ANIMAL SCIENCE

Molecular delimitation methods validate morphologically similar species of red snappers (Perciformes: Lutjanidae)

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Abstract: In this study, we tested the taxonomic validation of red snappers species (Southern red snapper *Lutjanus purpureus*; Silk snapper *L. vivanus*; Blackfin snapper *L. buccanella;* and Pacific red snapper *L. peru*) based on comparative analysis, using four methods for species delimitation. These methods were based on either genetic similarity or phylogenetic trees inferred from two mitochondrial (Cytochrome b and D-loop) and two nuclear (Myostatin and S7 introns) markers. On one hand, the genetic results corroborated the presence of four red snapper species, confirming their taxonomic validation despite their remarkable morphological similarity. On the other hand, few incongruencies in the species delimitation methods were observed according to the phylogenetic reconstruction method (maximum likelihood or Bayesian inference) when using. Based on the phylogenetic results, *L. buccanella* should represent a more ancient lineage in relation to the clade that encompasses *L. purpureus*, *L. peru* and *L. vivanus*. The single-locus phylogenetic analysis based on Cytb recovered each the red snapper species as a well-supported clade. Overall, this study provided a DNA-based validation of the traditional morphological taxonomy of red snappers.

Key words: ABGD, GMYC, *Lutjanus*, red snappers, species delimitation.

INTRODUCTION

The morphological identification of several species of *Lutjanus* Bloch, 1790 (family Lutjanidae), popularly known as snappers, is hindered by the overlapping of meristic or morphometric traits, especially during early life stages (Victor et al. 2009). The identification of adult specimens can also be quite difficult due to the overlapping of meristic and morphometric characters. (Allen 1985), as observed within red snappers. The "red snapper complex" are fish species of the family Lutjanidae with commercial importance and comprise species with very similar morphology. They usually have a sympatric distribution, especially those species that occur in the southern western Atlantic. An

exception is *Lutjanus peru*, distributed in the Pacific. (Allen 1985).

The red snappers Southern red snapper *Lutjanus purpureus* (Poey 1866), Pacific red snapper *L. peru* (Nichols & Murphy 1922), Silk snapper *L. vivanus* (Cuvier, 1828), and Blackfin snapper *L. buccanella* (Cuvier, 1828) share remarkable similar morphological traits, being hardly differentiated from each other. Molecular data indicated that *L. peru* is a sister species of *L. purpureus* (Gold et al. 2015), even though both taxa are geographically isolated by the Panama isthmus. The few external features that discriminate both species are related to color patterns, resulting in several cases of misidentification (Allen 1985, Cervigón 1993,

Cervigón et al. 1992). The "red snapper complex" is traditionally formed by *Lutjanus campechanus* (Poey 1860), *L. purpureus*, and *L. vivanus*. Recently, Veneza et al. (2019) recovered a well-supported clade composed of *L. purpureus*, *L. campechanus*, *L. vivanus* and *L. buccanella*, being similar to the classification proposed by Rivas (1966).

Most of phenotypic traits used to identify the species of red snappers are highly similar, such as dorsal and anal fin spines and rays, lateral line scales, and color pattern (Cervigón 1993, Cervigón et al. 1992). In addition, the genetic studies have provided conflicting results (Chu et al. 2013, Gold et al. 2011, Sarver et al. 1996, Veneza et al. 2019). According to Gold et al. (2011), the contradiction between these studies are derived from the utilization of different approaches and incorporation of plesiomorphic traits. Thus, defining the delimitation of these closely related species of red snappers using DNA-based species delimitation methods are important to organize their taxonomic status.

Several methods of species delimitation have been proposed. They are divided into those that identify new potential species within samples without a priori information (discovery) and those requiring that each sample should be putatively assigned to specific lineages (validation) (Ence & Carstens 2011). The tree-based species delimitation methods consider the genealogical information of individuals, being able to discriminate species efficiently (Fujita et al. 2012). The other methods usually rely on genetic similarity thresholds, being widely applied in studies of molecular identification and validation of species, particularly in cases of morphologically similar taxa (Bickford et al. 2007, Hebert et al. 2003).

