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Exploring the Diversity of Elopidae (Teleostei; Elopiformes) Using DNA Barcoding Analysis

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Abstract: Elopidae is the most speciose family within the Elopiformes, comprising seven valid species. Despite this reduced number of species, the family presents poorly resolved systematics, mainly owing to its wide distribution and highly conserved anatomic features. Therefore, we aimed to explore the species diversity of the Elopidae using species delimitation, genetic diversity, and phylogenetic analysis combined with DNA barcoding of the *COI* gene. The results from the delimitation analysis grouped the species into a single cluster, while the genetic diversity analysis among the groups showed a distance ranging between 1.29 and 2.78%. Both phylogenetic and haplotype network analysis grouped the species into four clades, associated with the distribution of the organisms. The lack of resolution in the species delimitation analysis might be directly associated with the recent radiation of the group, a hypothesis corroborated by both the low genetic diversity (close to the 2% threshold) and the few mutations that separate the haplotypes observed among the species. Interestingly, our data supported a new arrangement for the *Elops* species. In addition, the data available in public databases present taxonomic errors at several levels. Although some issues remain unsolved, our results can be used in the identification of taxa and provide information to assist taxonomic revisions of the Elopidae.

Keywords: *COI*; genetic distances; species delimitation; recent diversification

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1. Introduction

Elopidae comprises seven valid species in the single genus *Elops* and is the most diverse group within the Elopiformes [1,2]. Elopidae includes marine and estuarine fishes that are found globally in tropical and subtropical regions and are mostly allopatric in distribution, although some species may be sympatric [1–4]. For example, according to Adams et al. [1], *Elops senegalensis* (Regan, 1909) and *Elops lacerta* (Valenciennes, 1847) are sympatrically distributed in the eastern Atlantic, while *Elops saurus* (Linnaeus, 1766) and *Elops smithi* (McBride et al., 2010) are allopatrically distributed in the west, with an overlap of these two species observed in the Gulf of Mexico and southeastern United States. In addition, *Elops hawaiensis* (Regan, 1909) and *Elops machnata* (Forsskal, 1775) are sympatric in the Indo-Pacific, while *Elops affinis* (Regan, 1909) occurs only in the eastern Pacific (Figure 1).

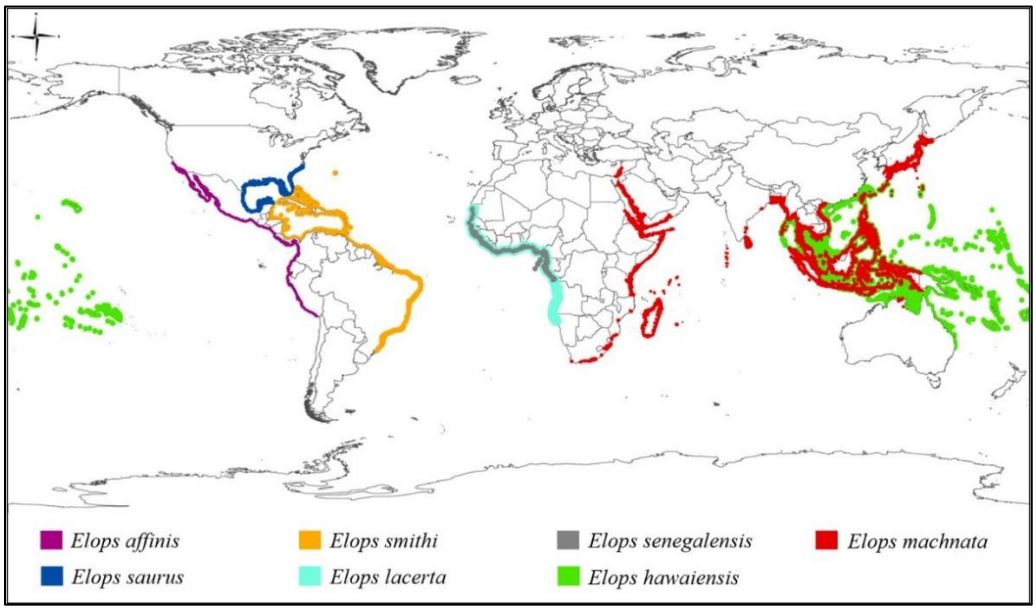

Figure 1. Geographical distribution map of *Elops* species by Adams et al. [1].

