

Article

Building a DNA Reference for Madagascar's Marine Fishes: Expanding the COI Barcode Library and Establishing the First 12S Dataset for eDNA Monitoring

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Academic Editor: Michael Wink

Received: 23 June 2025

Revised: 10 July 2025

Accepted: 11 July 2025

Published: 18 July 2025

Citation: Volanandiana, J.J.A.; Ponton, D.; Ruiz, E.; Fiadanamiarinjato, A.E.M.; Rieuvilleneuve, F.; Raberinary, D.; Collet, A.; Behivoke, F.; Jaonalison, H.; Ranaivomanana, S.; et al. Building a DNA Reference for Madagascar's Marine Fishes: Expanding the COI Barcode Library and Establishing the First 12S Dataset for eDNA Monitoring. *Diversity* **2025**, *17*, 495. <https://doi.org/10.3390/d17070495>

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Abstract

Madagascar harbors a rich marine biodiversity, yet detailed knowledge of its fish species remains limited. Of the 1689 species listed in 2018, only 22% had accessible cytochrome oxidase I (COI) sequences in public databases. In response to growing pressure on fishery resources, this study aims to strengthen biodiversity monitoring tools. Its objectives were to enrich the COI database for Malagasy marine fishes, create the first 12S reference library, and evaluate the taxonomic resolution of different 12S metabarcodes for eDNA analysis, namely MiFish, Teleo1, AcMDB, Ac12S, and 12SF1/R1. An integrated approach combining morphological, molecular, and phylogenetic analyses was applied for specimen identification of fish captured using various types of fishing gear in Toliara and Ranobe Bays from 2018 to 2023. The Malagasy COI database now includes 2146 sequences grouped into 502 Barcode Index Numbers (BINs) from 82 families, with 14 BINs newly added to BOLD (The Barcode of Life Data Systems), and 133 cryptic species. The 12S library comprises 524 sequences representing 446 species from 78 families. Together, the genetic datasets cover 514 species from 84 families, with the most diverse being Labridae, Apogonidae, Gobiidae, Pomacentridae, and Carangidae. However, the two markers show variable taxonomic resolution: 67 species belonging to 35 families were represented solely in the COI dataset, while 10 species from nine families were identified exclusively in the 12S dataset. For 319 species with complete 12S gene sequences associated with COI BINs (Barcode Index Numbers), 12S primer sets were used to evaluate the taxonomic resolution of five 12S metabarcodes. The MiFish marker proved to be the most effective, with an optimal similarity threshold of 98.5%. This study represents a major step forward in

documenting and monitoring Madagascar's marine biodiversity and provides a valuable genetic reference for future environmental DNA (eDNA) applications.

Keywords: fish diversity; DNA barcoding; reference library; eDNA marker taxonomic resolution

1. Introduction

Fishery resources are an indispensable source of income and subsistence in many regions, particularly in developing countries, where nearly 97% of the world's fishers are concentrated [1,2]. Marine resources provide essential protein and income for coastal communities, especially in the southwestern part of Madagascar [3]. In Madagascar, fishing constitutes the primary livelihood for many coastal communities. Due to recurrent droughts, some populations that traditionally depended on agriculture and livestock farming have shifted toward fishing, thereby increasing pressure on marine resources [4–6]. This shift has contributed to signs of resource overexploitation, prompting some fishers to adopt destructive practices such as mosquito net trawling and beach seining, which further accelerate ecosystem degradation. Over the past two decades, this growing pressure has led to a noticeable decline in both catch sizes [7] and total catch volumes [6]. This situation underscores the urgent need for sustainable management and conservation strategies. Interventions such as banning destructive gear and establishing marine protected areas (MPAs) could be effective, although their implementation may carry significant social implications.

Effective conservation efforts must be grounded in precise knowledge of the species being exploited, including their biology and ecology [8]. Accurate species identification and monitoring are essential for understanding the impacts of both anthropogenic and natural stressors on marine biodiversity. However, most current data are derived from fisheries-dependent monitoring or underwater visual censuses (UVC), both of which have significant limitations. These approaches often rely on morphological and meristic characters for species identification, which can be difficult to interpret, particularly for juvenile individuals (e.g., those collected with mosquito nets) or in underwater conditions where visual cues are limited.

In line with a global trend toward the integration of molecular tools into biodiversity monitoring, environmental DNA (eDNA) has increasingly been used as a complementary approach to traditional survey methods, including in the context of fisheries and marine protected area (MPA) monitoring (e.g., [9,10]). In Madagascar, eDNA offers promising opportunities to improve our understanding of fish diversity and community structure across coastal habitats by detecting species through genetic material shed into the environment via secretions, excretions, tissues, or carcasses. Over the past few decades, the technique has undergone rapid development [11] and is now widely applied for species detection and biodiversity monitoring [12–14]. Compared to traditional methods, eDNA is more sensitive and capable of detecting a broader spectrum of species, including cryptobenthic and rare taxa [15–17]. Nevertheless, several limitations persist, among which the incomplete taxonomic coverage of reference DNA barcode libraries remains a major constraint for assigning detected operational taxonomic units (OTUs) in water samples to species-level identities [18]. Despite global initiatives such as the International Barcode of Life (IBOL), which aim to establish comprehensive DNA barcode libraries for documenting biodiversity, the standard barcode marker for vertebrates (a fragment of the cytochrome c oxidase subunit I (*COI*) gene) has limited applicability for fish species detection via eDNA. Although *COI* is considered highly resolutive for species discrimination [19], its

relatively long fragment length renders it unsuitable for eDNA metabarcoding, where DNA is typically fragmented and degraded due to environmental conditions [20,21].

To overcome these limitations, alternative mitochondrial markers targeting shorter DNA fragments have been developed. Zhang et al. [22] reviewed 22 primer sets derived from four mitochondrial genes (*12S rRNA*, *COI*, *cytochrome b*, and *16S rRNA*) designed to amplify short variable regions for species discrimination while anchoring them in conserved flanking regions to enable broad taxonomic coverage [22,23]. Among these, markers within the *12S rRNA* gene have become particularly popular for fish eDNA surveys due to their balance of universality and taxonomic resolution [23–25].

