

Research Article

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DNA barcoding reveals deep divergent molecular units in *Pomatomus saltatrix* (Perciformes: Pomatomidae): implications for management and global conservation

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Abstract

Pomatomus saltatrix is a high-value marine pelagic coastal fish, that is fished throughout subtropical and temperate coastal waters around the world. Despite its large economic potential, there are no global data on its genetic diversity, which could compromise the conservation of the species. The aim of this study was to analyse the genetic-evolutionary structuring of the species, with the intention of evaluating different genetic *P. saltatrix* stocks that may indicate potential species. Based on 157 Cytochrome C Oxidase Subunit 1 sequences, the molecular delimitation analyses of species (distance and coalescence methods), as well as the haplotype network, found profound geographic structuring related to five distinct units with high and significant F_{ST} pairwise values. The divergence of these molecular units is mostly related to the Pleistocene glacial and interglacial cycles of climatic oscillations. It is hypothesized that one ancestral lineage, adapted to cold water environments, diversified into two lineages, with one more adapted to warmer environments. The high values of global genetic diversity ($\pi = 0.016$; $h = 0.96$) may be related to the existing profound genetic differentiation. Due to the presence of five Molecular Operational Taxonomic Units (MOTUs) within the species it is necessary to employ different regional management strategies. Traits of low haplotype richness and shallow population contraction were identified in the MOTUs V (Venezuela and Brazil) and III (Turkey and Australia), respectively, representing conservation priorities. Other molecular markers, as well as morphological data, should be explored with the aim of defining the taxonomic status of *P. saltatrix* stocks.

Introduction

The continuity of marine environments has resulted, historically, in the idea of genetic homogeneity in marine environments based on the idea of panmixia (Palumbi, 1992; Cowen & Sponaugle, 2009). In recent years, using molecular approaches, cases of hidden diversity and/or discontinued lineages of marine species have increased, resulting in the revision of the panmixia paradigm (Damasceno *et al.*, 2015; Luz *et al.*, 2015; Freitas *et al.*, 2017; Machado *et al.*, 2017b; Healey *et al.*, 2018; Hernández *et al.*, 2018; Chen *et al.*, 2020; Neves *et al.*, 2020). The detection of these Molecular Operational Taxonomic Units (MOTUs) is extremely important for species conservation, since distinct management practices may be necessary to prevent the mixture and/or loss of different gene pools (Frankham *et al.*, 2008). Therefore, investigating population structures, mainly of commercially exploited species, is a crucial step towards the conservation of their genetic heritage. *Pomatomus saltatrix* (Linnaeus, 1766), popularly known as the bluefish, stands out as a biological model for the evaluation of molecular units.



The only representative of the Pomatomidae family (Nelson *et al.*, 2016), *P. saltatrix* is a species of great economic importance across the world, with a pelagic habitat and occurring in coastal and oceanic, tropical and subtropical waters with temperatures varying between 18–27 °C (Tortonese, 1986; Juanes *et al.*, 1996, 2002). Its wide distribution can be associated with its high dispersal ability, especially through large group migrations of adult individuals during reproductive periods (Wilk, 1977; Miralles *et al.*, 2014a), which can be influenced by seasonal changes in water temperature (Hare & Cowen, 1996; Juanes *et al.*, 1996). This species has high commercial value and can reach 130 cm total length and weight of ~15 kg, acting as an important pelagic fishing resource, as well as a subject of industrial, recreational and artisanal fishing in different countries (Juanes *et al.*, 1996; Carpenter *et al.*, 2015).

The fishing pressure exerted on this species since the 1960s is reflected in the decline in the amounts of this fish caught and has resulted in the species being categorized globally as 'Vulnerable' (IUCN – International Union for Conservation of Nature; Carpenter *et al.*, 2015), with local assessments revealing different scenarios (Europe 'Near Threatened', Mediterranean and Gulf of Mexico 'Least Concern'; Bizsel *et al.*, 2011; Collette & Abad-Uribarren, 2015; Pina Amargos & Collette, 2015). Reported global landings of *P. saltatrix* had an increasing trend until 1983, before declining. Since then, negative oscillations were seen until the last data update in 2018 (Pauly *et al.*, 2020; additional details about catch values are available in Sea Around Us <seararoundus.org> as interactive graphics; the cut-out of this graphic also can be found in Supplementary Figure S1).

The wide distribution of this species covers different environmental and ecological conditions, revealing great plasticity in terms of spawning season, number of reproductive peaks along the year, and reproduction zones, for example (Juanes *et al.*, 1996). Although there is little information surrounding the genetic structure and diversity of this species, regional assessments have detected population differences in geographically related schools that may represent potential species (Goodbred & Graves, 1996; Turan *et al.*, 2006; Pardiñas *et al.*, 2010; Miralles *et al.*, 2014b). This scenario highlights the necessity of the identification of potential evolutionary units for the effective management of this species, with the aim of conserving its genetic patrimony. Furthermore, many monotypic genera, when widely distributed as *P. saltatrix*, can be divided into new taxa and/or evolutionary units (e.g. *Phalloceros caudimaculatus* – Lucinda, 2008; *Octopus vulgaris* – Amor *et al.*, 2017; *Pogonias cromis* – Azpelicueta *et al.*, 2019; *Ommastrephes bartrami* – Fernández-Álvarez *et al.*, 2020). Thus, the status of *P. saltatrix* as a monotypic species should be investigated mainly due to both the wide-ranging and ecological plasticity.

As such, using grouping methods in sequences of the Cytochrome C Oxidase Subunit 1 (COI) barcode region has proven to be an efficient tool for a better identification of species richness (Ward *et al.*, 2005; da Silva Oliveira *et al.*, 2017; Moraes *et al.*, 2019). There are different approaches based on distance (DNA barcoding) and coalescent methods. Distance methods are based on the barcoding gap hypothesis, where the intraspecific variation must be lower than interspecific variation (Hebert *et al.*, 2003; Meyer & Paulay, 2005). Coalescent methods combine population genetics and phylogenetics to delimit the MOTUs (Pons *et al.*, 2006). Both approaches are very useful to taxonomy, since traditional approaches based on morphological characteristics cannot identify cryptic species and may underestimate the real diversity of taxa (Bickford *et al.*, 2007; Pinto *et al.*, 2018) including fishes (Berbel-Filho *et al.*, 2018; Jacobina *et al.*, 2018, 2020). In addition to solving taxonomic uncertainties (Machado *et al.*, 2017a), the barcode region COI can be useful

for the identification of species from early stages of development (Hubert *et al.*, 2010; Almeida *et al.*, 2018), detecting market fraud (Barbuto *et al.*, 2010; Carvalho *et al.*, 2015, 2017), phylogenetic studies and can aid in management and conservation practices (van Velzen *et al.*, 2007; Healey *et al.*, 2018; Souza *et al.*, 2018; Zhao *et al.*, 2018; Kim *et al.*, 2020).