The taxonomy of *Elops* species has been the subject of debate in recent years, given their wide distribution and highly conserved anatomical features [3–5]. For several years, diagnostic criteria for these species have been based primarily on anatomical (e.g., number of vertebrae) and molecular data (genetic distance of the cytochrome b- *cytb* gene). However, these diagnostic criteria are subject to problems and inconsistencies, such as the overlap in the number of vertebrae in some species, which can lead to taxonomic errors. In addition, the values for genetic distance based on the *cytb* gene obtained in previous studies vary widely (Table 1) [1–8].

Table 1. Data used as diagnostic characters for *Elops* species, in studies by different authors.

	Number of Vertebrae				**Genetic Distance *Cytb***				**Genetic Distance *COI***		
Species \ Authors	**[7]**	**[8]**	**[3]**	**[5]**	**[3]**	**[5]**	[4]	[8]	[2]	[4]	[2]
E. smithi	–	–	73–80	–	2.3–2.9	0.9–2.2	1–12.1	1.9–2.9	1.8–3.3	–	2.3–2.6
E. saurus	78–79	73–80	79–87	–	2.3–2.9	1.7–2.2	1.3–12.3	1.9–3.2	1.8–3.2	1.4–2.1	1.8–2.6
E. affinis	79	73–80	–	–	–	–	12.0–12.3	2.8–3.2	3.2–3.3	2–2.3	1.8–2.3
E. machnata	63–64	63–64	63–64	63–64	–	–	–	–	–	0.3–2	–
E. hawaiensis	68–69	68–70	68–70	65–67	–	0.9–2.2	1–12.0	–	–	0.3–2.3	–
E. senegalensis	69	63–70	67	–	–	–	–	–	–	–	–
E. lacerta	74	63–70	–	–	–	–	–	–	–	–	–

De Sousa et al. [2] recently presented cytogenetic data as new potential markers for species identification and presented the first DNA barcode analysis for the Elopidae (cytochrome oxidase subunit I—*COI* gene). Although this study has presented new data for species diagnosis of *Elops*, the data were limited to the description of only one new karyotype for *E. smithi* and used *COI* data for only three species of the genus (*E. saurus*, *E. affinis*, and *E. smithi*).

Normally, a 650 bp segment of mitochondrial DNA sequence of *COI* is used to identify individuals by species-level barcoding by estimating interspecific genetic distances [9,10]. This marker is a remarkably effective tool for identifying the species diversity of different fish groups as well as for delimitation analysis of cryptic species, identification

of individuals at different developmental stages, and identification of species and their synonyms [10–12]. Moreover, the proportion of organisms that cannot be distinguished by this marker is estimated to be only about 2.5% [13].

In view of the above, and owing to the molecular information available for the family Elopidae being limited to barcode sequences stored in public sources, we aimed to explore the species diversity of the family Elopidae via DNA barcoding to obtain new information that can contribute to taxonomic classification and a better understanding of the evolutionary relationships of the group.

2. Materials and Methods

2.1. Database Assembly

For all analyses, a database was produced with *COI* sequences of *Elops* and *Megalops* species (Outgroup) obtained from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/, "accessed on 17 July 2022") and Boldsystems (https://www.boldsystems.org/index.php/, "accessed on 18 July 2022") platforms. All sequences were aligned using Muscle software ver. 3.8 [14], and were manually edited following alignment.

2.2. Species Delimitation Analysis

A total of three different methodologies were used for the species delimitation analysis: the ABGD method, where the *COI* sequences were submitted to the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/ "accessed on 24 July 2022") with the following combination of settings: parameter range of Pmin = 0.001, Pmax = 0.1, and gap width = 1.5 for a total of 20 steps using the Kimura 2-parameter (K2P) model.

The bPTP method was performed on the server (https://species.h-its.org/ptp/ "accessed on 24 July 2022") using the default settings and a Bayesian topology was generated using MrBayes software ver. 3.2.7 [15]; and the GMYC method, tested on a specific server (https://species.h-its.org/gmyc/ "accessed on 24 July 2022"), using an ultrametric tree as the input file (as described below).