In this context, building comprehensive *12S rRNA* reference libraries for marine fishes has become a priority, especially for supporting the implementation of eDNA-based biodiversity monitoring. In Madagascar, initiatives have recently emerged to develop DNA barcode libraries, with a focus on fish at early life stages [26] and on threatened species [27]. In 2022, of the 1689 marine and transitional water fish species recorded in Madagascar [28], 387 have *COI* barcodes available, and 307 have sequences generated from three mitochondrial genes (*COI*, *16S rRNA*, and *NADH2*). To date, however, no surveys have applied *12S rRNA* barcoding to assess fish diversity in Madagascar.

Relying solely on public databases such as GenBank for species-level assignment remains problematic due to issues such as taxonomic misidentifications and the absence of voucher specimens [29,30]. Accurate species identification from eDNA data requires a reference database that is both comprehensive and well-curated. In response, Blackman et al. [29] recommend the development of locally curated reference libraries and propose practical guidelines for enhancing the reliability of open-access databases through targeted curation.

The objectives of this study were fourfold. First, we aimed to enhance Madagascar's *COI* DNA barcode reference library for marine fish by expanding sampling efforts through the analysis of specimens obtained from artisanal fishers, with attention to fishing gear types and associated marine ecosystems. Second, we reviewed and curated previously assigned Barcode Index Numbers (BINs) to improve taxonomic accuracy within the existing DNA barcode database. Third, we generated the first *12S rRNA* reference library for Malagasy marine fishes by sequencing representative individuals from each BIN, thereby providing a foundational resource for future eDNA metabarcoding surveys. Finally, we assessed the taxonomic resolution of various *12S* metabarcodes to identify the most informative markers and to define optimal sequence similarity thresholds for accurate species-level assignment.

2. Materials and Methods

2.1. Tissue Sampling

To improve understanding of Malagasy marine fish diversity, this study utilized tissue samples collected during multiple research campaigns conducted in southwestern Madagascar (Table 1), consolidated under container MADFI (Reference DNA barcodes library of Malagasy marine fishes) in Barcode of Life Data Systems (BOLD). For the *COI* reference library, new samples were collected in Toliara and Ranobe Bays, with three field campaigns. This was sampling designed to increase the species representation in the database by expanding the geographic coverage, exploring diverse habitats, and employing a broader range of fishing gear than those used by Jaonalison et al. [26]. The first two campaigns followed artisanal pirogue fishers from eight villages in Toliara Bay (S23°25'0", E 43°42'0") between May 2018 and April 2019, and 12 villages in Ranobe Bay (E43°30', S23°00', E43°38', S23°18') from October 2021 to November 2022. Fishing methods included beach seine, mosquito net trawl, gillnet, handline, and speargun. These efforts were complemented by samples obtained in the seagrass beds and along the mangrove fringes of Sarodrano (March 2022 and April 2023),

using mosquito net trawl and gillnet. Tissue samples and photographs were collected monthly in Toliara Bay, bimonthly in Ranobe Bay, and twice monthly in the Sarodrano area.

Table 1. List of DNA barcoding projects grouped under the Reference DNA barcodes library of Malagasy marine fishes (MADFI) container on the Barcode of Life Data Systems (BOLD) platform. Each project publicly available through the BOLD system v5 corresponds to a sampling and sequencing campaign targeting marine fish species from Madagascar, for molecular species identification and biodiversity monitoring. The sample is expressed as the number of pirogues surveyed during the barcoding campaigns.

Project Name in MADFI Container	Sampling Period	Sampling Gear	Sampling Areas	Number of Samples	Reference
CALMA	March 2019 to May 2019	light traps	Great barrier reef of Toliara and Anakao reef	60	Collet et al. [31]
COMAD	October 2014 to March 2015	mosquito seine nets and light traps	Great barrier reef of Toliara and Anakao reef	92	Jaonalison et al. [26]
RFSIO	November 2016 to April 2017, and November 2017 to April 2018	light traps	Great barrier reef of Toliara and Anakao reef	200	Jaonalison et al. [26]
IFBIO	November 2016 to April 2017, and November 2017 to April 2018	mosquito seine nets and light traps	Fishing landings in Toliara Bay	78	Jaonalison et al. [26]
GEOFI	May 2018 to April 2019	beach seine, mosquito trawl net, gillnet, handline, speargun	Fish landings in the eight villages along Ranobe Bay	252	present study
ARMSR	October 2021 to November 2022	beach seine, mosquito trawl net, gillnet, handline, speargun	Fish landings in the 12 villages along Ranobe Bay	597	present study
SAROD	March 2022 to April 2023	gillnet, mosquito trawl net	Seagrass beds and along the mangrove edges of Sarodrano	130	present study

To construct the *12S rRNA* reference library, one or more representatives of the different Molecular Operational Taxonomic Units (MOTUs) or BINs from the MADFI database in BOLD were selected for analysis. All *12S* sequences generated in this study are original and unpublished. They were obtained from two sources of DNA: (i) DNA extracts previously used in earlier studies to generate *COI* sequences [26,32], and (ii) newly extracted DNA from specimens collected specifically for the present study to expand the *COI* database.

2.2. Collection of Tissue Samples and Photographs

The same methodological protocol was applied across all sampling campaigns. All fish landed by each pirogue were systematically considered for tissue and photographic documentation. Following each fishing trip, the catch was sorted into morphologically similar groups within each family. For each group, a representative specimen was photographed, and a fin clip (typically from the pelvic or caudal fin) was taken for DNA extraction. Each tissue sample was preserved in a cryotube filled with 90% alcohol, labeled with a unique code, and stored at -20°C . Photographs were imported into an image database using Adobe Lightroom, where image file names were renamed to match the corresponding tissue codes.