Thus, this study aimed to evaluate the evolutionary cohesion of geographically different *Pomatomus saltatrix* stocks, throughout its distribution in different oceans. We specifically asked if *P. saltatrix* is composed of more than one evolutionary unit, which introduces the hypothesis that it could be a genus composed of cryptic species, since different genetic groups have been identified in previous studies (Goodbred & Graves, 1996; Turan *et al.*, 2006; Pardiñas *et al.*, 2010; Miralles *et al.*, 2014b). For this, we used traditional genetic approaches and modern coalescent methods of species delimitation. Furthermore, we evaluated the historical mechanisms responsible for the genetic diversity and diversification of molecular evolutionary units of this species, as well as its genetic diversity, with the aim of contributing to future management plans.

Materials and methods

DNA extraction, amplification, sequencing and alignments

Samples of muscular tissue from six specimens of *Pomatomus saltatrix* from the Caribbean were obtained (Islas Margaritas, Venezuela), stored in 96% ethanol and kept at –20 °C. The total genomic DNA was extracted from each sample with the help of the *DNeasy Tissue* (Qiagen®) kit, following the protocol suggested by the manufacturer. The samples were visualized through electrophoresis in 1% agarose gel, coloured with Gelred™ and visualized under ultraviolet light. Given the abundance of COI barcode database, a region of ~650 bp was amplified, via PCR, in both directions using the universal forward FishF1 (5'TCAACCAACCA CAAAGACATTGGCAC3') and reverse FishR2 primers (5'ACTT CAGGGTGACCGAAGAATCAGAA3'), described by Ward *et al.* (2005). The reactions were performed with a final volume of 25 µl which comprised: 12.5 µl of 2× Tay Pol Master Mix (Vivantis®), 0.5 µl of each primer (10 mM), 0.5 µl of magnesium chloride (50 mM), 2 µl of genomic DNA (40 ng µl⁻¹) and 9 µl of ultrapure water. The amplification cycle consisted of an initial step of 2 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 2 min at 72 °C with a final extension of 10 min at 72 °C. The amplifications were purified using ExoSap-IT® (Affimetrix®), in accordance with the protocol provided by the manufacturer. The samples were sequenced in forward and reverse directions using the kit 'Bigdye Terminator v 3.1 Cycle Sequencing Ready Reaction' (Applied Biosystems), using a capillary sequencing technique using the automatic sequencer ABI 3500-Applied Biosystems. The generated sequences were deposited in GenBank with access codes MN199456–MN199461.

Furthermore, an additional 154 COI sequences that were obtained from NCBI/GenBank and included on BIN AA9142 (Barcode Index Number) of BOLD (Barcode of life database) were added to our dataset. The content of each database was compared rigorously to avoid duplicated sequences in BOLD and GenBank databases. However, the sequences EU263791, GBMND68177 and ERDK030 were removed due to noise in the alignment. In total, 157 *P. saltatrix* sequences were included in the analyses (Figure 1; Supplementary Table S1). These sequences were from the Western North Atlantic Ocean, Gulf of Mexico, Caribbean Sea, Western and Eastern South Atlantic Oceans, Mediterranean Sea, Sea of Marmara, Black Sea, Indian Ocean and South Pacific Ocean. This is the first global genetic diagnosis of *P. saltatrix*.

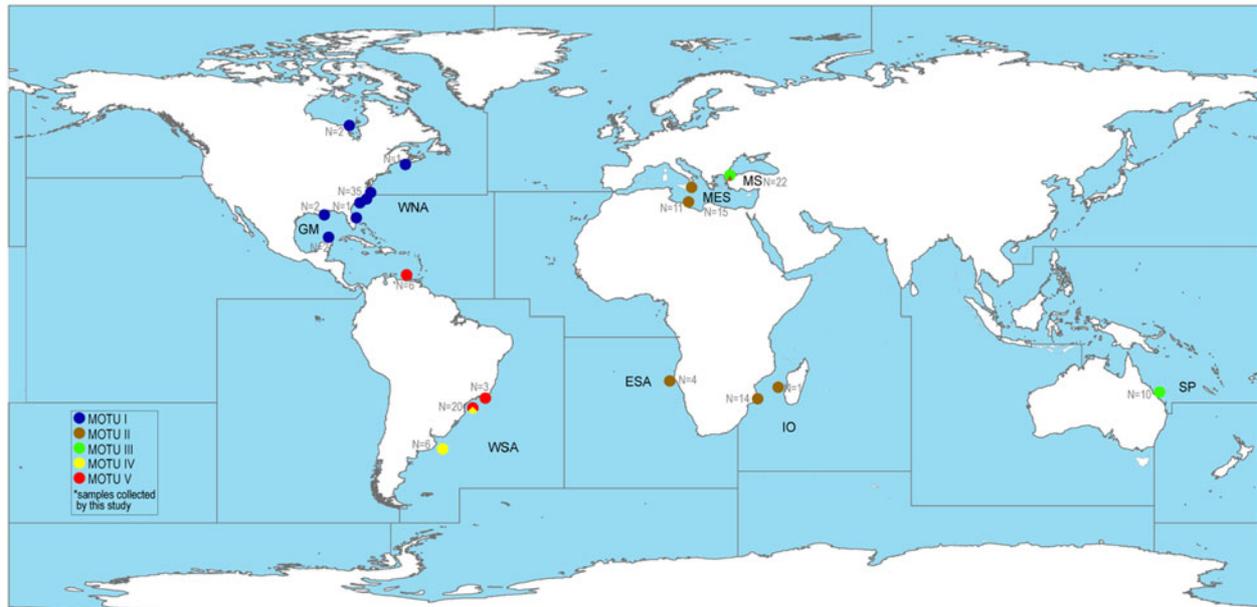


Fig. 1. Map of sampling locations and the geographic distributions of the five MOTUs for *P. saltatrix*. The different colours in the pie charts correspond to the five MOTUs (see Figures 2, 3 and Supplementary Figure S2 for more details about the MOTUs identifications), based on the FAOs regions. The grey values represent the sample size (N) of each site. (WNA: Western North Atlantic; GM: Gulf of Mexico; CS: Caribbean Sea; WSA: Western South Atlantic; MES: Mediterranean Sea; MS: Sea of Marmara; ESA: Eastern South Atlantic; IO: Indian Ocean; SP: South Pacific).

Species delimitation methods and time divergence estimates

All the sequences were aligned and edited using the ClustalW (Thompson *et al.*, 1994) algorithm in BioEdit Sequence Alignment Editor v.7.0. (Hall, 1999), through multiple alignments using the values 15 and 0.3 for gap openings and extensions, respectively (Hall *et al.*, 2011). To assess the cryptic diversity and identify possible MOTUs, we used two approaches: distance (traditional DNA barcoding and Assemble Species by Automatic Partitioning – ASAP) and coalescence (General Mixed Yule Coalescent – GMYC, Bayesian General Mixed Yule Coalescent – bGMYC, Bayesian implementation of Poisson Tree Processes – bPTP, and Multi-rate Poisson Tree Processes – mPTP). The MOTUs consisted of groups of sequences that acted as molecular entities which can or cannot represent a species (Blaxter *et al.*, 2005; Jones *et al.*, 2011). For all analysis, we used a multiple DNA sequences alignment for preserving the geographic information once we had shared haplotypes between different ocean basins. Because we employed different approaches to delimitate the MOTUs, the best species delimitation model for *P. saltatrix* will be determined from congruent results across methods and biological sense (geographic information, for example). We will also avoid MOTUs classified as singleton, which are composed of one sequence.