2.3. Molecular Analysis

For all analyses using the *COI* gene, evolutionary models were selected based on Bayesian information criterion using jModelTest2 software [16]. Bayesian inference (BI) analysis was performed in 10 million generations to obtain the posterior probability values for each of the clades. Furthermore, we performed a Maximum Likelihood (ML) analysis using the IQTREE software [17] with 1000 bootstrap pseudoreplicates. The HKY + G + I evolutionary model was used for both analyses.

To obtain the ultrametric tree, the package BEAST ver. 2.5 [18] was used to obtain the strict molecular clock with the HKY + G + I evolutionary model in a run of 10-million generations, sampling every 1-million generations. The convergence of parameters was visualized using Tracer 1.7.1 [19]. The consensus tree was determined after a burn-in of 10% of the original trees using TreeAnnotator [19]. All topologies were visualized using FigTree ver. 1.4.4 [20].

A haplotype network was constructed based on the topology obtained on the ultrametric tree using Haploviewer [21].

Inter and intraspecific genetic distances were calculated using Mega X software [22] based on the K2P model. The groups formed were based on the clusters obtained from the phylogenetic analysis.

3. Results

A 650 bp fragment was obtained for 97 *COI* sequences available in the databases used. These sequences correspond to seven species, five of which belonged to the genus *Elops* (*E. saurus, E. smithi, E. machnata, E. hawaiensis*, and *E. affinis*), and two species were used as outgroups (*Megalops atlanticus* and *Megalops cyprinoides*) (Table S1).

All three methods employed for species delimitation grouped *Elops* species into a single cluster (Figure 2). The ultrametric tree obtained from the *COI* gene database resulted in the formation of four clades with a posterior probability > 0.9, in which the species *E. machnata* and *E. hawaiensis* (clade *E. machnata+E. hawaiensis*) were grouped into a single clade, the sister group of the clade consisting of *E. smithi*, *E. saurus*, and *Elops* sp. (clade *E. smithi*) (Figure 2). Consequently, *E. affinis* formed a monophyletic group (clade *E. affinis*), a sister group to the clade that included *E. saurus* and *Elops* sp. (clade *E. saurus*) (Figure 2). In addition, the clades formed were directly related to the locality/collection patterns of the sequences analysed (Figures 2 and 3). Although BI and ML analysis showed low posteriori probability and support values, respectively, in most clades, the relationships between clades and tree topology were similar (Figures S1 and S2).