2.3. DNA Extraction, Amplification, and Sequencing

In the laboratory, genomic DNA was extracted from fin tissue samples using the Pure Link Genomic DNA Mini kit (Invitrogen, Waltham, MA, USA), following the manufacturer's protocol. Extracted DNA was stored at -20°C until further analysis. Two

mitochondrial gene regions were targeted for amplification: the mitochondrial *COI* gene and the ribosomal *12S rRNA* gene.

A 650 base pair fragment from the 5' region of the *COI* gene was amplified using the primer pairs FishF1 and FishF2 with FishR1, following the protocol established by Ward et al. [33]. PCR conditions for *COI* amplification included an initial denaturation at 98 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final post-extension step at 72 °C for 5 min.

For the amplification of the complete fragment of the *12S rRNA* gene, a ~1000 base pair fragment was amplified from representative specimens of each BIN in the local database. Amplification was performed using the primer pair XRMUPheF1 (5'-YAAAGCATAMCRCTGAAGATG-3') [34] and TeleoR (5'-CTTCCGGTACACTTACCATG-3') [35].

In cases of amplification failure, an alternative primer set, MiFish-U-F (5'-GTCGGTAA-AACTCGTGCCAGC-3') [24] and TeleoR, targeting a shorter ~600 base pair fragment, was employed. PCRs were carried out using the following thermal profile for 12S: initial denaturation at 92 °C for 5 min, followed by 35 cycles of 30 s denaturation, 30 s annealing (temperature ranging between 50 and 55 °C depending on primer set), and a final elongation step at 72 °C for 5 min. PCR products were visualized on 1% agarose gels stained with ethidium bromide, and successful amplifications showing clear and intense bands were selected for sequencing.

Selected PCR products were sent to GENOSCREEN (Lille, France) for Sanger sequencing, following procedures comparable to those described by Collet et al. [32] and Pham et al. [36]. The resulting sequences were retrieved, cleaned, aligned, and assigned taxonomic identities based on reference libraries.

2.4. Specimen Identification and Taxonomic Curation of the DNA Barcodes Library

All raw sequences obtained during this study were first examined and manually curated using Chromas software version 2.6.6 (<http://technelysium.com.au/wp/chromas/> (accessed on 20 October 2022)), or 4peaks (<https://nucleobytes.com/4peaks/index.html> (accessed on 11 January 2020)). Cleaned sequences were then exported in FASTA format and aligned using MEGA 7.0 [37], where they were compared with their closest sequences available in GenBank. Any discrepancies between the newly obtained sequence and the reference were verified by checking chromatograms using the corresponding AB1 files.

The cleaned *COI* and *12S rRNA* gene sequences were submitted to the BOLD database. Each *COI* sequence was automatically assigned to a BIN via the BOLD platform, based on the RESL (REFined Single Linkage) algorithm [38], which defines and delineates BINs in a standardized way. For each BIN, the associated names were reviewed (Figure 1).

When only one species name was associated with a BIN, and no other BINs were linked to that name, the species assignment was considered definitive. If multiple BINs were associated with the same species name, this raised the possibility of cryptic diversity or identification errors. These cases were further investigated using the “Taxon ID Tree” tool in BOLD, which allowed phylogenetic relationships to be reconstructed within relevant taxonomic groups (family or genus). In the resulting phylogenetic trees, each sequence was annotated with its geographic origin, BIN, GenBank accession number, and identifier, aiding in the critical assessment of the assigned names.

Each BIN was then taxonomically curated based on both molecular and phenotypic data. When identification was unambiguous, the sequence was assigned a Linnaean species name. In cases of uncertainty, the BIN was labeled at a higher taxonomic level (genus or family) followed by the BIN code. For species associated with multiple BINs, additional biogeographic analyses were conducted, including examination of their parapatric distribution and recent common ancestry. When possible, the type locality of each species was

verified via the Eschmeyer's Catalog of Fishes [39]. If the type locality corresponded to the BIN observed in Madagascar, the original Linnaean name was retained; otherwise, a “cf.” designation was used (e.g., cf. species name [BIN]).