DNA barcoding uses the genetic distance between the COI sequence pairs and assumes that interspecific differences are greater compared with intraspecific differences (Hebert *et al.*, 2003), due to the choice of an optimal threshold (OT). An OT was estimated from the present dataset through the localMinima function, implemented in the SPIDER package (SPeies IDentity and Evolution in R – Brown *et al.*, 2012) in R (R Core Team, 2017; <https://www.R-project.org/>). Once the OT was defined, the jMOTU v.4.1 software was employed (Jones *et al.*, 2011) for the delimitation of MOTUs. The ASAP method (Puillandre *et al.*, 2021) was performed on the program web-interface (<https://bioinfo.mnhn.fr/abi/public/asap/>), using K80 as the nucleotide substitution model, and all the other parameters were set as default. The ASAP delimitation was defined considering the partition showing the lowest ASAP-score.

The coalescent methods (GMYC, bGMYC, bPTP and mPTP) were based on phylogenetic trees and are related to the phylogenetic concept of the species (Eldredge & Cracraft, 1980; Nelson, 1989). The GMYC and bGMYC (Pons *et al.*, 2006; Reid & Carstens, 2012) are methods that aim to estimate the transition point, in an ultrametric tree, between the intraspecific (populational/coalescent) and interspecific (speciation/extinction) processes, based on branching rates over time. The pre-transitional nodes represent speciation events, and the post transitional nodes represent the coalescences within species (Pons *et al.*, 2006). The main difference between them is that the GMYC employed a consensus tree obtained from the software that implements Bayesian searches, while bGMYC uses multiple trees from the posterior distribution of trees from Bayesian analyses, which is an interesting approach due to the stochasticity of Markov Chain Monte Carlo search. For the reconstruction of the ultrametric tree, firstly, the nucleotide substitution model was estimated in jModelTest v.2.1.7 (Posada, 2008) under the Bayesian Information Criterion (K80 + I). The Bayesian Inference (BI) topology was reconstructed in BEAST v.2.4.7 (Bouckaert *et al.*, 2014) under the following conditions: relaxed molecular clock with a lognormal distribution and Yule speciation model. Three independent runs with 30 million MCMC, where trees and parameters were saved every 10,000 generations with a burn-in of 25%. The results were then combined using the LogCombiner v.2.4.7 of the BEAST software (Drummond *et al.*, 2012). The ESS values (Effective Sample Size; >200) for the convergence of the estimated parameters were verified used Tracer v.1.5 (Rambaut *et al.*, 2009). Posteriorly, the GMYC was performed using SPLITS (SPeies Limits by Threshold Statistics – Monaghan *et al.*, 2009) on the R platform (<https://www.r-project.org/>). We used the single threshold method, where a single point of transition between intra- and interspecific events and a default parameter interval is calculated (interval = $c(1.10)$). For bGMYC we sampled a set of 100 trees of posterior distribution of the BEAST runs using bGMYC package (Reid & Carstens, 2012) in R platform, following the settings recommended by the authors.

The other used methods were bPTP and mPTP (Zhang *et al.*, 2013; Kapli *et al.*, 2017) which, different to the above analysis,

used the number of substitutions to establish relationships within and between species. Thus, it assumes that the number of substitutions between species is greater than the number of substitutions within species. The analysis requires a non-ultrametric tree, which was generated using MrBayes v.3.1.1 (Huelsenbeck *et al.*, 2001; Ronquist & Huelsenbeck, 2003), from the 10 million MCMC and a burn-in of 25%. The bPTP method was performed using an online server (<http://species.h-its.org/ptp/>), using 400,000 MCMC generations with a thinning value = 100 and burn-in = 25%. The mPTP was also conducted in an online server (<https://mptp.h-its.org/#/tree>).

To understand the diversification processes of *P. saltatrix* MOTUs, the divergence times were estimated among the MOTUs. For this, we added a specimen of *Acanthocybium solandri* (Cuvier, 1829) as an external group to our dataset (Betancur-R *et al.*, 2013). Ultrametric Bayesian topology calibration was performed using two approaches: the first used a fossil (Purdy *et al.*, 2001) and the other used the mutational rate of the COI marker in *P. saltatrix* (1.2% per site per million years – Miralles *et al.*, 2014b). For this analysis, we used the HKY + G + I substitution model, relaxed clock with a normal distribution and Birth and Death model as a prior tree. The fossil record used to calibrate the topology was described from the 'Yorktown Formation' in Lee Creek Mine, NC, USA, which it is dated between 4.8 and 2.8 million years ago (Ma) (Hobbs, 2009). Since the fossil was described as *Pomatomus saltatrix*, we used it as a Most Recent Common Ancestor – MRCA – of *Pomatomus* (crown-group). For this calibration point, we implemented an exponential prior offset to 2.8 Ma (minimum age of the clade), with a mean of 0.7. We performed two independent runs of 600 million interactions, sampled at intervals of 100,000 generations with 25% burn-in. The convergence of parameter values was assessed in Tracer v.1.5 (Rambaut *et al.*, 2009). Following these procedures, the divergence times of the lineages were related to known climatic events, with the aim of elucidating which historical processes were responsible for MOTUs diversification.

Genetic diversity, population structure and demographic history analysis

The genetic diversity indexes (number of haplotypes (h), polymorphic sites (S), nucleotide (π) and haplotype diversity (Hd)) were obtained through DnaSP v.5.1 (Librado & Rozas, 2009). These values were calculated per sample site (oceanic basin) and by previously determined MOTUs. Because we had unequal sampling sizes, we applied a rarefaction method using the *rarefy* function in the R package *vegan* (Oksanen *et al.*, 2018) to provide an unbiased comparison in haplotype richness (Hr) among MOTUs. In the investigation regarding the relationships among haplotypes and their geographic distributions, a haplotype network was built in the software PopART using the TCS method (Clement *et al.*, 2002; Leigh & Bryant, 2015).

Genetic differentiation was tested using pairwise F_{ST} comparisons in ARLEQUIN v.3.0 (Excoffier *et al.*, 2005) and they were calculated using 1000 permutations ($P < 0.05$). The Analysis of Molecular Variance (standard AMOVA), using 1000 permutations ($P < 0.05$), was also performed in ARLEQUIN v.3.0. For this analysis, two structuring hypotheses were tested: (a) all the samples belong to a single unit (null hypothesis) and (b) all molecular units were determined by species delimitation methods. Furthermore, the genetic distances between the molecular units were calculated using the software MEGA X v.10.2.2 (Kumar *et al.*, 2018) employing the Kimura-2-parameter model (K2P; Kimura, 1980).

The Mismatch Distribution analysis was used to estimate expansions or population bottlenecks through DnaSP 5.1 (Rogers & Harpending, 1992; Librado & Rozas, 2009).