Recently, DNA based species delimitation methods have been applied to address an old taxonomic problem for species delimitation of red snappers *L. purpureus* and *L. campechanus*. Genetic data showed that these species represent two distinct evolutionary lineages, confirming the morphological delimitation already proposed by traditional taxonomy (Pedraza-Marrón et al. 2019, da Silva et al. 2020). This demonstrates the power of the molecular delineation tool for species, being particularly beneficial to conservation efforts (Carstens et al. 2013, Monaghan et al. 2009).

In general, the red snappers are commercially important being highly exploited across their distribution range. In fact, some species in this group are ranked as vulnerable according to the IUCN criteria (Anderson et al. 2015, Lindeman et al. 2016). In this sense, methods for the identification and delimitation of overexploited species become a reliable tool to regulate fisheries and to provide insights about conservation and management policies.

Because of the remarkable morphological similarity and evidence of close genetic relationship among red snappers, mitochondrial and nuclear molecular markers were used to validate the taxonomic status of red snappers following the classification proposed by Rivas (1966) and Allen (1985), in order to test the interspecific limits among *L. purpureus*, *L. vivanus*, *L. buccanella* and *L. peru*.

MATERIALS AND METHODS
Sampling

A total of 49 specimens were sampled, representing the four target species of this study (12 individuals of *L. purpureus*, seven of *L. peru*, 15 of *L. vivanus* and 15 of *L. buccanella*). Samples were obtained from several commercial seafood sites along the Brazilian coast (Table I). Fish from which samples were taken, were not collected exclusively for this study, specimens were captured by fishermen who provided the

Table I. Number of individuals (N) sequenced according to each genetic marker and sampling locality. The total number of haplotypes per species is shown in parentheses.

Species/Locality	Cytochrome b	D-loop	S7-2 intron	Myo-2 intron
L. vivanus (N = 15)				
Pará	1	-	-	1
Pernambuco	3	4	3	1
R. G. do Norte	2	1	-	3
Bahia	2	1	1	2
Sergipe	7	4	5	3
Ceará	-	1	-	2
Total	(3)	(9)	(10)	(8)
L. buccanella (N = 15)				
Pernambuco	2	3	1	2
R. G. do Norte	1	1	1	-
Sergipe	12	5	6	5
Total	(4)	(9)	(1)	(2)
L. peru (N = 7)				
México	(5)	(7)	(9)	(7)
L. purpureus (N = 12)				
Pará	6	4	5	5
Ceará	3	1	3	4
Bahia	3	1	2	1
Total	(6)	(6)	(17)	(12)

samples before sending the fish to markets. In this way, it was not possible to deposit specimens in a museum.

The nominal species were firstly identified by their morphological features (Allen 1985, Cervigón 1993, Cervigón et al. 1992, Menezes & Figueiredo 1980). Fragments of muscle tissue or caudal fins were excised from fish, stored in microtubes with 90% ethanol at -20°C, and used for analyses.

Ethical statement

All samples obtained in this study were acquired in commercial fish markets from already dead individuals, representing not endangered and or protected species. Therefore, there was no need to apply for a license for collection or approval by the Animal Ethics Committee.

Laboratory Procedures

Genomic DNA was isolated according to Sambrook and Russell (2001) or by using commercial kits (Wizard Genomic-PROMEGA). The genetic markers (mtDNA: D-loop and Cytochrome b (Cytb); nuDNA: S7-2 and Myostatin (Myo-2)) were amplified by Polymerase Chain Reaction (PCR) in 15 µL reaction volumes containing 125 µM of dNTPs, 10x PCR buffer, 3 mM of $MgCl_2$, 5 mM of each primer, about 50 ng of DNA template, 0.5 U of Taq DNA polymerase and ultrapure water up

to the reaction final volume. PCR were carried out under the following conditions: initial denaturation at 95ºC for 3 min; 35 cycles of denaturation at 94ºC for 30 seconds, annealing between 55 ºC and 60 ºC for 45 seconds, and extension at 72ºC for 1 min; a final extension was performed at 72ºC for 5 min. Loci-specific primers were used to amplify the mitochondrial and nuclear DNA regions as described in Table II.