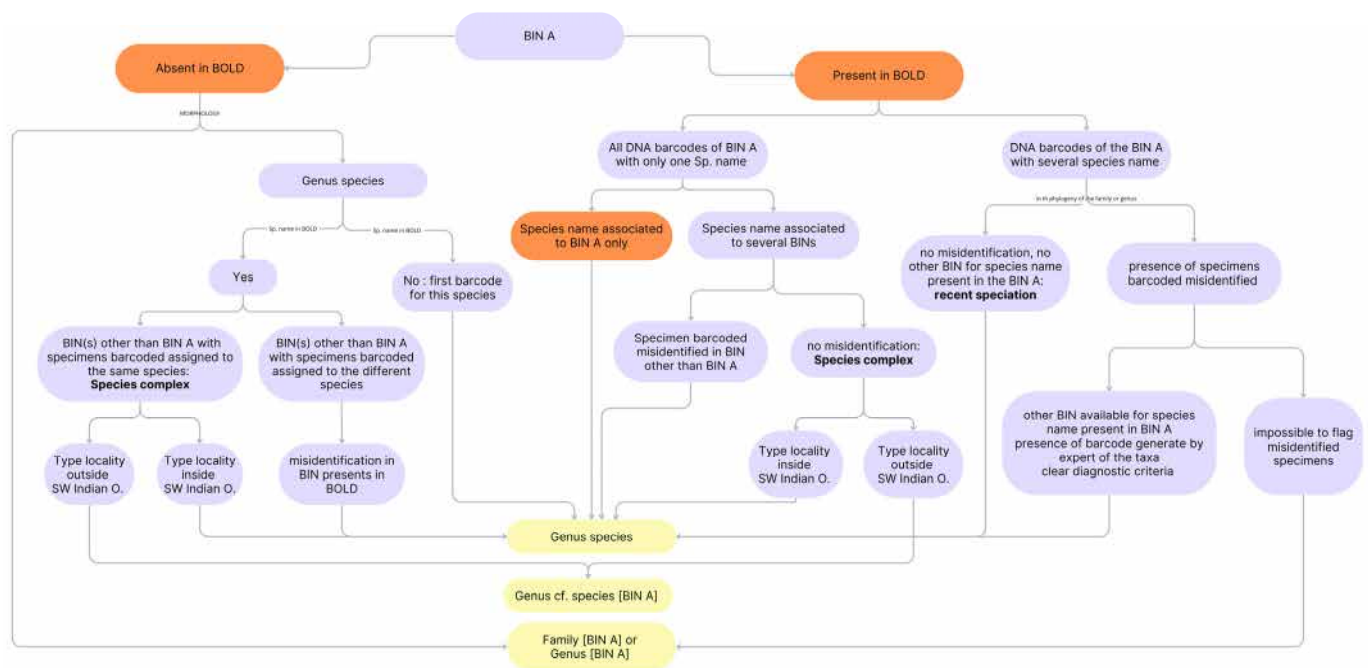


Figure 1. Decision tree illustrating the taxonomic identification method applied to each Barcode Index Number (BIN) for the curation of the reference database. Each BIN was initially generated automatically by the BOLD system. Taxonomic assignments (to species or genus) were then manually refined using an integrative approach combining: (i) morphometric traits and photographs of the barcoded specimens, (ii) the taxonomic composition within each BIN (consistency and frequency of species names), and (iii) phylogenetic relationships among BINs within the same genus or family (Table S1). This process allowed us to validate or correct initial identifications, resulting in a curated reference database suitable for reliable metabarcoding analyses.

For the 12S sequences generated in this study, the primary species identification was based on the BIN assignment of the corresponding COI sequence from the same specimen. This identification was then cross-validated using Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), accessed on 27 March 2023) results against GenBank. When COI sequences were unavailable, identifications relied on morphospecies-level phenotypic features and the BLAST match of the 12S sequence.

If identification remained uncertain, a conservative approach was taken:

- when sequence identity (seq ID) was greater than 99%, the species name was retained.
- when seq ID was between 95% and 99%, if only one genus appeared within the matches, the genus name was assigned. If multiple genera were listed, the identification was limited to the family level.
- when seq ID was between 90% and 95%, only the family name was used, provided it was consistently represented among the BLAST hits.

2.5. Evaluation of Taxonomic Resolution

To evaluate the taxonomic resolution of five 12S primer sets commonly used in environmental DNA (eDNA) based species detection, namely MiFish (171 bp), Teleo1 (63 bp), AcMDB (281 bp), Ac12S (389 bp), and 12SF1/R1 (106 bp) (see Zhang et al. [22] for primer details and arrangement), we followed the methodological framework proposed by Ruiz et al. [40] (submitted). This approach enables standardized comparison of taxonomic resolution

across different metabarcodes using the Barcode Index Number (BIN) system established in BOLD as a reference, which is based on *COI* sequence clustering.

Briefly, *in silico* extractions of each metabarcode region were performed on complete 12S gene sequences from individuals already assigned to BINs, using the novel methodology of Ruiz et al. [40]. We then carried out efficient (i.e., k-mer presorting) pairwise sequence comparisons of each metabarcode region across a range of similarity thresholds (90% to 99%), using default parameters of the program VSEARCH [41].

This sequence delineation was compared to the BIN reference framework to evaluate congruence. Two types of discrepancies were quantified: false negatives (where individuals from different BINs were considered similar) and false positives (where individuals from the same BIN were considered different) (Figure 2). The total error rate for each metabarcode was defined as the sum of the false-positive and false-negative rates at each similarity threshold, providing an objective measure of taxonomic resolution across markers.

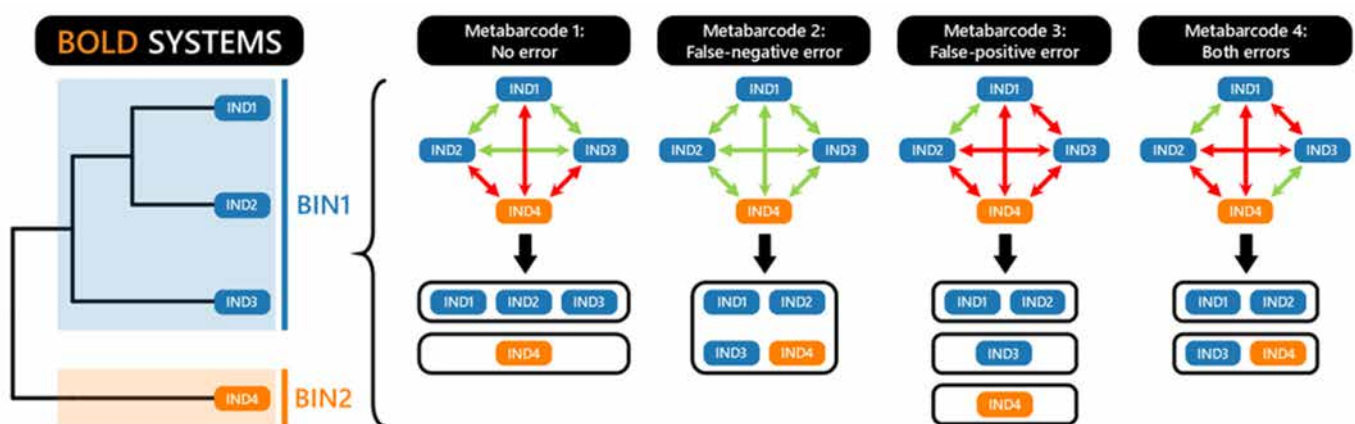


Figure 2. Illustration of different identification error scenarios in metabarcoding analyses, based on taxonomic clustering of Barcode Index Number (BINs). Green arrows indicate correct assignments, while red arrows represent errors. On the left, a phylogenetic tree shows the separation of four individuals (IND1 to IND4) into two BINs: BIN1 (IND1, IND2, IND3) and BIN2 (IND4). The four columns on the right represent different metabarcoding identification scenarios. Metabarcode 1 showed no error: all individuals were correctly identified as similar or different based on their BIN assignments. Metabarcode 2 showed a false-negative error, where IND4 (BIN2) was incorrectly considered similar to individuals from BIN1. Metabarcode 3 showed a false-positive error, where IND3 (BIN1) was incorrectly classified as different from other BIN1 individuals. Metabarcode 4 showed both error types: IND3 and IND4 were incorrectly identified as similar, and IND3 was also incorrectly considered different from other BIN1 individuals.

