic counts nor morphological descriptions were recorded. Prior phylogenetic studies using COI data had confirmed the separation of these species, but these studies were based on a single genetic marker and a single individual of *E. lineatus* (Kumar & Kunal, 2013; Santini et al., 2013). Given that morphological and meristic characters can distinguish *E. affinis* and *E. lineatus* individuals, for the moment we should be conservative and keep them separate. However, we recommend a re-evaluation of their status through the genomic characterization of representative samples of both species throughout their range, with a description of each specimen sampled.

Fishery management implications for *E. alletteratus*

As mentioned in the introduction, no management recommendation for *E. alletteratus* has been released by ICCAT (ICCAT, 2020). However, for *E. alletteratus* once we consider the North Atlantic and the TEA regions contain two evolutionary significant units, the regional characterization of these units using the mtDNA CR provides an initial insight into their genetic population structure. Characterization of the North Atlantic region revealed a clear pattern of population substructure, with two genetically distinct populations corresponding to the NWA and the NEA (Table 2). Additionally, based on mitochondrial information from temporal samples, we can acknowledge temporal stability on the genetic differentiation patterns within region. In sum, our results indicate two scales of differentiation in what is now considered a single population with no defined boundaries. The first, supported by the deep evolutionary differences between the North Atlantic and the TEA individuals. The second, based upon the significant differentiation between the Northeast and the Northwest Atlantic

regions. Accordingly, for management purposes the populations of *E. alletteratus*, should be divided into a minimum of two separate units: Tropical East Atlantic and North Atlantic and Mediterranean, with a further consideration of a third unit in the North West Atlantic.

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Author contributions JO and LVV performed the wet lab work. JO analysed the data and drafted the manuscript. JAB, NS, RMA and JV supervised the genetic analysis and contributed to the manuscript. GC, TYD, GH, PGL, RML, FNS and NCD provided the samples and contributed to the manuscript. JO and JV conceived the study.

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