PCR amplicons were sequenced by the dideoxy terminal method (Sanger et al. 1977), using Big Dye kit (ABI Prism TM Dye Terminator Cycle Sequencing Reading Reaction – Thermo Fisher) in automatic sequencer (ABI - Thermo Fisher). In the case of nuclear regions, each individual was sequenced bidirectionally (forward and reverse) to identify putative heterozygotes.

DNA Dataset

After sequencing, sequences were inspected, edited and aligned using BioEdit (Hall 1999). Cases of HIM (Heterozygous Indel Mutations) were resolved by the reconstruction of alleles in the software Codon-Code Aligner (Codon Code Corporation) followed by visual evaluation of electropherograms. The number of polymorphic

sites and the presence of stop codons were verified using the software MEGA X (Kumar et al. 2018).

The gametic phase of heterozygous in the nuclear markers was defined according to the algorithm Phase v.2.0 (Stephens & Donnelly 2003) under the default settings of DnaSP v.5.10 (Librado & Rozas 2009). Only haplotypes with probability values higher than 0.8 were used in the subsequent analyses. The dataset was subjected to a saturation test of evolutionary changes using the software DAMBE v6.4.81 (Xia 2017). The transition vs. transversion graphs indicated no saturation for the markers used here.

Selection of Evolutionary Model and Genetic Divergence

The best-fit model of nucleotide substitution for the present dataset was defined based on the Akaike Information Criterion (AIC) in the software jModelTest 0.1.1 (Posada 2008). Therefore, the following models were selected: TrN+I (Cytb); TrN+I+G (D-loop); HKY+I+G (S7-2) and TPM1uf+I (Myo-2). The p genetic distances within and between species and mean p-distances with K2P model (Kimura 1980) between species were estimated using MEGA X (Kumar et al.

Table II. Primers used for PCR, according to genetic markers in the present study and their respective annealing temperature.

Marker	Primers	Reference	Sequence (5'-3')	Annealing (°C)
Cytochrome b	FishCytbF	Sevilla et al. (2007)	ACCACCGTTGTTATTCAACTACAAGAAC	57
	TruckCytbR		CCGACTTCCGGATTACAAGACCG	
D-loop	Dloop A	Lee et al. (1995)	TTCCACCTCTAACTCCCAAAGCTAG	55
	Dloop G		CGTCGGATCCCATCTTCAGTGTTATGCTT	
Myostatin-2	Mio 2F	da Silva et al. (2017)	GCATCGAGATTAACGCCTTC	60
	Mio 2R		GGCCCTCTGAGATCTTCACC	
S7-2	S7RPEX2F	Chow & Hazama, (1998)	TGGCCTCTTCCTTGGCCGTC	59
	S7RPEX3R		AACTCGTCTGGCTTTTCGCC	

2018). Boxplots were built to represent the intra and interspecific genetic distances using the R package (R Core Team 2015).

Automatic Barcode Gap Discovery (ABGD)

This method relies on the presence of barcode gap in the distribution of pairwise genetic distances among individuals from distinct species (Puillandre et al. 2012). We used the online ABGD version available at https://bioinfo. mnhn.fr/abi/public/abgd/abgdweb.html. This algorithm, and other single-locus methods, were applied to the Cytb data because this marker has been widely used in phylogenetic studies of vertebrates and presents an appropriate level of genetic variance for identifying species (Avise 2000, Farias et al. 2001). Since the methods based genetic similarity are highly sensitive to the threshold of genetic divergence, two Pmax values (0.1 and 0.2) were selected for comparative purposes. The remaining parameters followed the default values.

Generalized Mixed Yule-Coalescent (GMYC)

This is a tree-based method that searches a transition point between coalescent and branching events using a combination of coalescence and speciation models under a maximum likelihood (ML) approach (Pons et al. 2006). Similarly, to ABGD, GMYC is a single-locus method and therefore only Cytb data were used in this analysis. The GMYC requires an ultrametric tree as input. The input tree was obtained in the software BEAST v1.8.0 (Drummond et al. 2005) after 50 million generations with sampling at every 5000 generations based on TrN + I substitution model and lognormal (uncorrelated) model of relaxed clock, and a Yule process as tree prior. The chain convergence was evaluated in Tracer v1.6 or according to graph inspection and ESS (Effective Sampling Size) values. Only convergence with values of ESS above 200 were

considered adequate. Finally, the sampled trees were summarized in the software TreeAnnotator v1.8.0 using a burn-in of 20%. The GMYC was run in the Splits package (Ezard et al. 2014), available in R platform.

