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PRIMARY RESEARCH PAPER

Different speciation processes in a cryptobenthic reef fish from the Western Tropical Atlantic

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Abstract The saddled blenny *Malacoctenus triangulatus* is a widely distributed species of cryptobenthic reef fish that occurs from the Caribbean to southeastern Brazil, including the oceanic islands. Subtle morphological differences have been observed between populations, suggesting some degree of structuring along its distribution, especially between insular and coastal environments. In this study, we conducted phylogeographic analyses of *M. triangulatus* based on mitochondrial (cytochrome oxidase I and cytochrome b) and nuclear (rhodopsin) genes, including sequences of *M. brunoi*, a closely related species endemic to the oceanic islands of southeastern Brazil. Three highly structured lineages were identified within the *M. triangulatus* complex: one restricted to the Caribbean province probably isolated by the Amazon barrier, and two in the Brazilian province, one in the northeastern oceanic islands (NOI) and another along the coast (including *M. brunoi*). This result indicates that divergent evolutionary processes have driven the evolution of the saddled benny in the Tropical Southwestern Atlantic: an ancient isolation of the NOI lineage during the Neogene and a recent ecological speciation event in the southeastern oceanic islands, which were connected to the coast during Pleistocene marine regressions. Together, these results

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provide insights on the evolutionary patterns and oceanographic barriers in the Western Tropical Atlantic.

Keywords *Malacoctenus triangulatus* · Phylogeography · Species complex · Shallow reef fish · Sea-level fluctuations · Labrisomidae

Introduction

Until the early 1980s, several species of reef fishes were considered to be widely distributed throughout the Western Atlantic (Rocha et al., 2008). Thus, Brazilian marine ichthyofauna was believed to comprise a subset of the Caribbean species that reached their southernmost limit in the southwest Atlantic along the Brazilian coast (Floeter et al., 2001; Anderson et al., 2015). However, despite sharing some of its species with the Tropical Northwestern Atlantic (TNA) province, there is considerable reef fish endemism along the Tropical Southwestern Atlantic (TSA) (Greenfield, 1988; Sazima et al., 1997; Rocha & Rosa, 1999; Floeter et al., 2008). Several studies have recognized the Amazon–Orinoco plume in the North Brazil Shelf (NBS) province as an influential barrier to the formation of pairs of sister species of shallow reef fishes in the Western Tropical Atlantic (Rocha, 2003; Floeter et al., 2008; Rodríguez-Rey et al., 2017).

Despite the increased knowledge about the biogeography and evolution of southwestern Atlantic reef fish (Floeter et al., 2008; Pinheiro et al., 2018), many questions about evolutionary processes and biogeographic patterns remain to be settled, especially regarding cryptobenthic reef fish, which have received far less attention than more conspicuous species due to their cryptic features (Ahmadia et al., 2012; Brandl et al., 2018). In addition, few studies have attempted to delimit inter- and intraspecific lineages using molecular data, which could improve our understanding of species boundaries, population structure, and geographical barriers of Atlantic marine organisms (Turchetto-Zolet et al. 2013).

Malacoctenus triangulatus Springer, 1959, a cryptobenthic fish whose distribution encompasses three biogeographic provinces (TNA, NBS, and TSA, sensu Spalding et al., 2007), is widely distributed in the Western Tropical Atlantic from the Bahamas in the Caribbean to Arraial do Cabo on the southeastern coast of Brazil (Feitoza et al., 2005; Guimarães et al., 2010), including the oceanic islands off northeastern Brazil (Mendes, 2006). However, its close association with the benthos, the low mobility of individuals as adults, its cryptic behavior, demersal eggs, and short larval phase (Brogan, 1994; Depczynski & Bellwood, 2003; Mendes, 2006) may affect the species' dispersal ability, which may lead to genetic structuring among its populations (Hohenlohe, 2004; Cowen & Sponaugle, 2009).

Springer (1959) described *M. triangulatus* from New Providence Island in the Bahamas based on a few differences in color pattern and sets of severely overlapping congeneric morphometric and meristic characters. Later, in a study of phenotypic variation within the species, Springer & Gomon (1975) found subtle differences (especially in the number of lateral line scales) between specimens from two Brazilian localities (Bahia coast and Fernando de Noronha oceanic island). However, the characters analyzed by Springer & Gomon (1975) could not distinguish between the populations of the Brazilian and the Caribbean provinces. Thus, for over 40 years *M. triangulatus* has been recognized as a single entity known to occur across the Western Tropical Atlantic. Nevertheless, Victor (2015) showed that *M. triangulatus* individuals from the TNA exhibit a degree of cryptic genetic diversity similar to that of other regional cryptobenthic species complexes such as the brightly colored complexes of cleaner and sponge gobies of *Elacatinus* (Taylor & Hellberg, 2005) and the well-defined *Starksia ocellata* species complex (Greenfield, 1979), whose species were recognized and named before the advent of molecular techniques. As has already been shown for some marine organisms, to date, no molecular studies have been conducted to test whether the morphological differences observed by Springer & Gomon (1975) between *M. triangulatus* specimens from the TSA are related to cryptic speciation (Santos et al., 2006; Rodríguez-Rey et al., 2017—fish; Barroso et al., 2010—polychaete; Mattos et al., 2018—crustacean), population structure (Santos et al., 2006; Neves et al., 2016; Machado et al., 2017—fish; Rodríguez-Rey et al., 2016; Mattos et al., 2018—crustacean), or phenotypic plasticity (Souza et al., 2015—fish).

On the other hand, *Malacoctenus brunoi* Guimarães, Nunan & Gasparini, 2010, a closely related congener endemic to Trindade and Martin Vaz oceanic islands in southeastern Brazil, was described based on morphological differences (Guimarães et al., 2010) and can be distinguished from *M. triangulatus* mainly by the number of lateral line scales and color pattern. These characters are the same ones identified by Springer & Gomon (1975) and which were attributed to population variation between *M. triangulatus* specimens from the TSA (mainland and oceanic insular shallow reef environments). In addition, based on molecular data, Pinheiro et al. (2017) suggested that *M. brunoi* is a non-monophyletic species that has evolved recently during Pleistocene sea-level changes, when it was intermittently connected with *M. triangulatus* from the mainland.