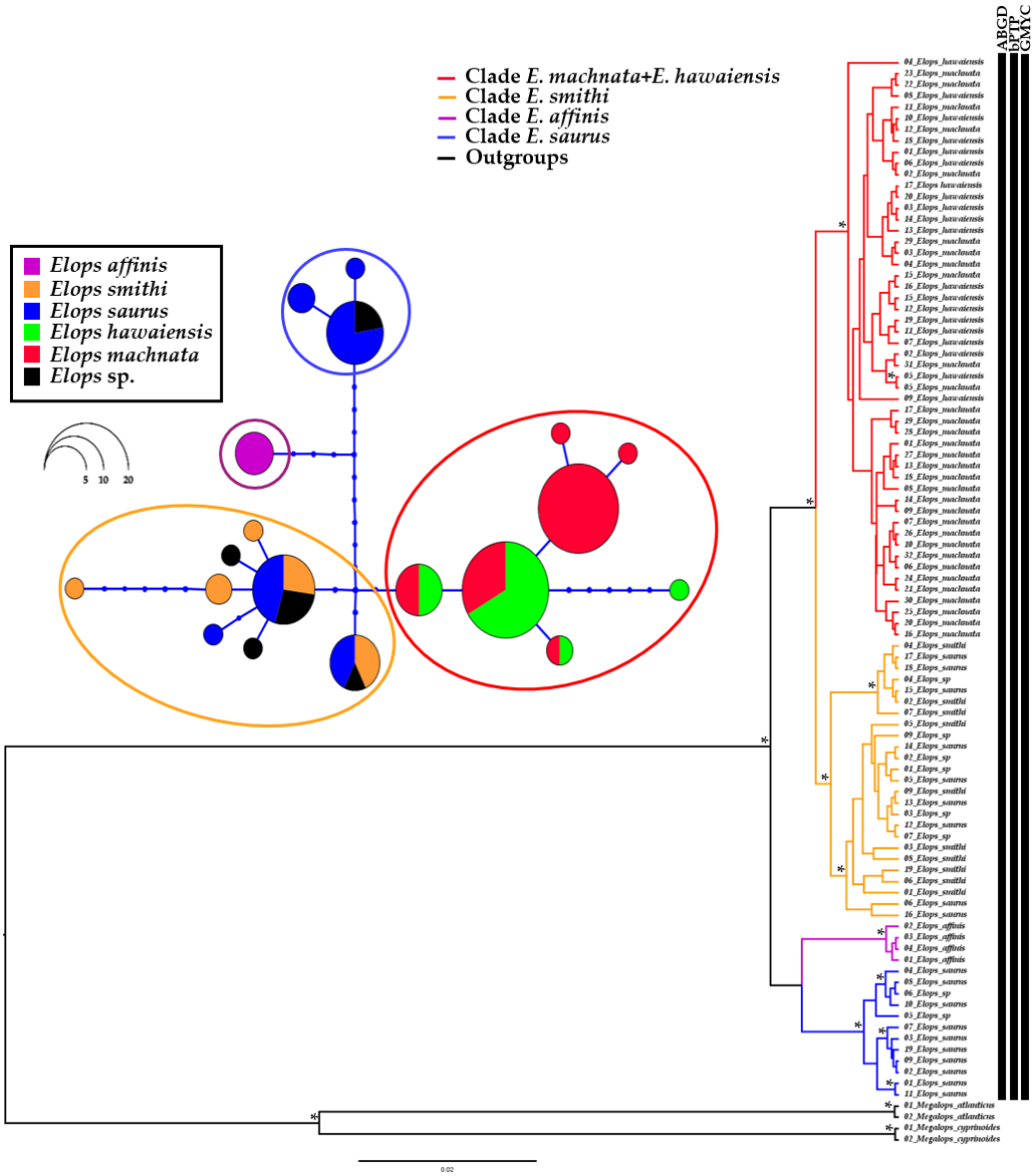

Figure 2. Ultrametric tree and haplotype network representing the relationships of *Elops* species. In the network, each circle represents one haplotype, and the size of the circles is proportional to the haplotype frequency. Color codes represent the species, and the color codes in line of the ultrametric tree represent the clades found in *Elops* species. Vertical bars (on the right) indicate the results of the delimitation analysis based on ABGD, bPTP, and GMYC, respectively. Asterisks (*) at nodes indicate posterior probability > 0.9.