3. Results

3.1. Taxonomic Coverage of *COI* Sequences

The MADFI project currently comprises 2146 *COI* sequences, of which 953 were generated during the present study (Table 2).

Using the RESL algorithm, 502 distinct BINs were delineated from the complete *COI* dataset (Figure 3a). These BINs span two classes, 25 orders, 82 families, each family containing between one and 44 BINs, and 424 valid species names according to the World Register of Marine Species (WoRMS) (Figure 3a). The families with the most BINs in our database were Labridae (43), Apogonidae (41), Gobiidae (29), Pomacentridae (26), and Carangidae (22) (Figure 3d).

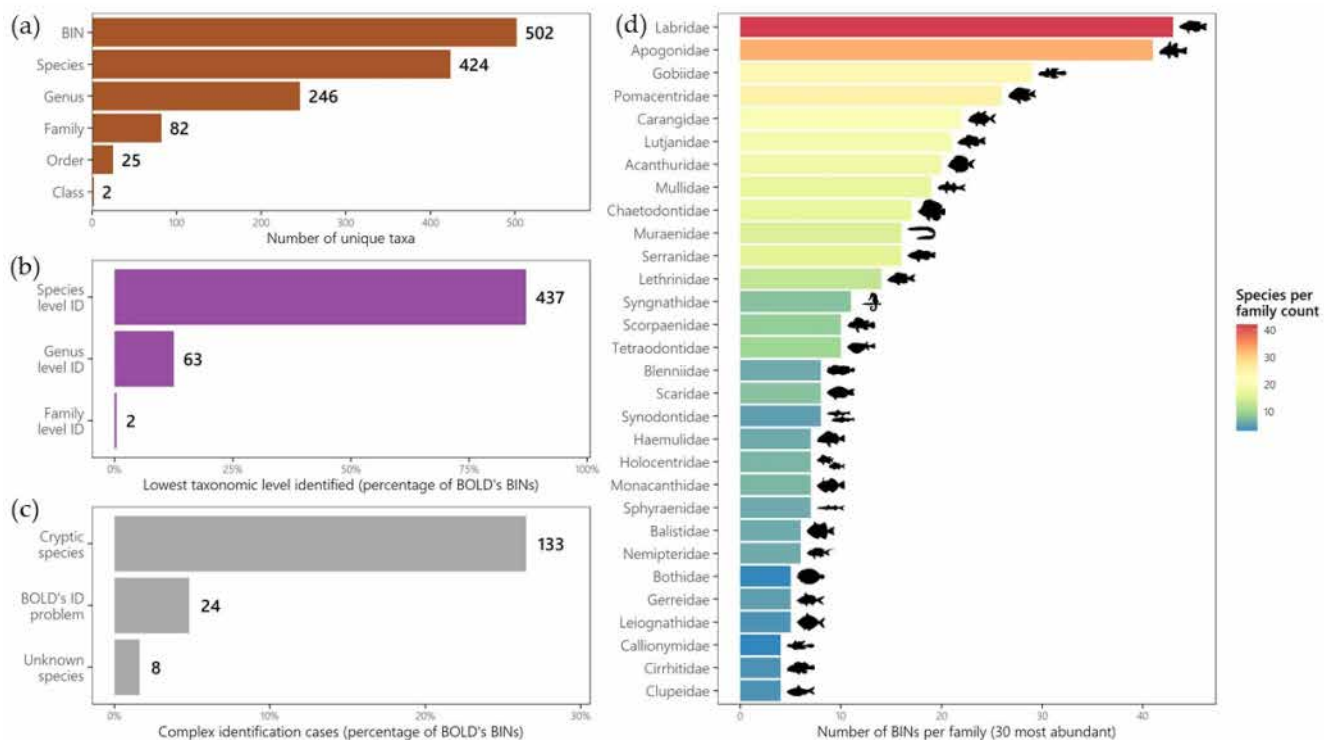


Figure 3. Overview of the taxonomic diversity and identification resolution of marine fish bar-coded in the MADFI (Reference DNA barcodes library of Malagasy marine fishes) project in BOLD. (a): Number of taxa identified for each BIN. (b): Proportion of BINs for which identification was achieved at species, genus, or family level. (c): Proportion of complex identification cases, including potential cryptic species, inconsistencies related to BIN identification issues in BOLD (specimens misidentified), and BINs not identified at the species level. (d): Number of BINs and species richness per fish family for the 30 most represented families. The barplot shows the number of BINs per family, with color shading indicating species richness: blue bars represent families with lower species diversity, and red bars represent those with higher diversity. Silhouettes illustrate the general morphology of representative species within each family.

Of the 502 BINs identified in this study, 488 correspond to BINs already registered in the BOLD database as part of the MADFI project, and 14 are newly generated BINs not yet present in BOLD. Among the 502 BINs, 437 were identified at the species level, including 133 cryptic species, 63 at the genus level, and two at the family level (Figure 3b,c). Among the 488 BINs already registered in BOLD, 26 BINs are newly recorded for the West Indo-Pacific, and 290 BINs for Madagascar (Figure 4c).

The 14 new BINs, spanning 10 families, were generated from the new samples and include the following families: Gobiidae (4), Clupeidae (2), Apogonidae (1), Blenniidae (1), Bothidae (1), Cynoglossidae (1), Diodontidae (1), Labridae (1), Pseudochromidae (1), and Syngnathidae (1). Among these, five BINs were identified at the species level (including one cryptic species), eight at the genus level, and one at the family level (Syngnathidae).