Thus, our hypothesis is that populations of the *M. triangulatus* species complex are genetically structured in four lineages, one occurring in the TNA and three within the TSA (Brazilian coast, northeastern oceanic islands, and southeastern oceanic islands of Brazil, the latter of which has been described as *M. brunoi*). This genetic subdivision is expected due to the influence of the Amazon–Orinoco plume in separating the TNA and TSA provinces, whereas within the TSA this subdivision is due to the geographic isolation between the northeastern and southeastern oceanic islands relative to the mainland.

Considering that the patterns and processes underlying the genetic distribution of marine organisms in South America are still largely unknown, enhancing our knowledge of evolutionary patterns in the *M. triangulatus* species complex can help understand macroecological patterns and the impacts of geological features on the evolution and diversification of Western Tropical Atlantic species.

The main objective of this study was to investigate phylogeographic patterns in the *M. triangulatus* complex in the Western Tropical Atlantic. The specific objectives were to (1) assess the genetic structure of populations in the *M. triangulatus* species complex across its entire geographic distribution and identify its main genetic lineages; (2) investigate the influence of the Amazon–Orinoco Plume barrier to the genetic structuring of *M. triangulatus*; and (3) evaluate whether the main genetic lineages identified in our study are supported by the morphological differences between TSA specimens (mainland and oceanic insular shallow reef environments) observed by Springer & Gomon (1975).

Materials and methods

Sampling, DNA extraction, amplification, and sequencing

Malacoctenus triangulatus individuals were collected using hand nets or plastic bags during daytime SCUBA and free dives in shallow waters, and occasionally from tidal pools. Samples were obtained between July 2012 and September 2014 at eight localities in the TSA: five along the Brazilian coast and continental islands—Ipojuca, state of Pernambuco [PE] (08°33′S 35°00′W; $N = 18$); Farol da Barra, state of Bahia [BA] (13°00′S 38°32′W; $N = 20$); Abrolhos Archipelago, off Bahia coast [AB] (17°57′S 38°42′W; $N = 20$); Franceses Island, state of Espírito Santo [ES] (20°55′S 40°45′W; $N = 18$); and Arraial do Cabo, state of Rio de Janeiro [RJ] (22°59′S 41°59′W; $N = 20$); and in the three northeastern oceanic archipelagos (NOI)—São Pedro and São Paulo [SS] (00°55′*N* 29°20′W; $N = 16$); Fernando de Noronha [FN] (03°50′S 32°25′W; $N = 19$); and Rocas Atoll [RA] (03°51′S 33°49′W; $N = 16$) (Fig. 1). Sample sizes at each locality ranged from 16 to 20 individuals, totaling 147 individuals (Online Resource 1).

Specimens were euthanized with a eugenol-alcohol solution prior to the removal of a small sample of muscle tissue, which was preserved in anhydrous ethanol. All specimens were deposited in the ichthyological collections of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ) and the Universidade Federal do Rio Grande do Norte (UFRN) (Online Resource 1). Samples were obtained under permits 10806/2011 and 37598-1/2013 issued by the Ministério do Meio Ambiente do Brasil/Instituto Chico Mendes de Conservação da Biodiversidade, Sistema de Autorização e Informação em Biodiversidade. *Malacoctenus triangulatus* samples from Manuel Luis reef [ML] (00°52′S 44°15′W; $N = 5$) (Fig. 1), the northernmost emerging reef along the Brazilian coast (Moura et al., 2016), are the only sample from the NBS, donated by SISBIOTA–MAR, and additional *Malacoctenus* sequences were obtained from GenBank, including sequences of *M. triangulatus* from six localities in the TNA: Belize [BZ]

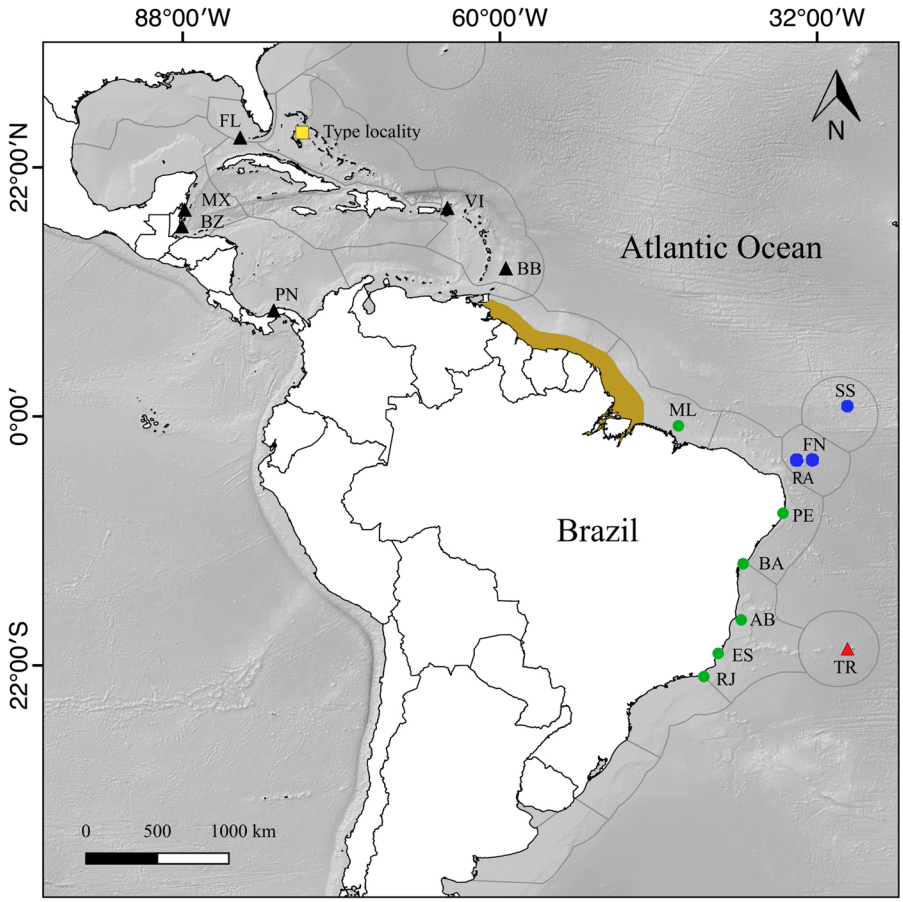

Fig. 1 Sampled sites along the distribution of the *Malacoctenus triangulatus* species complex. Yellow square = type locality (Bahamas); triangles = GenBank sequences; and circles = sampled sites on the Tropical Southwestern Atlantic. Sampling sites and their acronyms: Tropical Northwestern Atlantic (TNA clade—black triangles)—Belize = BZ, Mexico = MX, Barbados = BB, Virgin Islands = VI, Panama = PN, and Florida = FL; northeastern oceanic islands (TSA-NOI clade—blue circles)—São Pedro and São Paulo Archipelago = SS, Fernando de Noronha = FN, and Rocas Atoll = RA; Brazilian coast (TSA coast clade—green circles + *M. brunoi*—red triangle)—Manuel Luis reef = ML; Ipojuca, Pernambuco = PE; Farol da Barra, Bahia = BA; Abrolhos Archipelago, Bahia = AB; Franceses Island, Espírito Santo = ES, and Arraial do Cabo, Rio de Janeiro = RJ. Gray lines indicate marine ecoregions (Spalding et al., 2007). Brown area indicates the Amazon–Orinoco Plume barrier (Rocha, 2003)