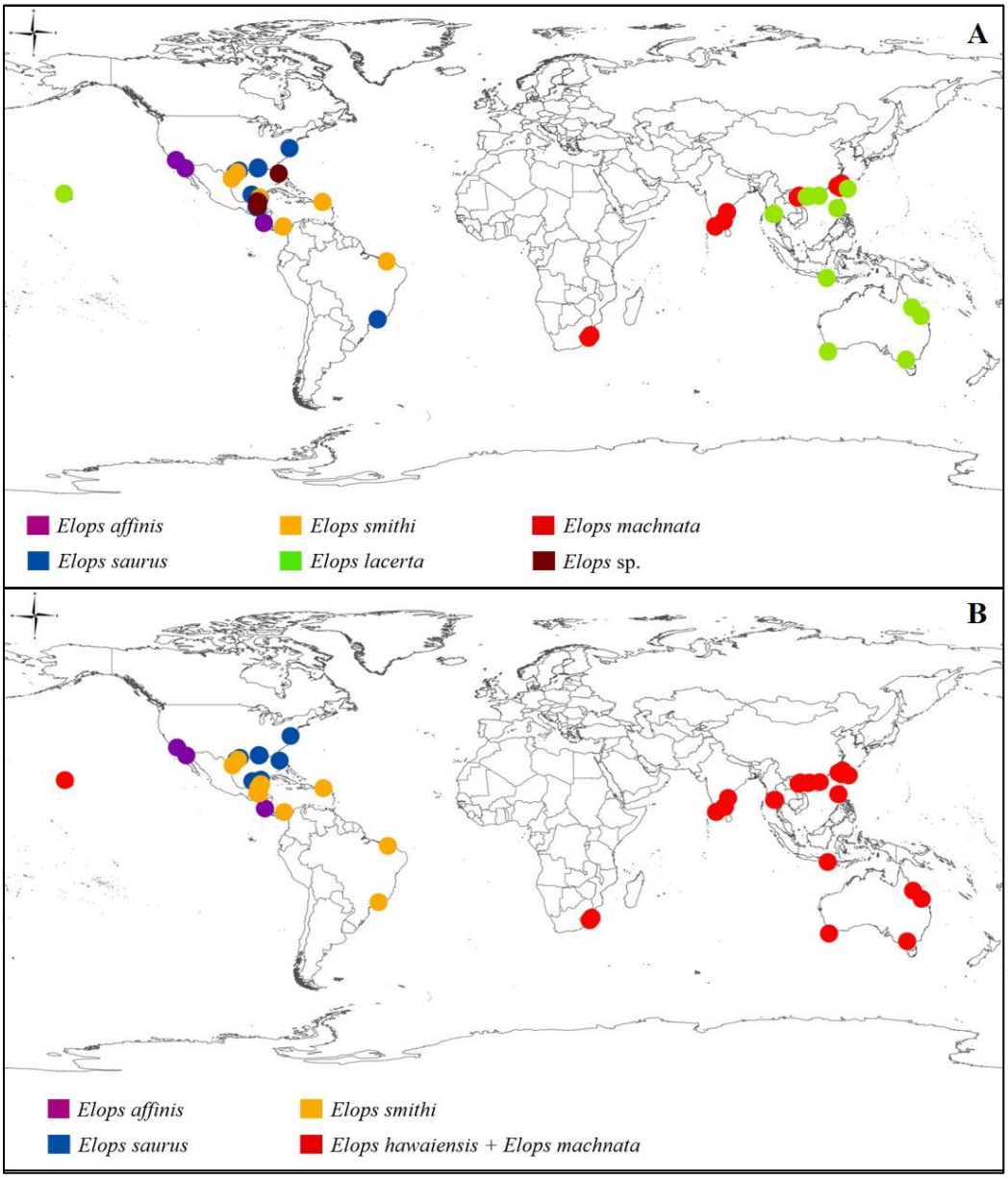

Figure 3. Geographical origin of the sequences of the species used in this study. (**A**) According to the taxonomic description of the databases. (**B**) According to the phylogenetic relationships.

The phylogenetic relationships observed in the preliminary analysis led us to infer that *E. hawaiensis* and *E. machnata* are paraphyletic and strongly related, showing a posterior probability < 0.9 and an intraspecific distance of 0.25%.

In addition, *E. saurus* could also be paraphyletic, as some sequences were grouped in the clade *E. saurus* while the majority of the sequences grouped within the clade *E. smithi*, with an interspecific distance of 2.78% between these clades. Finally, *E. affinis* and *E. smithi* are monophyletic, and it is worth mentioning that taxonomic disagreements were observed among some sequences for *E. smithi*, *E. saurus*, and *Elops* sp. from the databases (GenBank and Boldsystems) (Table S1).

The haplotype network analysis identified 19 haplotypes, directly related to the clades observed in the phylogenetic analyses of the ultrametric tree (Figure 2). Moreover, each clade presented some unique and/or exclusive haplotypes, while others haplotypes were shared among the distinct clades of *Elops* species.

Genetic distances within and between species were calculated based on the clades observed in the phylogenetic analysis. As shown in Table 2, genetic distances within the

groups formed by the *Elops* species ranged between 0 and 0.69%. The genetic distances between clusters of *Elops* ranged from 1.29% (clade *E. smithi* vs. clade *E. machnata*+*E. hawaiensis*) to 2.78% (clade *E. smithi* vs. clade *E. saurus*), whereas those between *Elops* and outgroups ranged from 18.74% (clade *E. affinis* vs. clade *M. atlanticus*) to 22.55% (clade *E. smithi* vs. clade *M. cyprinoides*) (Table 2).