Among the 488 BINs already registered in the BOLD database, 432 were assigned to the species level. These species include 132 BINs from 43 families corresponding to species belonging to cryptic species. The families most concerned with cryptic diversity were Apogonidae (23 BINs), Labridae (11 BINs), Gobiidae (10 BINs), Pomacentridae (6 BINs) and Serranidae (6 BINs), representing 26% of all putative species in the MADFI COI gene database. Additionally, 55 were identified only at the genus level, distributed across 31 families, mainly composed of Apogonidae (7), Gobiidae (3), Syngnathidae (3), and Synodontidae (3). Only one BIN was assigned at the family level (Exocoetidae).

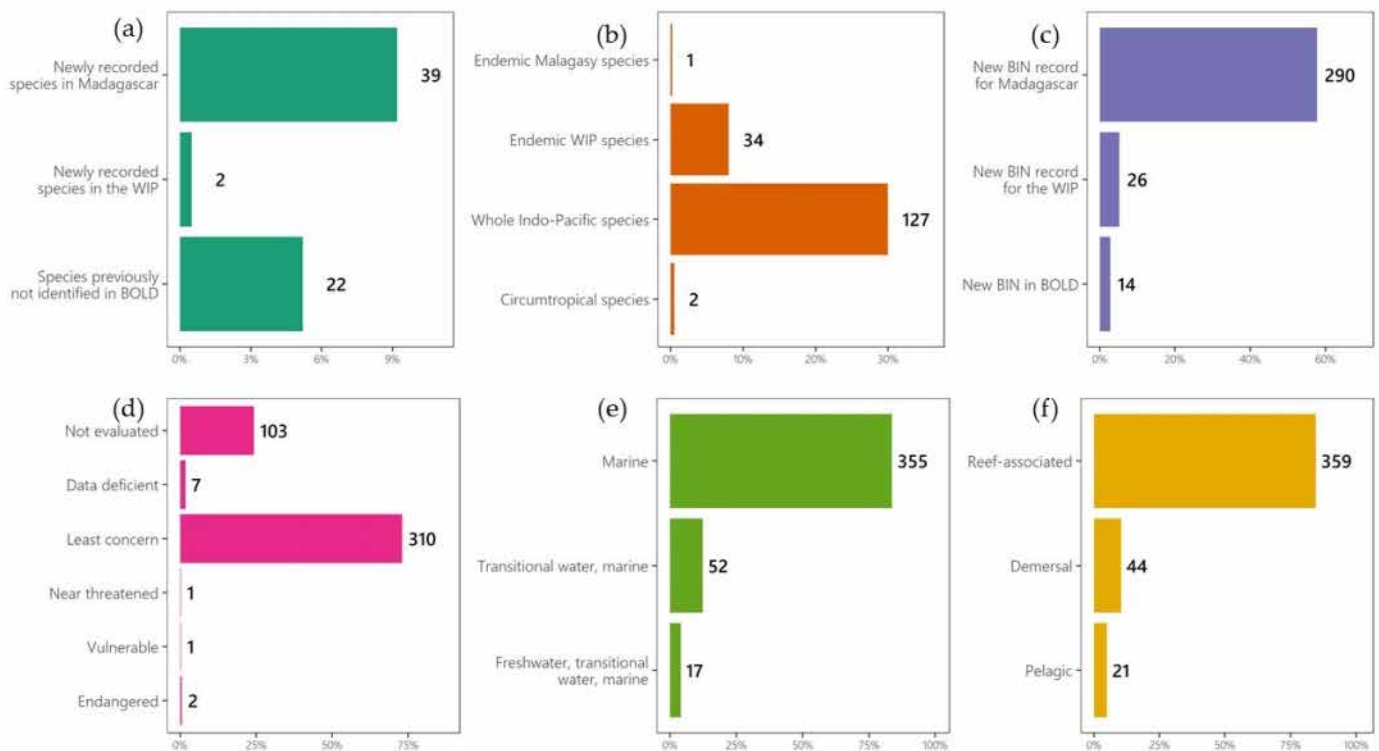


Figure 4. Summary of main characteristics of the 424 valid fish species (according to WoRMs) and 502 BINs recorded in the MADFI project. (a): Number of species newly recorded for Madagascar, in the Western Indian Pacific (WIP), or not previously identified in BOLD. (b): Biogeographic distribution of recorded species across four categories (endemic Malagasy, WIP endemic, whole Indo-Pacific, and circumtropical). (c): Novelty of BINs in BOLD (BINs newly recorded for Madagascar, the WIP, or globally). (d): IUCN Red List status of recorded species. (e): Habitat range based on FishBase. (f): Ecological lifestyle of the recorded species based on FishBase. (a,b,d–f): The X-axis in all panels represents the percentage of valid species ($n = 424$) as recognized by WoRMS (the X-axis in panel (c) represents the percentage of BOLD-registered BINs ($n = 502$)).

A detailed analysis of specimens assigned in BOLD systems to the 488 BINs revealed that:

- 243 BINs included specimens with a unique species name (one BIN, one species name). Among them, 236 were identified at the species level. Of these species names, 125 BINs were associated with a single BIN and could therefore be directly linked to the same species name, including only one cryptic species (*Ariosoma cf. meeki* [BOLD:ADD3087]). The remaining 118 BINs were associated with more than one BIN. Phylogenetic reconstructions at the family or genus level revealed that 87 species most probably corresponded to species complexes, accounting for 17% of all BINs. For 24 of the remaining BINs, after verification using the phylogenetic tree, they turned out to be misidentified specimens. However, seven BINs could only be assigned at the genus level.
- 212 BINs contained specimens with different species names. Phylogenetic reconstructions and further manual verifications confirmed that these specimens were misidentified. Among these, 188 BINs had another correct BIN available for the species name and a barcode generated by a taxonomic expert, with clear diagnostic criteria. All 188 BINs were identified at the species level, including 40 cryptic species. For the remaining 24 BINs, it was not possible to resolve the misidentifications, and their identification remained at the genus or family level.
- 33 BINs were either associated with a genus name or were not identified in the BOLD database. Among them, 25 were identified at the genus level, and eight at the species level, including four cryptic species.