Bayesian Poisson Tree Process (bPTP)

This method is similar to GMYC because it also searches a transition point between intra- and interspecific events based on likelihood inferences (Zhang et al. 2013). Nonetheless, the bPTP considers the number of events of branching/speciation, thus modelling the speciation according to this parameter while the GMYC relies on the time of ramification (Zhang et al. 2013). For comparative analyses, we tested the effects of different input trees based on maximum likelihood (ML) or Bayesian inference (BI). A tree based on Cytb data inferred from maximum likelihood under GTR GAMMA model and 10 replicates was built in RAxML v.8.29 and used as input. We generated a MrBayes (Ronquist et al. 2012) tree for bPTP input, using 10^7 generations, sampled every 10^4, and HKY + G model. The BEAST tree used in GMYC was also used as input for bPTP. The analyses were then carried out using the web bPTP server (https:// species.h-its.org/), using 100000 of MCMC and 0.2 of burn-in.

Bayesian Phylogenetics and Phylogeography (BPP)

The BPP was the only multiloci method used in this study. Nuclear markers were not suitable for single-locus approaches (e.g. as we observe when comparing intra-and interspecific distances). In this way, we used these markers (and mtDNA) in BPP analysis. BPP algorithm relies on a Bayesian approach and a multispecies coalescent model, estimating the distribution probability of species delimitation models based on rjMCMC from the alternative species distribution

models compatible with a defined guiding tree (Rannala & Yang 2003, Yang & Rannala 2010). Therefore, the BPP requires a specified guiding tree, as well as an input file with the nominal species as defined by the user and a multiloci dataset (Cytb, D-loop, S7-2 and Myo-2). In this study, we tested the following topology, based on the results of previous studies (Veneza et al. 2019, Gold et al. 2011): (((*L. purpureus*, *L. peru*), *L. vivanus*), *L. buccanella*). The BPP analysis comprised 100,000 generations with sampling at every five generations and using a burn-in of 40,000.

RESULTS

Characterization of the Dataset

We obtained two DNA datasets for either mitochondrial (Cytb and D-loop) or nuclear (S7-2 and Myo-2) markers. After editing and alignment, the Cytb comprised 776 bp with 77 polymorphic sites. The D-loop sequence alignment encompassed 817 bp with 160 polymorphic sites. The intron 2 of S7 and Myostatin-2 genes alignments comprised 716 bp (65 polymorphic sites) and 440 bp (32 polymorphic sites), respectively.

The number of sequenced specimens for each marker per species as well as the number of total haplotypes are shown in Table I.

Molecular Taxonomy and Species Delimitation

The lowest interspecific mean genetic distance (p distance) based on Cytb sequences was 0.019 between *L. peru* and *L. purpureus* and the highest was 0.072 between *L. peru* and *L. buccanella*. In the case of D-loop, the interspecific mean distance ranged from 0.064 between *L. vivanus* and *L. peru* to 0.111 between *L. purpureus* and *L. buccanella*. The mean p-distance ranged from 0.019 between *L. vivanus* and *L. purpureus* to 0.034 between *L. vivanus* and *L. buccanella* for the S7-2

marker. The Myo-2 sequences showed a genetic distance from 0.003 between *L. buccanella* and *L. peru* to 0.012 between *L. vivanus* and *L. peru*. The highest mean interspecific genetic distance values included *L. buccanella*, as observed in three out of the four selected markers, except for the myostatin intron that showed low genetic variation. Therefore, *L. buccanella* should be regarded as the most genetically divergent species of red snappers from the analyzed dataset.