Table 2. Interspecific and intraspecific nucleotide distance (K2P) found for the *COI* gene of Elopidae species. The values are the percentages, and bold indicates values less than 2% (threshold).

Species	1	2	3	4	5	6
1- *E. affinis*	**0**					
2- *E. hawaiensis* + *E. machnata*	2.11	**0.25**				
3- *E. saurus*	**1.83**	2.47	**0.33**			
4- *E. smithi*	2.32	**1.29**	2.78	**0.69**		
5- *M. atlanticus*	18.74	19.41	19.31	19.35	**0**	
6- *M. cyprinoides*	21.54	22.42	21.85	22.55	15.38	**0**

4. Discussion

4.1. Low Genetic Diversity Resulted from Recent Radiation

Elopiformes is one of the oldest lineages of Teleosts, characterized by a leptocephalus larval stage and high anatomical conservatism [3,23]. Studies focused on the taxonomic classification of *Elops* species are limited and report conflicting results. In addition, analysis using DNA barcoding does not fully encompass the diversity of *Elops*. Therefore, it is not surprising that the taxonomy of the species of this genus is problematic, primarily due to anatomical similarities among them [2–4].

COI-based species delimitation methods are widely used in fish studies despite their limitations, which are primarily attributable to the stochasticity of the coalescent process; however, these methodologies have provided remarkably accurate results [24,25]. Nevertheless, the low divergence observed in *Elops* analysed using molecular species delimitation methods indicates that such approaches are ineffective for this particular group.

According to criteria based on DNA barcoding, the low diversity in *Elops* suggests that this group of species could be a single species. However, systematic, anatomic, and biogeographical aspects of the species of this genus have already been discussed in several studies, and the fact that some organisms are geographically isolated (allopatry) contradicts this hypothesis. A recent radiation of species, the occurrence of introgressive hybridization, and rapid evolution and/or genetic drift might be responsible for the low genetic diversity of DNA barcoding in groups of organisms that present well-defined systematic criteria, such as those observed in *Elops* [26–29].

In fact, according to inter and intraspecific genetic distance analysis, the hypothesis of a recent diversification is more plausible, considering that despite the low genetic distance, all clades presented values near or above the threshold for species definition (2%) [9]. Furthermore, the few mutations separating the identified haplotypes reinforce the hypothesis of recent diversification. In the case of the *Elops* species, a recent diversification would explain the lack of resolution in the delimitation analysis, as well as the genetic diversity values observed among the groups.

Low genetic diversity among closely related species is commonly observed in fishes and is frequently found in basal lineages of nonteleostean and teleostean fishes [28–30]. For instance, species belonging to Lepisosteidae and Scombridae present clear morphological and ecological criteria for their taxonomy; however, their molecular distances based on *COI* are approximately 0.9% and 1.4%, respectively [26,31].

Tree-based approaches are valuable tools for visualizing species delimitation [32]. In the case of the DNA barcode, which is commonly used for species identification [29–32], we must emphasize that phylogenetic inferences may be seen with caution. In the present study, the phylogenetic relationships grouped the species in an arrangement that seems

to reflect their geographic distribution, with *E. affinis* being restricted to the west coast of the Americas in the Pacific Ocean, *E. hawainsis* and *E. machnata* on the coasts of the Indian Ocean and eastern Pacific, *E. saurus* on the east coast of North America and part of Central America in the Atlantic Ocean, and *E. smithi* on the coast of South America and part of Central America [1]. In addition, the presence of unique and exclusive haplotypes shared only among the species of the clade reinforces the separation of the species based on their geographic distribution. Interestingly, new information regarding the relationship between *Elops* clades demonstrated that *E. smithi* is phylogenetically closer to the clade formed by *E. machnata* + *E. hawaiensis*, with a genetic distance of <2% between the groups (1.29%). The relationship between the clades *E. affinis* and *E. saurus* agrees with that proposed by De Sousa et al. [2] and suggests that they are sister groups.