(16°48′*N* 88°04′W; *N* = 6), Mexico [MX] (18°16′S 88°50′W; *N* = 2), Barbados [BB] (13°05′S 59°28′W; *N* = 3), Virgin Islands [VI] (18°25′*N* 64°35′W; *N* = 3), Panama [PA] (09°22′S 82°57′W; *N* = 5), and Florida [FL] (24°38′S 82°55′W; *N* = 1); and sequences from *M. brunoi*, a closely related species from the TSA, from Trindade and Martin Vaz Island [TR] (20°30′S 29°19′W) (Fig. 1, Online Resource 1).

Genomic DNA was extracted from muscle tissue samples of each specimen using a modified salting-out protocol (Rodríguez-Rey et al., 2016). Two fragments of mitochondrial DNA (cytochrome oxidase I [COI] and cytochrome b [Cytb]) and one fragment of nuclear DNA (rhodopsin, Rho) were amplified using the following primers: COI—Fish F2 and Fish R2 (Ward et al., 2005), Cytb—CytbGobyF (5′ GTG ACT TGA AAA ACC ACC GTT G 3′) and CytbGobyR (5′ AAC AAA AAG TAT CAT TCG GGC TTG ATG 3′) the newly designed primers, and Rho—Rod-F2w and Rod-R4n (Sevilla et al., 2007).

Amplification reactions included 1 µl of DNA, 10 µl Taq Master Mix (Promega®), 5 µl Miliq water, and 2 µl of each primer in a final volume of 20 µl. All reactions were performed with an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C,

and 1 min of extension at 72°C; and a final extension of 5 min at 72°C. Both strands of the PCR products were sequenced in an ABI 3730xl capillary sequencer (www.htseq.org). The forward and reverse sequences obtained were edited using Seqman 5.01 (DNASTAR Inc., http://www.dnastar.com), aligned by the ClustalW algorithm implemented in MEGA 6.0 (Tamura et al., 2013), and checked manually for misalignments. The phase of diploid nuclear sequences was reconstructed using PHASE (Stephens et al., 2001) as implemented in DnaSP 5.10 (Librado & Rozas, 2009), using default settings and including only allelic states with 100% probability. All haplotype sequences were deposited in GenBank (Online Resource 1).

Phylogenetic analyses

The phylogenetic analysis was performed by Bayesian speciation birth–death model to allow for inter- and intraspecific variations in the dataset following Ritchie et al. (2016), using BEAST 1.7 (Drummond et al., 2012). The analysis was conducted with a relaxed lognormal clock and uncorrelated substitution rates among branches, using default priors and run for 20 million generations, with sampling every 2.000 generations. The convergence among Markov Chain Monte Carlo (MCMC) was checked using Tracer 1.7 (Rambaut et al., 2018), with the first 15% of trees removed as burn-in, and a consensus tree assessing the posterior probability values of each clade was generated using TreeAnnotator 1.8.1 software (Drummond et al., 2012). For the molecular clock, relaxed mutation rates of 1.5% (COI) and 1.3% (Cytb) per million years were obtained from closely related taxa following Pinheiro et al. (2017). The nucleotide substitution model was selected using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1 (Posada, 2008), which suggested the HKY + G model for the COI and Cytb datasets, and HKY + I for the Rho dataset. The dataset included all the COI, Cytb, and Rho haplotypes of *M. triangulatus* and *M. brunoi*, and additional haplotypes of *Malacoctenus delalandii* (Valenciennes) and *Paraclinus spectator* Guimarães & Bacellar, selected as outgroups (Online Resource 1). Nucleotide divergences were estimated in MEGA 6.0 (Tamura et al., 2013) using the *p*-distance (Nei & Kumar, 2000).

Two species delimitation methods were implemented on the COI and Cytb datasets, the Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) and the Poisson Tree Processes (PTP; Zhang et al., 2013). The ABGD method was calculated using K80 distance in the online version (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html), whereas the PTP method was estimated in the web interface of Bayesian PTP (bPTP) (http://species.hits.org/ptp/, last accessed 26 August 2018), using the estimated maximum-likelihood (ML) trees as the input tree and 5×10^5 MCMC generations.

Phylogeographic analyses

The genealogical relationships among haplotypes were assessed by constructing haplotype networks with PopART 1.7 (Leigh & Bryant, 2015) using the TCS method (Clement et al., 2002). The haplotype networks were individually constructed for each marker (COI, Cytb, and Rho).

The genetic diversity parameters of the NBS + TSA lineages of the *M. triangulatus* complex, including number of haplotypes (H), number of polymorphic sites (S), haplotype diversity (h), and nucleotide diversity (π), were estimated in ARLEQUIN 3.5 (Excoffier & Lischer, 2010).

The population genetic structure of *M. triangulatus* was first estimated by the fixation index (F_{ST}), with 1000 random permutations. Six different a priori hypotheses of population structure were tested using the analysis of molecular variance (AMOVA) on the COI dataset (broader geographical representation). Firstly, using all samples we tested (1) a hypothesis based on the influence of the Amazon–Orinoco plume as a barrier to gene flow of shallow-water reef fishes between Caribbean and Brazilian provinces (TNA/NBS + TSA); next, using samples from the Brazilian provinces (NBS + TSA) we tested the following hypotheses based on (2) the major clades recovered in the phylogeny analyses (SS + FN + RA/ML + PE + BA + AB + ES + RJ + TR); (3) the six ecoregions sampled (SS/FN + RA/ML/PE/BA + AB + ES + RJ/TR) as proposed by Spalding et al. (2007); (4) the five subprovinces (SS/FN + RA/ML + PE/BA + AB + ES + RJ/TR) as proposed by Pinheiro et al. (2018); (5) the geographical discontinuity between the coast and all oceanic islands (SS/FN + RA/ML + PE + BA + AB + ES + RJ/TR); (6) the geographical discontinuity between the coast and the northeastern oceanic islands, despite

their intermittent connectivity caused by repeated exposure of seamounts between Trindade and Martin Vaz Islands and the Brazilian coast (SS/FN + RA/ML + PE + BA + AB + ES + RJ + TR) as reported by Pinheiro et al. (2017). The F_{ST} values and AMOVA were calculated in ARLEQUIN 3.5 (Excoffier & Lischer, 2010).