Demographic oscillations for each MOTUs were also investigated using a Bayesian Skyline Plot (BSP – Drummond *et al.*, 2005) performed on BEAST v.2.4.7 (Bouckaert *et al.*, 2014), using the same mutational rate as used for the calibration (1.2% per site per million years – Miralles *et al.*, 2014b). The best evolutionary model for each MOTU was determined in jModelTest v.2.1.7 (Posada, 2008), using the Bayesian Information Criterion. All MOTUs were set to evolve by the K80 model, except MOTU I, whose model was K80 + I. Three independent runs of 10 million MCMC interactions, using a burn-in of 25%, were performed for all MOTUs, except for MOTU V, which had four runs, due to chain convergence issues. These runs (log and trees files) were combined using LogCombiner in BEAST v.2.4.7, and the effective sampling size (>200) was checked in Tracer v.1.7.1 (Rambaut *et al.*, 2009) and the BSP reconstructed.

Results

Molecular Operational Taxonomic Units and divergence time estimation

The final alignment of the COI sequences had 570 bp containing 63 polymorphic sites, of which 41 were parsimoniously informative. Insertions, deletions and stop codons were not observed in the final alignment, indicating that pseudogenes were not present, avoiding paralogue analyses.

For the species delimitation analysis based on the traditional distance approach, the estimated OT was 0.0074 (0.74%), equivalent to 4 bp for the dataset. Five MOTUs were identified from this value as the intra- and interspecific limits (Figure 2) which belong, generally, to different geographic locations: (a) MOTU I: Western North Atlantic cold waters, covering the coasts of the USA, Canada and the Gulf of Mexico, (b) MOTU II: Mediterranean Sea and adjacent seas, covering Turkey, Tunisia and part of southern Spain, Eastern South Atlantic, the coast of Namibia, the Indian Ocean and the coast of South Africa, (c) MOTU III: Southern Pacific Ocean (Australia) and Sea of Marmara (Turkey), (d) MOTU IV: South Atlantic, represented by the State of São Paulo (Brazil) and Buenos Aires, Mar del Plata (Argentina), and (e) MOTU V: Caribbean Sea (Venezuela), represented by the State of Nueva Esparta, and the Brazilian South Atlantic, represented by the States of São Paulo and Rio de Janeiro. The ASAP distance method found the same pattern with the lowest ASAP-score (Figure 2).

In analyses based on the coalescent methods, only GMYC corroborated the distance analyses, identifying five units (Figure 2), with the maximum likelihood value (ML = 1485.702) for this model significantly higher ($P < 0.05$) than the null model (ML₀ = 1359.036). On the other hand, the bGMYC model only identified three MOTUs (Figure 2). MOTU A was equivalent to the grouping of the Western North and South Atlantic and Mediterranean Sea, Indian Ocean and South Pacific Ocean (joining MOTUs I, II and III). MOTUs B and C were equivalent to MOTUs IV and V, respectively, identified in previous analyses. For both methods, singletons (MOTUs represented by a single sequence) were not identified. The mPTP method grouped all sequences in one single species. The bPTP showed a lot of singletons (105 singletons), and, therefore, we did not include these results. Our best species delimitation model based on congruence across methods and absence of singletons revealed five MOTUs. Although in IB topology the deep nodes are not supported, the five MOTUs had probabilities over 0.9. Estimates of *Pomatomus saltatrix* divergence time among the five identified MOTUs indicated that the processes of differentiation began in the Pliocene (~3.3 Ma, IC = 2.8–4.37). This showed a dichotomous event, where two clades were formed: (a) Western North Atlantic,

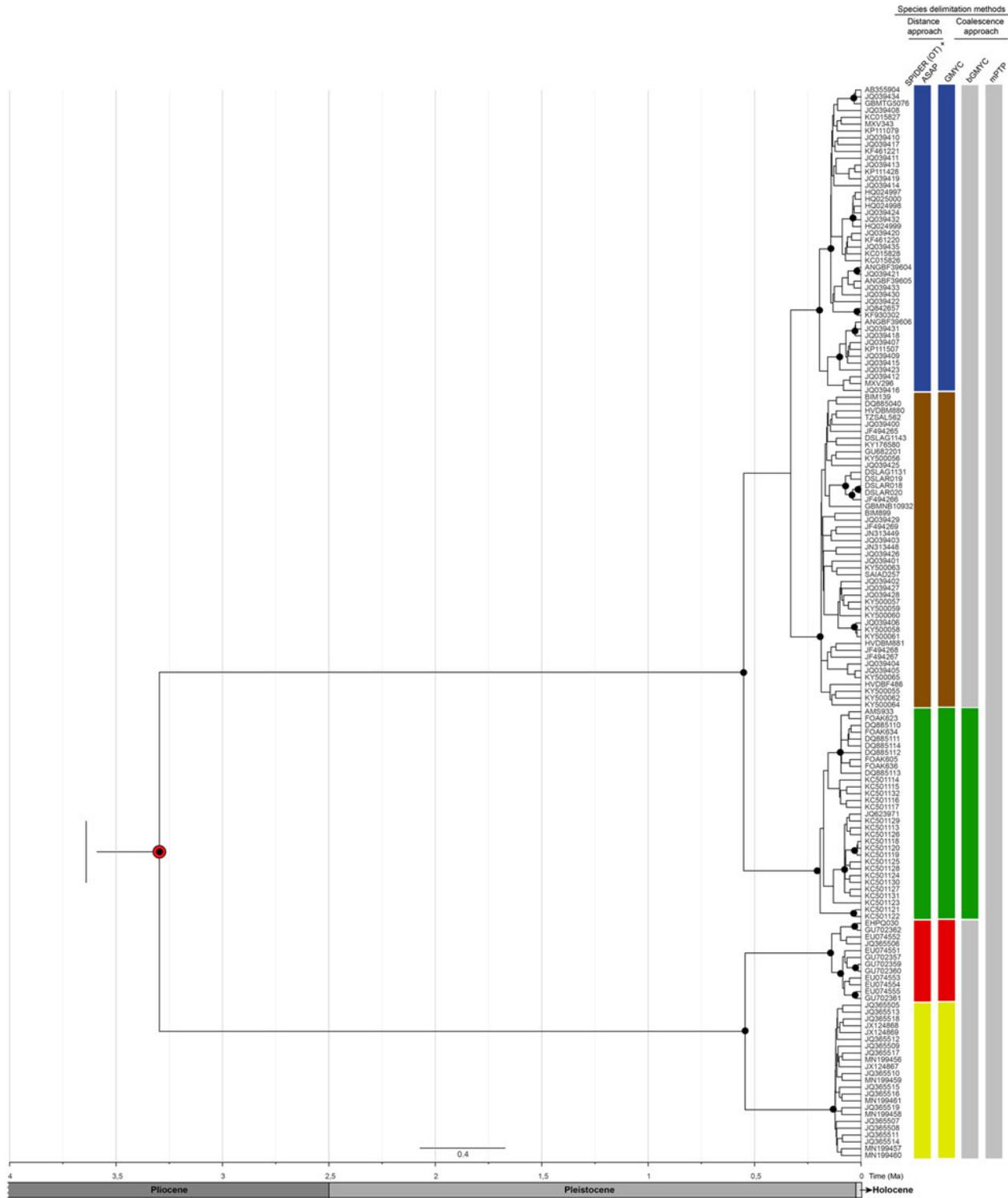


Fig. 2. Bayesian inference calibrated topology of *Pomatomus saltatrix* based on COI sequences and species delimitation approaches based on distance (SPIDER and ASAP) and coalescence (GMYP, bGMYP and mPPT) methods. The different coloured bars represent the MOTUs (for more details about MOTUs distribution, see Figure 1). The black circles represent posterior probabilities higher than 0.9. The red circle represents the calibrated node based on *P. saltatrix* fossil (Purdy *et al.*, 2001).