4.2. Taxonomic Conflicts among Elopidae Species

The phylogenetic analysis, in combination with the genetic distance between the clades obtained, indicates the presence of some inconsistencies in the taxonomy of the species. The genetic distance values observed in this study are comparable to the data obtained by De Sousa et al. [2]. However, our results differ considerably from those observed by Ramanadevi and Thangaraj [4] (see Table 1), and this discrepancy is possibly a result of the use of taxonomically incorrect sequences and the lack of sequences of particular species, such as *E. smithi*, in the study.

The primary issue in studies on Elopidae is species misidentification [2,3]. For example, sequences that have been assigned to *E. saurus* are observed in the *E. smithi* clade, despite the location of the sample collection being consistent with the occurrence of only *E. smithi* (Figure 3a and 3b). Therefore, the *E. smithi* clade should technically be formed solely and exclusively by *E. smithi*, not including *E. smithi*, *E. saurus*, and *Elops* sp. as observed in our analysis. Such taxonomic problems involving these two species are not new, and De Sousa et al. [2] have already highlighted recurrent misidentification and reported the persistent misclassification of *E. smithi* as *E. saurus*, even in areas where *E. saurus* is not found.

In addition, the existence of contact zones between these two species, and their high dispersive capacity, where some species are capable of migrating to regions where other organisms occur, can also lead to possible systematic problems. For example, there are reports of *E. saurus* in the Mediterranean Sea and Pacific Ocean [33,34]. However, as shown in the distribution of the species (Figure 3b), the possibility of sympatry for these two species exists only on the east coast of Central America, and it is uncommon for *E. smithi* to occur on the east coast of North America and *E. saurus* to be found on the east coast of South America. Therefore, both *E. smithi* and *E. saurus* should be monophyletic species, contrary to what is reported in the literature; these discrepancies probably occur due to the introduction of taxonomic flaws when depositing sequences of these species in databases.

The phylogenetic relationship observed between *E. hawaiensis* and *E. machnata* was quite peculiar, and similar results were observed by Ramanadevi and Thangaraj [4], where both the phylogenetic relationship, the shared haplotypes among both species and the intraspecific genetic distance between the two species, revealed that they should be considered a single species. Although there exists an isolated branch with five samples of *E. hawaiensis* in our ultrametric tree, the lack of relationship by locality, the low support value in this node of the tree, and the low genetic distance of only 0.449% observed between these and other *E. hawaiensis* and *E. machnata* samples indicate that this branch is not strongly supported.

In this scenario, although more robust analyses using different methodologies (molecular, morphological, and ecological) are needed to validate the relationship between these two species, certain evidence, such as the occurrence of these organisms in sympatry, the high morphological similarity, and the molecular data presented herein, indicate that these two taxa might be subspecies belonging to a single and exclusive species.

Therefore, it is essential to clarify the taxonomic status of *Elops* species using analysis that allows the accurate identification of organisms (in terms of morphology, genetic distance, and cytogenetics) before making information available in public databases to avoid errors in future studies that utilize such data and to circumvent inconsistencies between results, such as those observed in the present study.

5. Conclusions

The information obtained using distance methods, haplotype network, and phylogenetic tree constructions led us to infer that *COI* is a useful marker for species classification in the family Elopidae, and is helpful for identifying taxa that require further analysis, such as *E. hawaiensis* and *E. machnata*, which might be synonymous species, to assist in rectifying existing taxonomic errors in databases.

Likewise, our data provided basic information regarding these species, which can represent good comparison models in phylogeographic studies. Furthermore, it is imperative that we reinforce multiloci analysis integrated with traditional taxonomy. This combinatorial analysis might present the most appropriate approach for the delimitation and taxonomy of *Elops* species and will aid in resolving systematic uncertainties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14111008/s1, Table S1: Data on the species analysed in this study, their respective GenBank and Boldsystems accession codes, as well as their geographic origins. Figure S1: Maximum likelihood tree representing the relationships of *Elops* species. Color codes in line represent the clades found in *Elops* species. Asterisks (*) at nodes indicate support values >90%. Figure S2: Bayesian inference tree representing the relationships of *Elops* species. Color codes in line represent the clades found in *Elops* species. Asterisks (*) at nodes indicate posterior probability > 0.9 [35–48].

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