In addition, to infer multilocus genetic structure in the NBS + TSA lineages of the *M. triangulatus* species complex, Bayesian assignment tests were performed using both mitochondrial (COI and Cytb) and nuclear (Rho) genes in the R package GENELAND (Guillot et al., 2005; R Development Core Team 2006). The following parameters, ten independent runs, 300,000 Markov chain Monte Carlo iterations of K from 1 to 10, sampling all 300 iterations, and the first 300 samples removed as burn-in, were used to determine the most probable number of populations and detect possible spatial genetic breaks among lineages without previously assigning individuals to any cluster.

Results

Main lineages in the *Malacoctenus triangulatus* complex

We obtained 570-bp sequences for COI from 77 individuals in the *M. triangulatus* complex, resulting in 75 polymorphic sites and 35 haplotypes; 662-bp sequences for Cytb from 132 individuals, with 87 polymorphic sites and 70 haplotypes; and 464-bp sequences for Rho from 36 individuals, with seven polymorphic sites and six phased alleles (Online Resource 2).

Phylogenetic analyses of COI and Rho markers showed congruent topologies forming three major geographical lineages: one comprised the samples from the TNA in the Caribbean (TNA clade), which may be referred to as *M. triangulatus* sensu stricto and forms the sister group of a group that split into two clades, one comprising the samples from the northeastern oceanic islands (NOI) (TSA–NOI clade) and the other comprising the samples from the Brazilian coast including ML samples from NBS province and samples of *M. brunoi* (represented only by COI sequences) from the southeastern oceanic islands (TSA coast + *M. brunoi* clade) (Fig. 2a, c).

Similarly, results of the phylogenetic analyses of Cytb sequences from the TSA were consistent with the analyses of COI and Rho sequences, recovering the same two lineages in the TSA (TSA–NOI and TSA coast + *M. brunoi* clades) (Fig. 2b). The Cytb gene also included *M. brunoi* sequences (not available for Rho). Surprisingly, neither mitochondrial genes recovered the monophyly of *M. brunoi* as might be expected, considering it is an endemic species to the southeastern oceanic islands (SOI) of Brazil (Fig. 2a, b). The divergence time between samples of the Caribbean (TNA) and Brazilian provinces (NBS + TSA) was approximately 3.5 million years ago (COI) (Fig. 2a), whereas the split between the TSA–NOI and TSA coast + *M. brunoi* clades was approximately 1.6 and 2.1 million years ago for Cytb and COI, respectively (Fig. 2a, b).

The *p*-distance ranged 6.4–8.2% for COI and 1.1–1.5% for Rho between the TNA and NBS + TSA, and 3.6–4.8% for COI, 3.6–5.1% for Cytb, and 0.6–1.1% for Rho between the NBS + TSA clades. For *M. brunoi*, we found low genetic divergence (0–1.6% for COI and 0–1.5% for Cytb) in relation to the remaining haplotypes of the TSA coast clade and high genetic divergence (3.6–4.6% for COI and 3.9–4.8% for Cytb) in relation to the TSA–NOI clade and the TNA clade (6.4–7.5% for COI) (Online Resource 3).

In addition, the two species delimitation methods (ABGD and bPTP) based on COI and Cytb datasets recovered the same two lineages (TSA–NOI and TSA coast + *M. brunoi*) in the *M. triangulatus* complex. The recursive ABGD analysis identified two distinct lineages given a series of prior values from 0.021 to 0.036 indicating that all individuals from the NOI (SS + FN + RA) belong to the same lineage (NOI clade), whereas *M. brunoi* and *M. triangulatus* individuals from the Brazilian coast belong to the same lineage (TSA coast + *M. brunoi* clade) (Fig. 2a, b). Similarly, the bPTP method also identified the same two distinct lineages consisting of the same individuals, with a posterior probability of 0.914 and 0.483 for the NOI clade and 0.978 and 0.992 for the TSA coast + *M. brunoi* clade for COI and Cytb, respectively.

Conversely, the ABGD and bPTP methods for the COI dataset showed contrasting results for individuals from the Caribbean province (TNA clade). Considering a prior maximum divergence of intraspecific