Eastern South Atlantic, Indian Ocean, Mediterranean Sea, Sea of Marmara and South Pacific and (b) Caribbean Sea and Western South Atlantic.

Genetic diversity, population structure and demographic history

The 157 sequences were composed of 68 haplotypes of which 44 were unique and, in general, the species showed high levels of haplotype and nucleotide diversity (Table 1). The haplotype

richness for all MOTUs was corrected for sample size and evidenced that MOTUs III and V had the lowest rarefaction haplotype richness (Supplementary Figure S2). The rarefaction curves of haplotype richness showed that our sampling was incomplete, especially for MOTUs I, II, and IV. The haplotype network showed that these haplotypes were distributed across five distinct haplogroups (Figure 3; Supplementary Figure S3) equivalent to the identified MOTUs.

The AMOVA considering the existence of a single group, presented a F_{ST} value of 0.73 ($P < 0.05$), demonstrating a greater

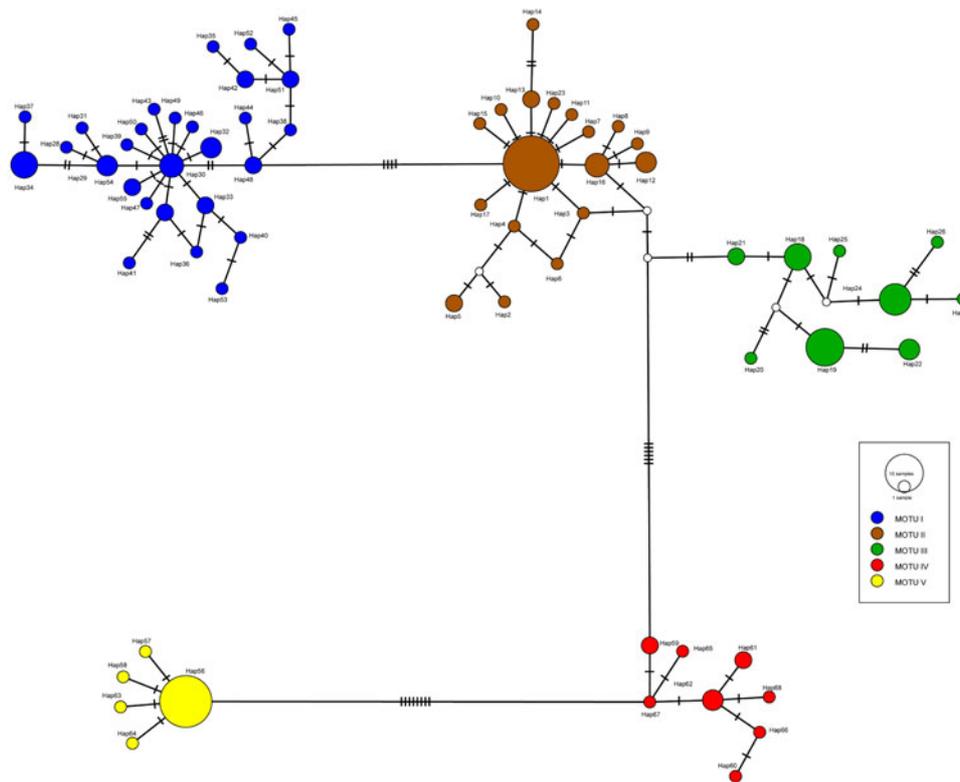


Fig. 3. Haplotype network based on TCS method generated in PopART of *Pomatomus saltatrix*. The circles represent the haplotypes, and different colours represent the MOTUs (for more details about haplotype distribution, see Supplementary Table S1 and Figure S3). Lines between the haplotypes represent the mutation steps and white circles are missing or unidentified haplotypes.

difference between the sample sites (73.04%) (Table 2). Similarly, considering the presence of five distinct units, the analysis presented a F_{ST} value of 0.814 ($P < 0.05$), demonstrating a greater difference between the groups (77.4%) (Table 2). The pairwise F_{ST} between the five MOTUs presented significant values varying between 0.67–0.91, whereas the genetic distances were 1.4–2.86% (Table 3).

Due to the profound genetic differentiation among MOTUs, the demographic analyses were performed one by one, separately. The Mismatch distribution analysis, for both models and units, presented a unimodal distribution, except for MOTU V, with a possible contraction in population size (Supplementary Figure S3). In general, the BSP results agreed with the Mismatch analyses, where MOTUs I and II presented a recent population expansion and MOTUs IV and V showed a stable pattern. Retraction in the population size was identified only for MOTU III (Figure 4).

Discussion

Cryptic diversity, genetic variation and demographic history of Pomatomus saltatrix

The sequences are grouped into a single BIN (AA9142) in BOLD, which consists of a sequence cluster defined algorithmically, based on distance method, in the BOLD database. The high threshold employed by BIN (2.2% – Ratnasingham & Hebert, 2013) merged all sequences in a single MOTU. Despite that, the species delimitation methods (distance SPIDER and ASAP, and coalescence GMYC) and genetic population analyses demonstrated the presence of five molecular units in *Pomatomus saltatrix*. This evidence, therefore, sustains our hypothesis that this species, although distributed worldwide, comprises different genetic pools, sufficiently different to flag a potential complex of cryptic species.

The bGMYC identified that the Western North Atlantic (MOTU I) and part of the Mediterranean Sea (MOTU II) formed a single molecular evolutionary unit. However, Miralles *et al.* (2014b) and Pardiñas *et al.* (2010), using a similar approach involving data from two mitochondrial markers (Cytb and COI), found a clear separation between these locations. As well as the other analysis, MOTU III was recovered and morphometric data suggest differentiation between the Sea of Marmara (part of MOTU III) and the Mediterranean Sea (part of MOTU II) (Turan *et al.*, 2006), despite mPTP recovery only one general unit. The results obtained by Miralles *et al.* (2014b) and Pardiñas *et al.* (2010) are similar to those found in our analyses using the distance methods and GMYC, which indicated three distinct units: MOTUs I, II and III. Nevertheless, the pairwise F_{ST} values were very high and significant between the five defined units for the other methods used in our analyses, suggesting a high genetic-evolutionary differentiation and the possible absence of gene flow. Gene flow has been recognized as one of the main mechanisms which determines how populations evolve in an independent way (Slatkin, 2018). Thus, the comparison of all the data obtained here with data found in the literature, reinforces the genetic-evolutionary structure of five distinct molecular units in *P. saltatrix*.