3.2. Overall Profile of Fish Species in the MADFI Database According to the Fishbase

Among the 424 species validated through World Register of Marine Species (WoRMS) (Table S2), 39 are newly recorded for Madagascar, two are newly recorded for the entire Western Indo-Pacific (WIP) region, and 22, although already known, had not yet been identified in BOLD (Figure 4a). Regarding their biogeographic distribution, the majority of species (127) are widely distributed across the Indo-Pacific, 34 are endemic to the WIP, one species is strictly endemic to Madagascar, and two species have a circumtropical distribution (Figure 4b).

In terms of conservation status according to the International Union for Conservation of Nature (IUCN) via WoRMS, most species (310) are classified as “Least Concern,” while 103 species have not yet been assessed. Very few are threatened: two are endangered (*Cheilinus undulates* and *Lethrinus mashena*), one is vulnerable (*Hippocampus histrix*), and one is near threatened (*Favonigobius melanobranchus*) (Figure 4d).

From an ecological perspective, 355 species are strictly marine, 52 occur in both transitional and marine zones, and 17 are euryhaline (present in freshwater, brackish, and marine environments) (Figure 4e). Finally, regarding ecological lifestyle, the majority of species (359) are reef-associated, 44 are demersal, and 21 are pelagic (Figure 4f).

3.3. Taxonomic Coverage of 12S Sequences

The 12S barcode library comprises 524 sequences, of which 89% correspond to near full-length 12S rRNA sequences (~1000 bp), obtained with long-range primers. The remaining 11% are shorter sequences (~600 bp), generated using internal primers when full-length amplification failed, likely due to mutations at primer binding sites or DNA degradation. These sequences represent 446 species from 78 families. The five most diverse families in terms of species were Labridae (40), Apogonidae (37), Gobiidae (26), Acanthuridae (19), and Pomacentridae (19) (Table 2). Among all BINs in the database, 86% (434 BINs from 76 families) were sequenced for the 12S gene. The complete set of fish DNA sequences for these two genes covered 513 species distributed across 84 families, including 436 species from 75 families with sequences available for both the *COI* and 12S genes. Conversely, 67 species from 35 families were available only for the *COI* gene, while 10 species from nine families were available exclusively for the 12S gene.

3.4. Taxonomic Resolution of Five Different 12S Primers

A total of 319 species have complete 12S gene sequences associated with BINs defined by the *COI* marker. For these species, the taxonomic resolution of five sets of primers used for taxonomic assignments was evaluated by comparing their level of polymorphisms to species delimitation based on *COI* polymorphisms (BINs). For all metabarcodes, lowering the similarity threshold (ST) from 99.5% to 90% led to a reduction in false-negative errors, as expected (Figure 5). When the ST was set at 99% or less, both MiFish and ACMDB showed an increase in false-positive errors (overestimation of the number of species compared to the number of BINs). This false-positive error appeared at a lower ST (97%) for Teleo1, while no false-positive errors were observed for the AC12S and 12SF1R1 metabarcodes. As a result, all metabarcodes had different ST values at which false-negative and false-positive errors were well-balanced. Optimal ST values were 99.5% for Ac12S and 12SF1R1, 98.5% for AcMDB and Mifish, and 95.5% for Teleo1.

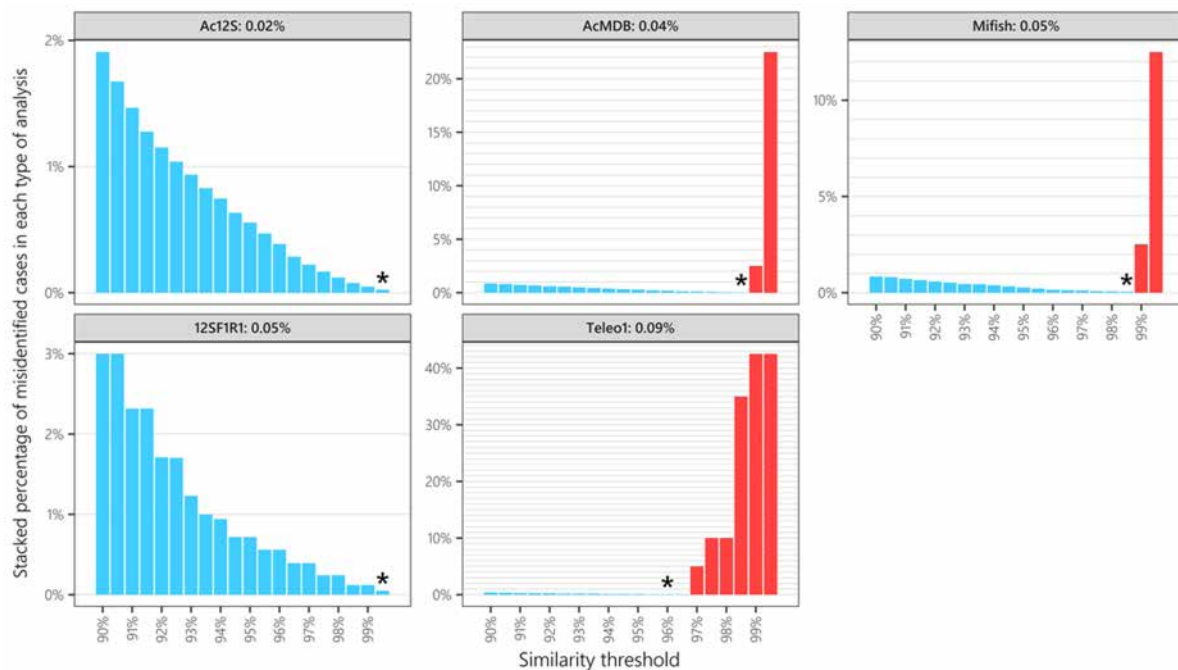


Figure 5. Proportions of false-positive errors (in red) and false-negative errors (in blue) for each metabarcode depending on similarity thresholds ranging from 99.5% to 90% for the five different metabarcodes (Ac12S, AcMDB, Mifish, 12SF1R1, and Teleo1). The black star represents the optimal threshold for each metabarcode, while the minimal error rate achieved at this threshold is indicated in the top panel.