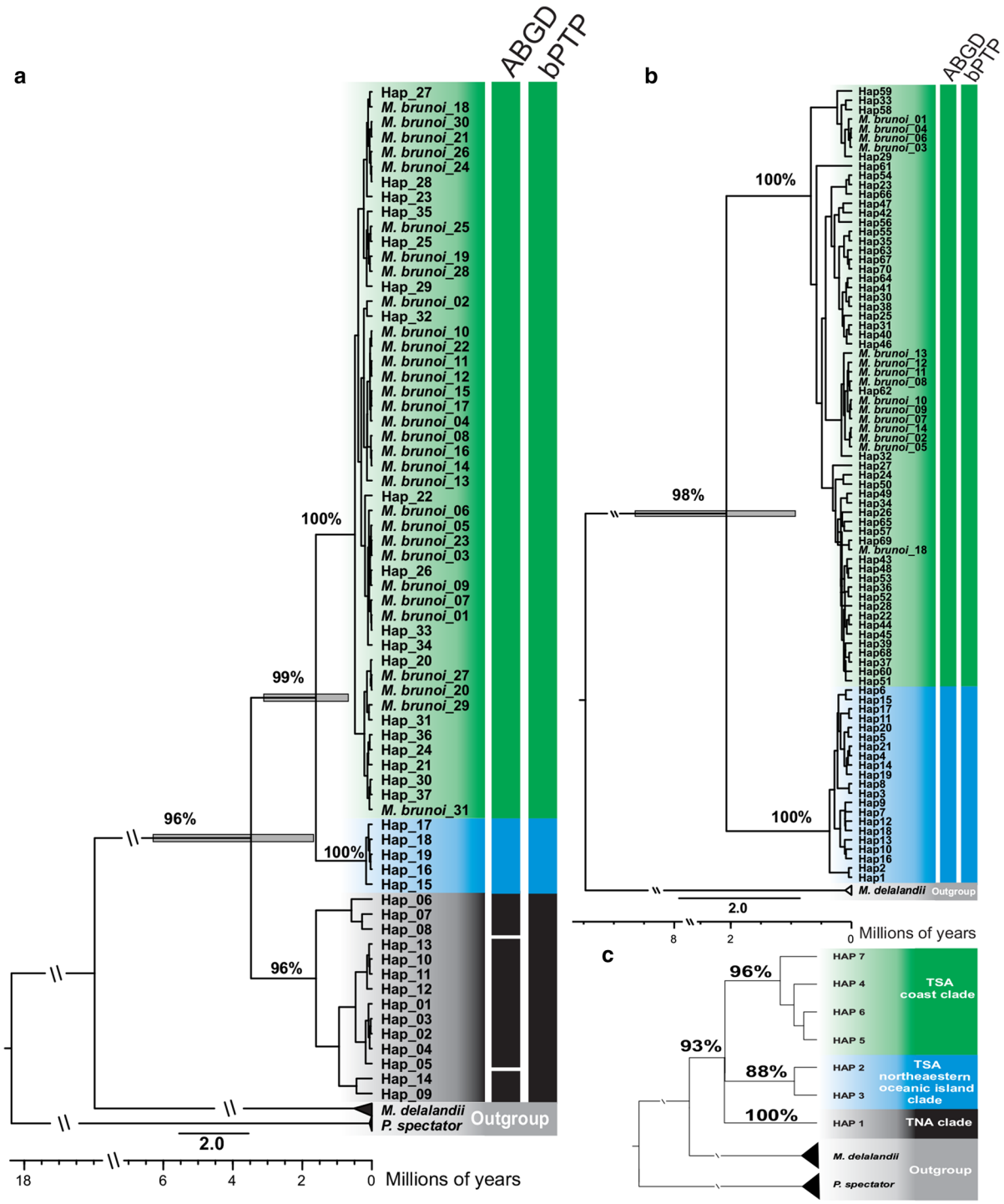

Fig. 2 Bayesian phylogenies of *Malacoctenus* based on COI (**a**), Cytb (**b**), and Rho (**c**) with estimates of divergence times (horizontal bars) and a posteriori probability values for the existence of each clade. The horizontal gray bars indicate 95% credibility intervals for node age estimation. TSA–NOI clade, samples from oceanic islands of northeastern Brazil in blue; TSA coast clade, samples from the coast and from oceanic islands of southeastern Brazil (*M. brunoi*) in green, TNA clade, samples from Caribbean in black; and outgroups in gray

diversity (P) of 0.001–0.0057, the ABGD method indicated that the individuals from the TNA clade belong to three different lineages, whereas the bPTP method indicated that these individuals belong to the same lineage, with a posterior probability of 0.463 for the TNA clade.

The haplotype network showed the lack of shared haplotypes between samples from the TSA–NOI clade and the TSA coast clade for all markers, as well as shared haplotypes for COI and Cytb between samples from the TSA–SOI (*M. brunoi*) and TSA coast clade.

The COI and Rho haplotype networks showed substantial separation between the TNA and TSA clades. In addition, different COI haplogroups were recovered in the TNA clade. A deep separation between the TSA coast clade and the TSA–NOI clade was also observed in all networks, as evidenced by the phylogenetic analyses. In the TSA–NOI clade, an exclusive haplogroup was recovered from São Pedro and São Paulo Archipelago with one (COI) and two (Cytb) haplotypes separated by three or four mutational steps (Cytb), and one step (COI) from the remaining northeastern oceanic islands, Fernando de Noronha and Rocas Atoll. The COI and Cytb networks shared haplotypes between *M. brunoi* and the TSA coast clade and also had exclusive haplotypes in all localities. For COI, *M. brunoi* shared four haplotypes with all localities of the TSA coast clade (except RJ), whereas for Cytb a single haplotype was shared with three localities of the TSA coast clade (PE, BA, and RJ) (Fig. 3).

The pairwise F_{ST} values revealed significant differences between the TSA–NOI localities and all other localities (F_{ST} = 0.904–0.978), between SS and the other NOI islands (F_{ST} = 0.686–0.921), between RJ and three other coastal localities (ML, PE, and ES) (F_{ST} = 0.173–0.338), and between Trindade and Martin Vaz Islands and the extreme coastal localities of Arraial do Cabo (RJ) and Manuel Luis reef (F_{ST} = 0.219–0.244) (Table 2).

The AMOVA performed with the COI dataset showed statistically significant results for all hypotheses tested; however, when all samples were included, most of the genetic variation (K = 2, Φ_{CT} = 67.76%) was distributed between groups (TNA and NBS + TSA), providing support for the Amazon–Orinoco barrier hypothesis. The populations within the TNA and NBS + TSA provinces also showed geographical structuring, although a smaller percentage of genetic variation was distributed among populations within groups (Φ_{SC} = 26.72%). Indeed, within the NBS + TSA provinces, the genetic variation was best explained by partitioning groups into major clades (K = 2, Φ_{CT} = 89.11%), followed closely by geographical discontinuity B (K = 3, Φ_{CT} = 88.81%), which recognized São Pedro and São Paulo Archipelago (SS) as a distinct group from the remaining TSA–NOI clade, while grouping *M. brunoi* within the TSA coast clade (Table 1). Differences between populations within groups accounted for 1.44–3.46% of the genetic variation, whereas within-population variation was $\leq$ 16.48%.

In addition, the multilocus GENELAND analysis performed with NBS + TSA clade samples suggested two genetically different groups. K = 2 has the highest posterior probability in all the ten runs (46.3–55%), consistent with the phylogenetic TSA–NOI and TSA coast clades (K = 2, SS + FN + RA/ML + PE + BA + AB + ES + RJ + TR). Within the TSA coast clade, the Manuel Luis reef (ML) is less likely to belong to the cluster (0.5%), probably due to the low number of samples from this locality (n = 3) (Fig. 4). The analysis also identified *M. brunoi* as belonging to the same group of samples from the coast of Brazil.

Discussion

The phylogeographic analyses of the *Malacoctenus triangulatus* species complex revealed the presence of at least three lineages along the Western Tropical Atlantic, one in the Caribbean province (TNA) and two closely related lineages in the Brazilian provinces (NBS + TSA). However, the biodiversity of the group in the TNA province may still be underestimated, as suggested by Victor (2015) who found a similar degree of cryptic genetic diversity when comparing two other regional cryptobenthic reef fishes species complexes, whose species were recognized and named before the development of molecular techniques.