As COI represents the most conservative gene region in the mitochondrial genome, it may not be sensitive to intraspecific events (same species, but different populations), especially recent ones (Hebert *et al.*, 2003; Satoh *et al.*, 2016), being more suitable for molecular species identification or cryptic diversity. These events are highly interconnected, since cryptic diversity can be originated from distinct populations that have spent a long time isolated, resulting in gene flow loss (Mayr, 1893; Fišer *et al.*, 2018), which seems to be the case of *P. saltatrix*. Therefore, despite the COI marker not being ideal for phylogeographic approaches, inferences can and should be made about the

Table 1. Genetic diversity indexes for COI marker of *Pomatomus saltatrix* for the sampling locations (ocean basins) and the five MOTUs identified by distance and GMYC methods

| Ocean basins/MOTU | Initials | N | H | S | h | π |
|------------------------|---------------------|-----|----|----|-------|--------|
| Western North Atlantic | WNA | 39 | 27 | 26 | 0.97 | 0.007 |
| Gulf of Mexico | GM | 4 | 4 | 4 | 1 | 0.0035 |
| Mediterranean Sea | MES | 25 | 14 | 14 | 0.91 | 0.0032 |
| Sea of Marmara | MA | 22 | 6 | 11 | 0.75 | 0.004 |
| Black Sea ^a | BS | 1 | 1 | – | – | – |
| Eastern South Atlantic | ESA | 4 | 1 | 0 | 0 | 0 |
| Indian Ocean | IO | 15 | 6 | 5 | 0.65 | 0.0025 |
| South Pacific | SP | 10 | 4 | 5 | 0.533 | 0.0017 |
| Caribbean Sea | CS | 6 | 3 | 2 | 0.6 | 0.0012 |
| Western South Atlantic | WSA | 29 | 11 | 17 | 0.73 | 0.009 |
| MOTUs | MOTU I | 45 | 28 | 27 | 0.97 | 0.0064 |
| | MOTU II | 46 | 18 | 18 | 0.77 | 0.0023 |
| | MOTU III | 31 | 9 | 12 | 0.83 | 0.005 |
| | MOTU IV | 12 | 8 | 7 | 0.92 | 0.003 |
| | MOTU V | 23 | 5 | 4 | 0.32 | 0.0006 |
| | Global ^b | 157 | 68 | 62 | 0.96 | 0.016 |

N, Sample size; H, Haplotype number; S, Polymorphic sites; h, Haplotype diversity; π , Nucleotide diversity; MOTU, Molecular Operational Taxonomic Unit.

^aSince the Black Sea has only one sample, the genetic diversity parameters could not be calculated; ^bThe final database was composed by 157 samples, with two without geographic information.

Table 2. AMOVA for COI marker of *Pomatomus saltatrix* considering all samples in one unit (null hypothesis) and the five units identified by distance and GMYC approaches (MOTUs)

| Group | One unit | Five units |
|----------------------|----------|------------|
| Variation source (%) | | |
| Between groups | – | 77.34 |
| Between populations | 73.04 | 4.03 |
| Within populations | 26.96 | 18.63 |
| Fixation index | | |
| F_{SC} | – | 0.178* |
| F_{ST} | 0.73* | 0.814* |
| F_{CT} | – | 0.773* |

The italic values represent the major variation font. *Significant values ($P < 0.05$).

mechanisms that lead to diversification of the different units found here.

These five MOTUs established their current configurations in ~0.25 Ma during the Pleistocene glacial cycles (Levin, 2009). The MRCA of *P. saltatrix* dates from the Pliocene (~3.3 Ma, IC = 2.8–4.37), during the global cooling of the planet (Herbert *et al.*, 2016; Karas *et al.*, 2017). At ~3.3 Ma there was cooling in the northern hemisphere and Pacific, and warming in the southern hemisphere (see Karas *et al.*, 2017). This palaeoclimatic event indicates the origin of two lineages of *P. saltatrix*: a more plastic lineage, which adapted to cold and warm environments (hypothetical ancestor of MOTUs I, II and III) and another lineage which was restricted to warmer environments (hypothetical ancestor of MOTUs IV and V). At ~0.3 Ma (Pleistocene; IC = 0.03–0.59), during the preglacial Riss cycle (Levin, 2009), there was an increase in global temperatures which was associated with the other cladogenetic episode detected here (MOTUs I and II).

Table 3. Pairwise F_{ST} differentiation (below diagonal) and mean genetic distance percentages for the K2P model (above diagonal) for the five molecular units of *Pomatomus saltatrix* COI marker identified by distance and GMYC approaches

| MOTU | 1 | 2 | 3 | 4 | 5 |
|-------------|-------|-------|-------|-------|-------|
| 1. MOTU I | – | 1.41 | 1.95 | 2.5 | 2.86 |
| 2. MOTU II | 0.67* | – | 1.4 | 2.05 | 2.054 |
| 3. MOTU III | 0.70* | 0.75* | – | 2.33 | 2.3 |
| 4. MOTU IV | 0.77* | 0.86* | 0.81* | – | 1.7 |
| 5. MOTU V | 0.84* | 0.90* | 0.84* | 0.91* | – |

*Significant F_{ST} values ($P < 0.05$).

This dichotomy can be related to migrating events to colder waters.

A peculiar relationship between the Sea of Marmara and the South Pacific Australian coast was found in MOTU III. Although having been established at ~0.2 Ma (IC = 0.02–0.432) during the Pleistocene, the MRCA dates to the Pliocene, when the Red Sea had not yet been formed, thereby allowing free passage between these oceans (see Herold *et al.*, 2012). As there is no connectivity between these regions under their current configurations, the pattern found here can be treated as retention of ancestral genetic polymorphisms.

One of the most intriguing findings was the division of *P. saltatrix* along the Western South Atlantic (Brazil and Argentina) in two genetic-evolutionary isolated units (MOTU IV and V), with the possible absence of gene flow between them. This result is also reflected in the high and significant pairwise F_{ST} value (0.914), where the region around the Rio de Janeiro and São Paulo states (south-eastern Brazil) appears to be the possible location of a phylogeographic break. Genetic evidence, combined with distribution modelling, demonstrated that the latitude of ~25°S (coast of Rio de Janeiro/São Paulo) represents a water temperature