The pattern of intra and interspecific divergence for each genetic (Supplementary Material - Figure S1), revealing that the highest values of genetic distance referred to mtDNA sequences. In this sense, the separation between intra- and interspecific divergence or barcoding gap was more evident in mtDNA, where the lowest interspecific distance was higher than the highest intraspecific distance. Conversely, this pattern was not observed within the nuDNA markers as some values of inter and intraspecific distances were overlapped (Figure S1).

The results from ABGD using Pmax = 0.1 and Pmax = 0.1 were similar, seven and six partitions were observed respectively for these values (Figure S2). When using Pmax = 0.1, the first five partitions in the results indicated four groups (putative species), and the last two partitions indicated two groups. For Pmax = 0.20, four groups were recovered in the first five partitions, and two groups were recovered in the last one.

In GMYC, the single threshold model (-log ML = 434.24) had the best adjustment to the genetic dataset than multiple thresholds. Like ABGD, GMYC results recovered four independent groups corresponding to those previously defined by morphological features (Fig. 1).

A subtle difference was observed in bPTP inference when input trees based on ML or BI methods were used. In the case of the ML tree, five operational taxonomic units (OTUs)
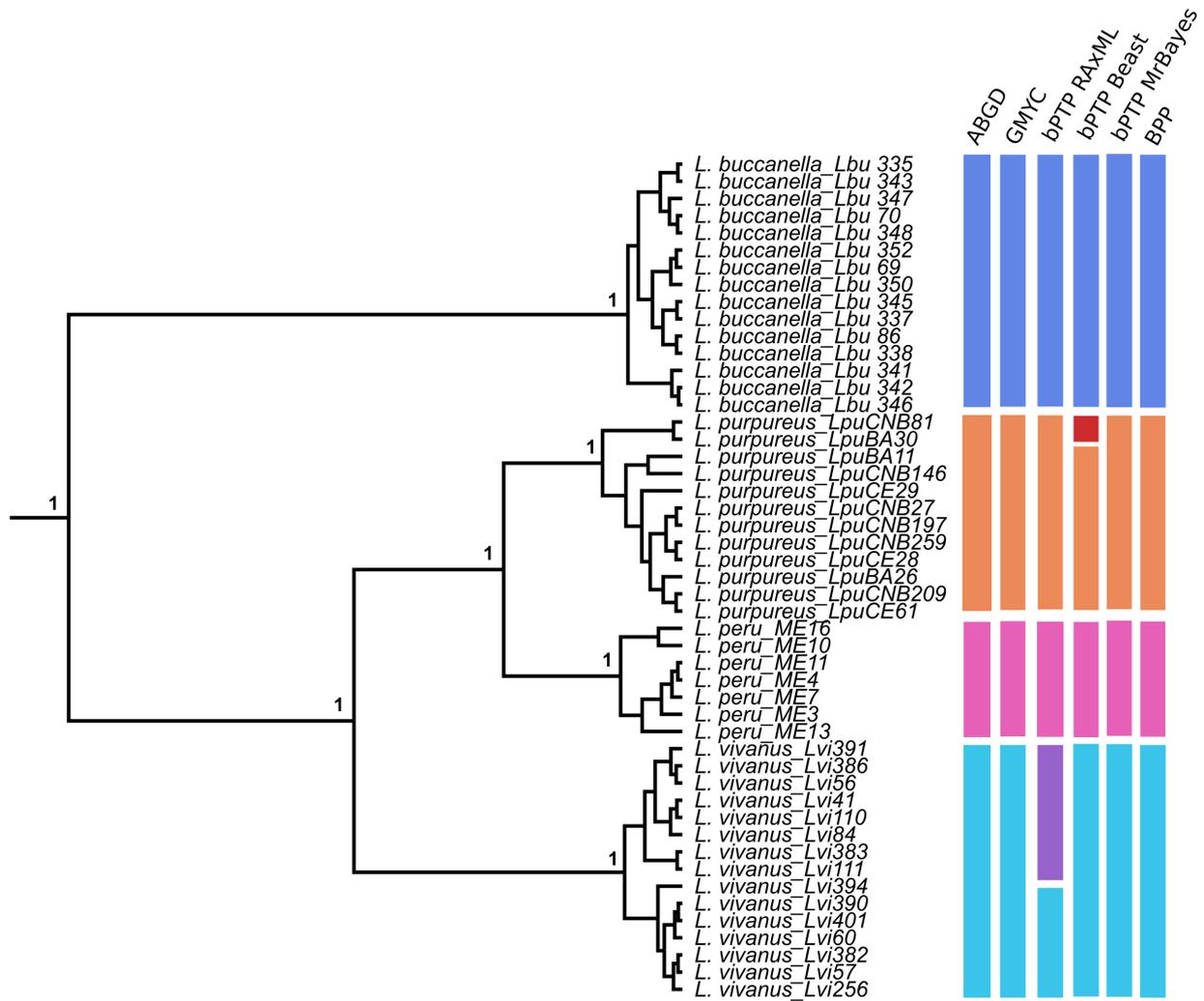
Figure 1. Ultrametric tree inferred from Bayesian inference in BEAST based on Cytochrome b sequences, highlighting species delimitation approaches. The results based on ABGD (four first partitions), GMYC, bPTP (based on input trees inferred from BI and ML approaches) and BPP (multiloci analyses with a mean value of θ) are indicated. The posteriori probability values above 0.9 are shown on each node.