Of the two Brazilian lineages (NBS + TSA clade), one is restricted to the northeastern oceanic islands (TSA–NOI clade) and the other is distributed along the Brazilian coast (including ML from NBS) and southeastern oceanic islands (TSA coast + *M. brunoi* clade). Even though the coast of Brazil is a highly heterogeneous environment influenced by different marine currents and numerous freshwater and

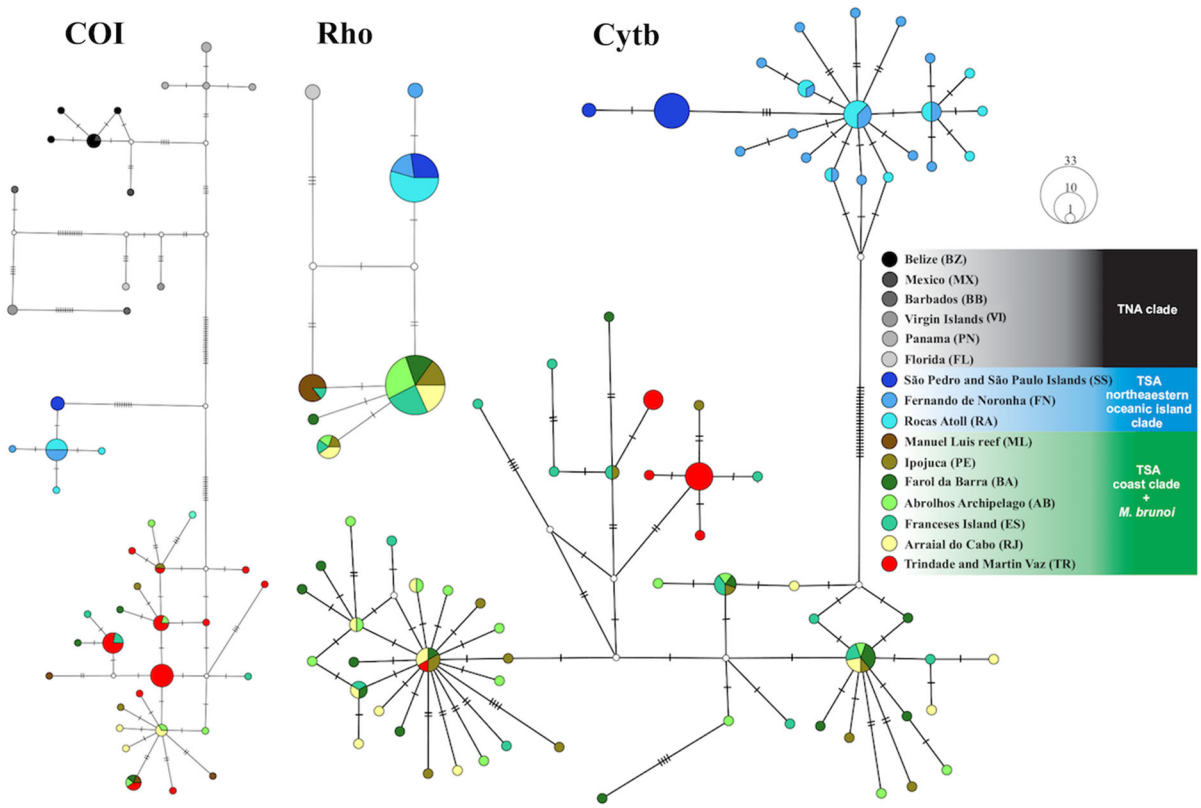

Fig. 3 Haplotype networks obtained from TCS analysis using 95% probability of parsimony for mtDNA (COI and Cytb) and nuDNA (Rho) from specimens in the *Malacoctenus triangulatus* species complex from the Western Tropical Atlantic. Each haplotype is represented by a circle, with the size of the circle proportional to haplotype frequency

Table 1 Analysis of molecular variance (AMOVA) evaluating different hypotheses for mtDNA cytochrome c oxidase subunit I (COI) in the *Malacoctenus triangulatus* species complex

Hypothesis (number of groups)	Group composition	ΦCT	ΦSC	ΦST
All samples				
Amazon barrier (2)	TNA/NBS + TSA	67.76*	26.72*	5.53*
Within the Brazilian provinces (NBS + TSA)				
Major clades (2)	SS + FN + RA/ML + PE + BA + AB + ES + RJ + TR	89.11*	1.52*	9.31*
Ecoregions (6)	SS/FN + RA/ML/PE/BA + AB + ES + RJ/TR	80.77*	2.75	16.48*
Subprovinces (5)	SS/FN + RA/ML + PE/BA + AB + ES + RJ/TR	80.12*	3.46*	16.42*
Discontinuity A (4)	SS/FN + RA/ML + PE + BA + AB + ES + RJ/TR	81.36*	2.99	15.65*
Discontinuity B (3)	SS/FN + RA/ML + PE + BA + AB + ES + RJ + TR	88.81*	1.44*	9.75*

Location acronyms are provided in Fig. 1. Details of the hypotheses are presented in the text. Discontinuity A refers to isolation between the coast and oceanic islands, whereas discontinuity B refers to the connectivity between Trindade and Martin Vaz Islands (TR) and the coast caused by exposure of seamounts

*$P < 0.05$

sediment flows, overall, no genetic structure was evident from our data over its extension. The only exception was for the southernmost locality (RJ), which showed a weak sign of structuring in relation to other localities (ML, PE, and ES) (Table 2).