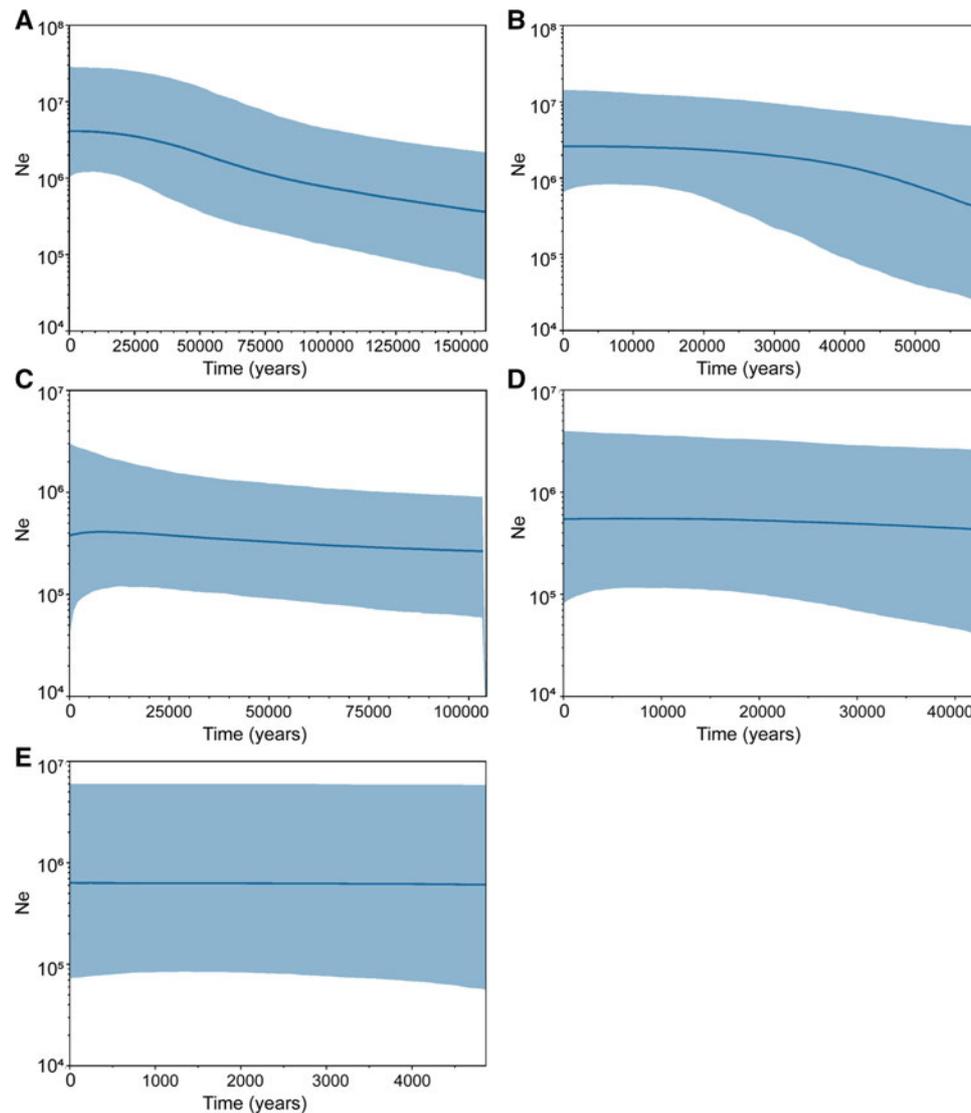


Fig. 4. BSP of each *Pomatomus saltatrix* MOTU. The y axis represents population size, and the x axis the time in years. The blue line is the mean population size \times generation time, and the blue bar corresponds to the 95% confidence intervals. (a) MOTU I, (b) MOTU II, (c) MOTU III, (d) MOTU IV, (e) MOTU V.

transition region, which cools in a southern direction (Acha *et al.*, 2004; Anderson *et al.*, 2017). This is due to the contrast of warm water from the north (Brazilian Current) and cold water coming from the south (the branch of the Antarctic Circumpolar Current that moves northwards along the Patagonian shelf). This water temperature gradient has already been associated with the fixation of local adaptations in various marine organisms that co-exist along the Brazilian coast (*Macrondon ancylodon* – Santos *et al.*, 2006; *Epinephelus itajara* – Benevides *et al.*, 2014; *Chaetodipterus faber* – Machado *et al.*, 2017b; *Mussismilia hispida* – Peluso *et al.*, 2018; *Scartella cristata* – Araujo *et al.*, 2020; *Galeocerdo cuvier* – Andrade *et al.*, 2021), a phenomenon known as environmental isolation (Wang & Bradburd, 2014). Variations in temperature intervals represent factors that can mould the genetic structure of organisms (Crow *et al.*, 2007; Freitas *et al.*, 2017; Hoey & Pinsky, 2018). Therefore, this would explain the split between MOTU IV, which occupies zones of colder waters, and MOTU V, which occupies warmer waters.

Regions with distinct climatic conditions can also create abiotic barriers to gene flow (Nielsen *et al.*, 2004; Crow *et al.*, 2007; Machado-Schiaffino *et al.*, 2010; McKeown *et al.*, 2020). This is observed between the Gulf of Mexico and the Western North Atlantic (location of MOTU I), and the Caribbean Sea and the Western South Atlantic. Although these regions are

near to each other, the sharing of haplotypes between these regions was not observed. The transition between tropical and temperate climates, from the region of the Caribbean Sea is a known barrier of gene flow for many marine species (DeBiase *et al.*, 2016; Nunes *et al.*, 2017; Mattos *et al.*, 2019), even for long distance migratory species such as *P. saltatrix*.

The close relationship between the Caribbean Sea and the Atlantic Ocean (MOTU V) represents a paradigm break. The Amazon River Plume is known as a gene flow barrier between Caribbean and Brazilian regions (Araujo *et al.*, 2020), due to its ever more intense modification of the marine landscape (Floeter *et al.*, 2008; Luiz *et al.*, 2012; Gouveia *et al.*, 2019). However, there is an extensive rich coral reef corridor in the twilight zone at the Amazon River Plume, which allows the transit of species, connecting the Caribbean and the South Atlantic (Moura *et al.*, 2016; Liedke *et al.*, 2020). *Pomatomus saltatrix* could, therefore, overcome this barrier possibly using this route of deeper marine waters, which would explain the genetic-evolutionary lack of differentiation of MOTU V.

Corroborating the data found here, phenomena of evolutionary diversification in the marine environment, due to Pleistocene climatic oscillations, have been documented in the literature (Hofreiter & Stewart, 2009; Stewart *et al.*, 2009; Gaither & Rocha, 2013; Ludt & Rocha, 2015; Piñeros & Gutiérrez-Rodríguez,

2017; Domingues *et al.*, 2018; Chen *et al.*, 2020). Furthermore, the profound differentiation found for *P. saltatrix* can be associated with different environmental conditions which the species experiences in each of the regions where the MOTUs are found (see Juanes *et al.*, 1996). Therefore, these data need to be better evaluated from an adaptive point of view (i.e. RNA sequencing).

The levels of genetic diversity varied between 0.0006 (MOTU V) to 0.007 (MOTU I) and between 0.32 (MOTU V) to 0.97 (MOTU I) for nucleotide and haplotypic diversity, respectively. This parameter is of extreme importance since it represents the potential of populations to evolve and to adapt, in the face of selective pressures (Frankham *et al.*, 2008). According to the IUCN, *Pomatomus saltatrix* is a species classified as 'Vulnerable', whose population size is declining. Despite the high global values found in this study for nucleotide and haplotypic diversity (0.0016 and 0.96), these values can be masked by the profound evolutionary differentiation found in this species. Furthermore, the high genetic diversity values may be characteristic of this species. In general, species with wide distribution, high migratory capacity and long larval periods are characterized by high genetic diversity (Planes, 1988). Thus, as this species presents distinct genetic pools throughout its areas of occurrence, the concern surrounding its conservation should not diminish. This is especially critical for MOTU V, which presents the lowest rarefied haplotype richness compared with the others.