were indicated in the dataset, representing the morphologically identified species, except for *L. vivavus* specimens that were split into two groups. When using a tree inferred with BEAST, five putative species were recovered. Sequences of *L. purpureus* were separated intro two clusters. When an input tree inferred from MrBayes was used, bPTP results recovered four species corresponding to the morphological identification (Fig. 1).

The BPP inferences were based on four models of species delimitation from a guiding tree (((*L. purpureus*, *L. peru*), *L. vivanus*), *L buccanella*). Therefore, collapsing and branching of nodes were possible to occur at only three nodes considered the predefined topology. Accordingly, four OTUs with statistical support above 0.95 were indicated by this approach, being congruent with the taxa established a priori by the traditional taxonomy (Fig. 1). Similar results were obtained using either values of 0 and 1 in the algorithm. The analysis using BPP were congruent with most of the results based on single-locus methods used here.

DISCUSSION

In this study, we evaluated the molecular taxonomy of four species of red snappers (Lutjanidae) from Western Atlantic (*L. purpureus*, *L. vivanus* and *L. buccanella*) and Pacific (*L. peru*) oceans based on single-locus analysis of Cytb and multilocus analysis, comprising both mitochondrial and nuclear genome regions. The genetic data corroborated consistently the taxonomic status of the four species herein analyzed regarding their morphological similarity and shared putatively diagnostic features (Allen 1985, Cervigón et al. 1992).

Mitochondrial DNA in the delimitation of red snappers

When the genetic divergence in mtDNA sequences was compared within and between species, a conspicuous barcode gap became evident, indicating that mitochondrial markers were highly effective for the molecular delimitation of species based on values of genetic distance. However, no barcode gap was observed in the nuclear DNA regions, suggesting that these markers are relatively less informative to discriminate species at least for distance-based methods (e.g. DNA Barcode or ABGD). The higher effectiveness of the mtDNA compared to the nuDNA for the identification of snappers is likely attributed to the low evolutionary rates of nuclear sequences. Moreover, the effective population size of mtDNA is smaller than that of nuDNA, i.e., the coalescence process and the discrimination of lineages in the latter takes longer than in the former (Avise 2000). Accordingly, the mtDNA has been used in several studies for identification of species (Hebert et al. 2003, Ward et al. 2005).

Even though the mtDNA is inherited as a single molecule, distinct regions of the mitochondrial genome show different evolutionary rates. For instance, the high variation in the D-loop region could account for the high interspecific genetic divergence observed in red snappers using this marker. This genetic behaviour has encouraged the utilization of the D-loop region in discriminating or identifying species of teleosteans (Pedrosa-Gerasmio et al. 2012, Wu & Yang 2012).

Red snappers: how many species?

In general, the distinct methods of molecular delimitation of species provided similar results, since most of them delimited the species following the morphological taxonomy. Few exceptions were observed when we varied the method of phylogenetic inference for bPTP input tree.