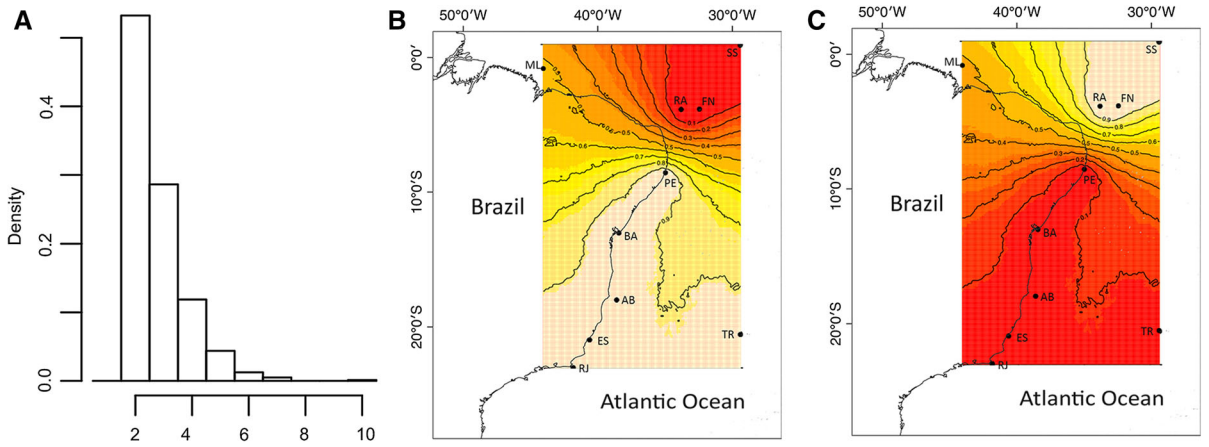

Fig. 4 Bayesian cluster analysis output from GENELAND. **a** The main histogram shows the frequency of inferred K-value across runs. **b–c** Maps of posterior probabilities for belonging to one of $K = 2$ clusters for samples of the *Malacoctenus triangulatus* species complex from the Brazilian provinces; **b** TSA coast with TSA–SOI and **c** TSA–NOI. The axes indicate latitude and longitude. The black dots correspond to sampling sites and the location acronyms are provided in Fig. 1

Table 2 Pairwise F_{ST} values for cytochrome c oxidase subunit I (COI) of the *Malacoctenus triangulatus* species complex in the Brazilian provinces (NBS + TSA)

	SS	FN	RA	ML	PE	BA	AB	ES	RJ
FN	0.717*	–	–	–	–	–	–	–	–
RA	0.825*	−0.007	–	–	–	–	–	–	–
ML	0.921*	0.935*	0.935*	–	–	–	–	–	–
PE	0.942*	0.947*	0.950*	0.122	–	–	–	–	–
BA	0.911*	0.929*	0.928*	−0.154	−0.083	–	–	–	–
AB	0.917*	0.930*	0.930*	−0.018	−0.076	0.115	–	–	–
ES	0.894*	0.914*	0.913*	0.165	0.100	0.091	0.167	–	–
RJ	0.978*	0.971*	0.976*	0.173*	0.288*	0.168	0.089	0.338*	–
M. brunoi	0.904*	0.912*	0.911*	0.219*	0.057	0.008	0.086	0.077	0.244*

Location acronyms are provided in Fig. 1

*$P < 0.05$

The influence of the Amazon–Orinoco Plume barrier to speciation of reef fishes

The classic model of allopatric speciation appears to be the most likely explanation for the differentiation of *M. triangulatus* lineages from the TNA and NBS + TSA provinces, with the Amazon–Orinoco Plume likely acting as a geographical barrier. This riverine complex has already been recognized by several authors as an influential barrier to the formation of pairs of sister species of shallow-water reef fishes in the Western Tropical Atlantic (Rocha, 2003, 2004; Bernal & Rocha, 2011).

Thus, using AMOVA with the mtDNA COI dataset we found support for this scenario, with 67.76% of all genetic variance being partitioned between the TNA and Brazilian provinces (NBS + TSA). Moreover, the divergence between *M. triangulatus* lineages from the TNA and NBS + TSA occurred approximately 3.5 Mya (Fig. 2a), shortly after the Amazon river began to flow into the Atlantic in the Neogene approximately 6.8 to 4.5 Mya (Hoorn et al., 2010), supporting the hypothesis of allopatric speciation and the influence of

the Amazon–Orinoco Plume barrier on the speciation of shallow-water reef fishes.

Different evolutionary processes drive the speciation of saddled blenny from the TSA oceanic islands

The *M. triangulatus* species complex is found in all oceanic islands of the TSA, suggesting a greater dispersal capacity than its congener *M. delalandii*, which is restricted to the mainland (Guimarães et al., 2010). The TSA has two important seamount chains which can act as stepping stones for species dispersion between the mainland and four of the five oceanic islands of Brazil: Fernando de Noronha and Rocas Atoll in the northeast, and Trindade and Martin Vaz in the southeast. Despite their similarity, our results suggest that these two groups of seamount chains have played different evolutionary roles in the speciation of the saddled blenny.

Large genetic divergence was observed between the northeastern oceanic islands (FN, RA, and SS) and the mainland lineage, despite the presence of the seamounts of the Fernando de Noronha Chain, which are currently less than 100 m deep (Almeida, 2006) and may have played a critical role in marine evolution of the northeastern oceanic islands, mainly by intermittently providing stepping stones for island colonization during a period of recurrent sea-level changes (Ludt & Rocha, 2015). The large genetic divergence between the northeastern oceanic islands and the mainland lineage can be attributed to an early Pleistocene event of allopatric speciation, which occurred approximately 2.1 and 1.6 million years ago for Cytb and COI, respectively (Fig. 2a, b), probably as a result of the large distance (a minimum 267 km) between the islands and the coast (Campos et al., 2007). The populations of cryptobenthic reef fishes can quickly become isolated from each other by the appearance of new physical barriers to gene flow or the occurrence of long-distance dispersal events that take individuals to distant localities. Moreover, reproductive incompatibility may evolve rapidly due to rapid generation turnover, and barriers to gene flow do not need to be completely impermeable or permanent and can consist of strong or temporally ephemeral surface currents or temporary land barriers exposed during glacioeustatic sea-level fluctuations (Cowman & Bellwood, 2011; Brandl et al., 2018).

In relation to northeastern oceanic islands, small genetic divergence as measured by pairwise genetic distances (*p*-distance: 0.2–0.4% COI; 0.5–0.9% Cytb; and 0.0–0.2% Rho) was observed between two ecoregions (Fernando de Noronha and Rocas Atoll, and São Pedro e São Paulo) despite the large geographical distance ($\sim$ 630 km). Nevertheless, F_{ST} values revealed strong evidence of structuring (Table 2) in these two ecoregions, which are cohesive ecological units that are likely to affect the most sedentary species due to geographic isolation (Spalding et al., 2007). Structuring among the two TSA–NOI ecoregions and the Brazilian coast was also found in the shallow-water reef fish *Entomacrodus vomerinus* (Valenciennes), but the molecular differences of the markers (COI, Cytb, and Rho) were much subtler (Neves et al., 2016) probably reflecting higher larval dispersal capacity.