The study of the demographic history of species is extremely important for their conservation, since bottlenecks or population expansions can affect their adaptation potential, as effective population size reflects the magnitude of non-neutral genetic gain fixed by selection (Frankham *et al.*, 2008). MOTUs I and II presented slight population expansions, dating from the Pleistocene at ~160,000 and 60,000 years, during the Riss glacial and Riss-Wurm interglacial cycles, respectively, remaining stable to the present day. MOTU III was the only one to present a slight drop in Holocene population size, at ~5000 years. Besides MOTUs IV remained stable throughout time, and MOTU V presents a pattern that may reflect a population size retraction for Mismatch distribution analysis, caution is needed with the BSP results, since the plain line may not reflect stable population sizes, but rather be due to lack of genetic information (Grant, 2015). The Pleistocene was a period marked by temperature and water level fluctuations (Chappell & Shackleton, 1986; Adams *et al.*, 1999; Reis *et al.*, 2013) and for some marine species, glacial and interglacial cycles have been associated with the expansion and diversification of effective population sizes (Díaz-Viloria *et al.*, 2012; Da-Silva *et al.*, 2015; Souza *et al.*, 2015). Thus, these results reinforce a scenario of palaeoclimatic oscillations influencing the population history of several marine species.

Conservation implications

Species delimitation approaches and knowledge about the distribution of genetic diversity, are important tools for the definition of management units (Batista, 2010; Zhao *et al.*, 2018). Despite being one of the most conserved mitochondrial regions, the COI gene recovered a deep and ancient diversification, revealing five molecular units and flagging potential cryptic species within the nominal species *Pomatomus saltatrix*. Although we reinforce the necessity to include more robust data (e.g. multiloci approach and/or integrative taxonomy), for now, we suggested to consider these molecular units for management. For conservation, the species is divided into six stocks, but not all are formally managed (Carpenter *et al.*, 2015): (1) USA, (2) Brazil, (3) North-east Atlantic and Mediterranean, (4) Eastern Central Atlantic, (5) Angola and South Africa and (5) Australia. Not all these groups correspond to those identified by the present data.

At a global level, there are some conservation initiatives for this taxon. Populations in the USA are managed by the Atlantic States Marine Fisheries Commission and Mid-Atlantic Fishery Management Council, which only consider the existence of the stock found in this region (Carpenter *et al.*, 2015). The data obtained here support these management strategies, since all the populations in this region form a single molecular unit (MOTU I). The use of molecular information has been used to aid such strategies in the management of marine species (Mamet *et al.*, 2019; Nykänen *et al.*, 2020). In South Africa, the initiative involves interruption in the capture of species coinciding with the spawning period, as well as the implementation of a minimum size of fish that can be captured (30 cm; Carpenter *et al.*, 2015). Additionally, in this region, the establishment of a marine protected area has been recommended (Dunlop & Mann, 2012). The association of these data from the literature with that obtained in this study, which defined MOTU II, allows for the observation that the establishment of a marine protected area in South Africa would favour the maintenance of that genetic stock found on the west and east African coasts, as well as the stock in the Mediterranean Sea. Since these regions were defined here as belonging to the same genetic-evolutionary unit, there is a need for congruence in the management forms in these regions.

On the Australian coast, the current management plan includes a definition of minimum sizes that can be fished (between 23–35 cm), and temporary pauses in fishing during spawning periods, to avoid the capture of individuals which have not yet reached sexual maturity. These strategies have already demonstrated positive effects (Broadhurst *et al.*, 2012; Schilling *et al.*, 2019). However, in these regions, fishing is mainly artisanal and for sporting purposes (Juanes *et al.*, 1996; Ceyhan *et al.*, 2007) and it has already been demonstrated that this exploitation strategy is also detrimental to stocks (Bender *et al.*, 2014; Giglio *et al.*, 2015). However, despite the regulations described above, MOTU III (Sea of Marmara – Turkey + Southern Pacific – Australia) was the only one to present a reduction in effective population size in this study. Thus, the combination of all this evidence draws attention to the exploitation of this evolutionary lineage, in order to avoid further compromising its effective population size.

In the Sea of Marmara, which represents the largest catch volumes in the Mediterranean basin, the fishing regulations are not respected, as individuals smaller than 25.5 cm (age of first maturation) are captured (Soykan, 2019). This, therefore, can potentially over-exploit this resource (Cengiz *et al.*, 2013; Ulman, 2014). In the Australian region, Juanes *et al.* (1996) previously demonstrated that a low catch (less than 1000 tonnes year⁻¹) would reflect a small population size. Corroborating these data, this stock showed the second lowest rarefied haplotype richness, reflecting the need for attention surrounding the maintenance of the evolutionary potential and effective population size of this stock. Furthermore, they indicate that the state of 'Least Concern' by the IUCN in the Mediterranean basin (Bizsel *et al.*, 2011), should be viewed with caution.

In Brazil, the two molecular units found should be managed as different stocks. The MOTU V, which presented the lowest genetic diversity values, based on the rarefied haplotype richness, covered two Brazilian marine ecoregions (Figure 1) that suffer from intense human-related disturbance pressures (Magris *et al.*, 2020). Despite the commercial value of this species in Brazil and the evidence of significant declines in capture rates in the region (Haimovici & Krug, 1996; Pauly *et al.*, 2020), this species is not managed (Carpenter *et al.*, 2015). However, a reduction in general capture force and in the capture of juveniles has already been recommended (Silvano & Begossi, 2010) to maintain population renewal cycles. Thus, considering this scenario of low genetic diversity with high exploitation rates in an ecoregion suffering from

environmental disturbance pressure, it can be predicted that the rapid depletion of the stock in this region is very critical.

Final remarks

Considering the results obtained, we concluded that *Pomatomus saltatrix* has five Molecular Operational Taxonomic Units. These five units represent potential candidates to be cryptic species. However, it is necessary to combine this information with other markers using a multi-loci approach, to confirm this hypothesis. Additionally, an integrative approach is also necessary, comparing molecular, morphological and ecological data aiming to obtain a more robust result for the taxonomic status of the species. If this species is considered as one single species, these units should not be managed as one set, due to the profound degree of divergence between them, with high F_{ST} values which indicate the apparent absence of gene flow and high genetic distances. For future management plans, greater attention should be given to MOTUs III and V, especially the populations from Brazil and Turkey. These populations do not have robust management plans, in addition to their MOTUs presenting the lowest rarefied haplotype richness and shallow signals of population contractions.

Furthermore, the results reinforce the influence of Pleistocene oscillations of the sea level on the structure of marine populations and contribute to the break in the panmixia paradigm in the marine environment, reinforcing that more phylogeographic histories, especially in species that are widely distributed, ought to be investigated.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0025315422000236>.

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