There is no perfect delimitation method and each one is limited by their specific simulation conditions (Carstens et al. 2013). Therefore, several authors have recommended to combine distinct methods to discriminate species instead of using a single approach. In this study, we integrated different strategies of species delimitation using molecular data to analyze closely related group of four amphi-American species of *Lutjanus*.

Among the tested algorithms, ABGD was the only based on a threshold of sequence similarity. The four recognized red snapper species were supported by this method. Regardless of the differences of ABGD variables, the results were similar and congruent with the morphological taxonomic status of the analyzed snappers. The inferences of independent evolutionary lineages or species based on genetic distance or barcode gap has been criticized because it might lead to biased results (Carstens et al. 2013). This criticism has been related to the inability of this method in incorporating the variance associated with genetic processes (Carstens et al. 2013) besides disregarding the uncertainties of gene trees (Mallo & Posada 2016). Nevertheless,

distance-based approaches continue to be an important tool in biodiversity studies, and have been widely applied in species identification, including studies in lutjanids (Benzaquem et al. 2015, Brandão et al. 2016, Veneza et al. 2018).

All molecular-based species delimitation methods were congruent with morphology-based identification, except for some results of bPTP. The taxonomic status of the four snapper species was further by the bPTP analysis when using the MrBayes inference. But, when a ML input tree was used, bPTP indicated five potential species. However, the RAxML is limited to the GTR model. Thus, the utilization of this single substitution model is likely to affect the estimation of branch lengths during the phylogenetic reconstruction of ML tree. As a result, this could overestimate the splitting in species delimitation analyses, as presently observed within *L. vivanus*. No additional evidence has been reported indicating the presence of distinct population in *L. purpureus* along the Brazilian Coast (da Silva et al. 2016, Gomes et al. 2012), as suggested by bPTP when using a BEAST input tree. It can be concluded that when using a MrBayes tree as input tree for bPTP, the results tend to be more conservative in accordance with morphology-based classification of red snappers, and the other single locus methods applied (GMYC and ABGD).

Our BPP results providing evidence to validate the taxonomic status of the four red snapper in the present dataset. Several studies have successfully used BPP for discriminating species or independent lineages (McKay et al. 2013, Silva et al. 2020). Despite sharing high morphological similarities, and being difficult to identify using morphology, *L. peru*, *L. purpureus*, *L. vivanus* and *L. buccanella* are genetically distinct. Therefore, the present results corroborate the effectiveness of the molecular methods for the delimitation or identification of closely related species, such as red snappers.

CONCLUSIONS

Based on DNA data, this study validated the four species of red snappers analyzed herein. The taxonomic status based on morphology has proved to be completely congruent with the molecular data for these fish group. The few diagnostic morphological traits that have been used by fish taxonomists and the molecular delimitation proved to be useful and informative to provide effective identification of otherwise controversial taxa.

The methods for species delimitation were useful to define the boundaries between the species of red snappers. In addition, some variables such as the phylogenetic reconstruction method affected the delimitation, and the number of groups inferred (bPTP was more sensitive when using Beast or RAxML input trees), leading to minor differences when compared to the other methods used.

As for the comparison between divergence patterns between nuclear and mitochondrial DNA sequences, no clear barcode gap was observed in the former. The intra and interspecific genetic distance values in mtDNA sequences were significantly different, being particularly informative for the molecular identification of species. Thus, mitochondrial markers are more suitable than nuclear markers for the species identification of red snappers.

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the contribution of each author was fundamental for the accomplishment of this work. Danillo Jorge Figueiredo da Silva, the main author of the work, contributed with ideas, generation, analysis and interpretation of data, text production and manuscript preparation; Ivana Barbosa Veneza contributed with the collection of most samples, data generation and manuscript preparation; Raimundo Darley Figueiredo da Silva contributed with the collection of most samples, generation and analysis of data and manuscript preparation; Maria Iracilda da Cunha Sampaio contributed financial support for the collection expeditions of samples, laboratory infrastructure and manuscript preparation; and Grazielle Fernanda Evangelista Gomes contributed with the full orientation to the work, including analysis and interpretation of data, text production, critical review of the manuscript and laboratory infrastructure support.