Conversely, results for both mitochondrial genes (COI and Cytb) indicated haplotype sharing between *M. brunoi* from the SOI and *M. triangulatus* from the coast. These results are consistent with the findings of Pinheiro et al. (2017) who found no evidence of reciprocal monophyly.

Despite the subtle morphological differences that characterize *M. triangulatus* (Springer & Gomon, 1975), a deep genetic divergence was observed between NOI and coastal samples (maximum *p*-distance of 4.8% for COI and 5.1% for Cytb). Meanwhile, this deep genetic divergence was not observed when the coastal samples of *M. triangulatus* were compared to *M. brunoi* from the SOI—where a pattern of low genetic divergence (maximum *p*-distance of 1.6% for COI and 1.5% for Cytb) was found—even though they are recognized as two different species (Guimarães et al., 2010). These findings point to different evolutionary patterns for the *M. triangulatus* species complex in the TSA (Fig. 5; Online Resource 3).

Testing of population structuring hypotheses using AMOVA revealed support for the hypothesis of geographic isolation between the coast and oceanic islands, except between Trindade and Martin Vaz Islands and the coast as reported by Pinheiro et al. (2017). This hypothesis suggests the occurrence of phylogeographic breaks such as the geographic isolation between TSA–NOI and the TSA coast and within the TSA–NOI clade (between ecoregions: Fernando de Noronha and Rocas Atoll, and São Pedro and São

Fig. 5 Morphotypes of the *Malacoctenus triangulatus* species complex photographed in: **A** Punta Cana, Dominican Republic, photo by A. Carvalho Filho; **B** Fernando de Noronha, northeastern oceanic islands, photo by L.F. Mendes; **C** Arraial do Cabo, Brazilian coast, photo by R.M. Dias; and **D** *Malacoctenus brunoi*, Trindade and Martin Vaz oceanic islands, southeastern oceanic islands, photo by F. Lima

Paulo) and, despite their current geographic isolation (Table 1), intermittent connectivity between TSA–SOI and the TSA coast caused by repeated aerial exposure of seamounts during a Quaternary period of sea-level changes (Pinheiro et al., 2017). In addition, multilocus analysis also indicated a lack of structuring between *M. brunoi* and the TSA coast lineage. Similarly, a lack of structuring between the TSA–SOI and the coast of Brazil was also found in the shallow-water cryptobenthic reef fish *Ophioblennius trinitatis* Miranda Ribeiro, 1919 using mitochondrial gene Cytb (Lastrucci et al., 2018), supporting the hypothesis of recent intermittent connectivity between the two regions. However, there was weak structuring among TSA–SOI (i.e., *M. brunoi*) and the coast, with significant values found only at both extremes of the distribution, Arraial do Cabo (RJ) to the south and Manuel Luis reef to the north (Table 2), suggesting that the two lineages (TSA–SOI and TSA coast) are undergoing speciation.

Thus, the conspicuous morphological differences between *M. brunoi* and *M. triangulatus* from the TSA coast appear to be related to phenotypic plasticity in response to environmental or biological differences between the insular and coastal environments. Conversely, cryptobenthic reef fishes exploit a range of niches that are inaccessible to larger fishes, and their ability to use a diverse range of microhabitats efficiently may have enabled the diversification of the lineages (Brandl et al., 2018). Thus, it is possible that a recent ecological speciation event in Trindade and Martin Vaz Islands has provided insufficient time for complete lineage sorting of ancestral polymorphisms. In fact, *M. brunoi* is believed to be one of the latest species to reach the archipelago and may have evolved by ephemeral ecological speciation in the last million years (Pinheiro et al., 2017).

Taken together, our results suggest that the evolutionary diversification of saddled blenny lineages in the TSA oceanic islands has been driven by distinct evolutionary processes, i.e., allopatric speciation in the oceanic islands of northeastern Brazil and ephemeral ecological speciation in the oceanic islands of southeastern Brazil. Additionally, an allopatric speciation event triggered by the Amazon–Orinoco plume might also explain the large divergence between the NBS + TSA and TNA lineages.

Taxonomic implications

Large genetic distances within traditionally recognized species, usually in combination with morphological, geographical, and other subtle differences, have led to the discovery of hidden diversity in various types of organisms and habitats (Bickford et al., 2007). Thus, several species complexes have been found in such unrelated marine organisms as fish (Santos et al., 2006; Rodríguez-Rey et al., 2017), polychaetes (Barroso et al., 2010), and crustaceans (Mattos et al., 2018).

The three main lineages in the *M. triangulatus* species complex (TNA, TSA–NOI, and TSA coast) show deep and consistent differentiation for both mitochondrial and nuclear markers, suggesting that these lineages should have their taxonomic status verified. Considering that the type locality of *M. triangulatus* is in the Bahamas, the nominal species should remain attached to the TNA clade. The molecular differences observed between the TNA lineage and both NBS + TSA lineages results might explain the morphological differences observed by Springer and Gomon (1975) as putative intraspecific variation. Interestingly, these authors had previously suggested possible differentiation in the populations of *M. triangulatus* from Fernando de Noronha (TSA–NOI) and Bahia (TSA coast), both localities investigated in our study. Thus, the differences in the number of lateral line scales and color pattern reflect diagnostic characters that may be useful for the description of a species endemic to the TSA–NOI, whereas the highly variable meristic characters found in many populations from the Caribbean (Springer & Gomon, 1975) may also be attributed to a species complex in the TNA.

The most intriguing pattern was found in the TSA coast clade. Even though our results indicate that the TSA coast lineage cannot be considered a taxonomic entity distinct from *M. brunoi*, the hypothesis of incomplete lineage sorting and ephemeral ecological speciation proposed by Pinheiro et al. (2017) cannot be ruled out. Thus, the delimitation of the coastal lineage is somewhat subjective and largely determined by the adherence of the researcher to one or another school of thought, splitting or lumping. Accordingly, the TSA coast lineage can be considered the same taxonomic entity as *M. brunoi* (lumpers) or an as yet undescribed species based on traditional taxonomy (splitters), the latter of which would agree with our previous hypothesis of four lineages comprising the *M. triangulatus* complex. Assuming there are four lineages would result in two undescribed species for the TSA (coast and NOI), which together with *M. brunoi* from the SOI and *M. triangulatus* from the TNA would comprise the *M. triangulatus* species complex. Further studies based on integrative taxonomic approaches should help improve species delimitation and resolve the taxonomy of this species complex.

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