Using similarity thresholds of 97% and 98%, AcMDB and MiFish metabarcodes were the most effective at discriminating BINs, while Ac12S and 12SF1R1 discriminated the most BINs at 99% similarity. For all three similarity thresholds (97%, 98%, and 99%), Teleo1 remained the least discriminating metabarcode (Figure 5).

The ability to distinguish different BINs within a family varied according to the metabarcodes used (Figure 6). An example of this variation is observed in the Carangidae family: no BIN discrimination errors were detected for the Ac12S and Mifish metabarcodes; one pair of BINs was not discriminated by both the AcMDB and 12SF1R1 metabarcodes; and four pairs of BINs were not discriminated for the Teleo1 metabarcode. The number of families (among 48) with BIN discrimination errors was 14, 15, 16, 16, and 19 for the Ac12S, Mifish, AcMDB, 12SF1R1, and Teleo metabarcodes, respectively. Pearson's correlation analysis revealed a moderate positive relationship between the number of non-discriminated BIN pairs and the number of BINs within each family, with a highly significant p -value ($r = 0.46$, $p < 0.001$). Regardless of the metabarcode, BIN discrimination errors were detected in the following 13 families (Figure 6). Outside of these 13 families, each metabarcode showed BIN discrimination errors in specific families. The Ac12S metabarcode showed errors in the Priacanthidae family. Errors were detected in several families across the different metabarcodes: AcMDB showed errors in Carangidae, Ephippidae, and Exocoetidae; MiFish in Ephippidae and Serranidae; and 12SF1R1 in Carangidae, Priacanthidae, and Serranidae. Finally, the Teleo1 metabarcode showed errors in the Carangidae, Ephippidae, Exocoetidae, Muraenidae, Pomacentridae, and Sphyraenidae families. The families with the highest number of non-discriminated BIN pairs were Lethrinidae for the Ac12S metabarcode, Labridae for the MiFish metabarcode, and Chaetodontidae for the metabarcodes AcMDB, 12SF1R1, and Teleo1.

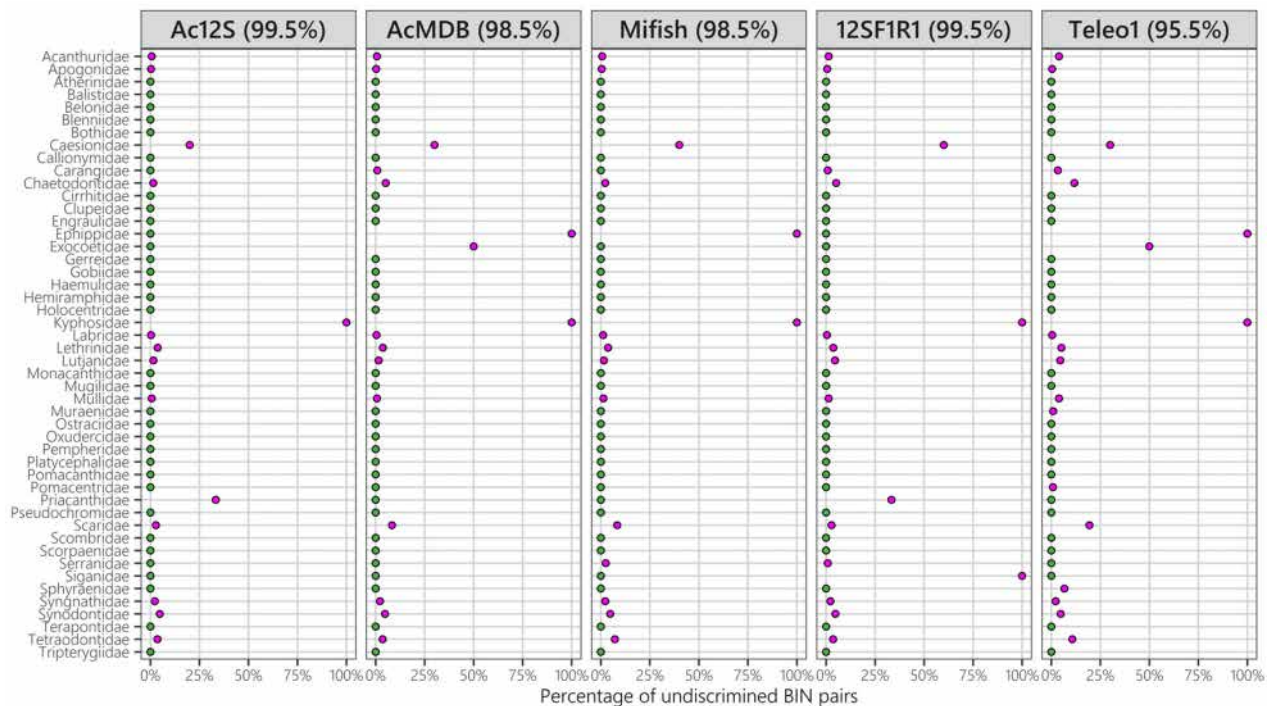


Figure 6. Percentage of BIN pair discrimination by family for each primer. The percentage indicated for each primer corresponds to its optimal similarity threshold used to assess taxonomic resolution across fish families. Ac12S (99.5%), AcMDB (98.5%), Mifish (98.5%), 12SF1R1 (99.5%), and Teleo1 (95.5%), Green indicates families for which all BINs are discriminated by the primer. Magenta indicates families containing BINs not discriminated by each primer.

4. Discussion

This study provides the most comprehensive molecular inventory to date of marine fish diversity in Madagascar. The newly assembled *COI* and *12S* reference libraries comprise 514 species spanning 84 families, 28 orders, and 2 classes. It constitutes the largest-scale DNA barcode reference dataset derived from specimens collected with a wide array of artisanal fishing gear over an extended sampling period exceeding 2 years. Despite earlier efforts in the same region of Toliara, this study stands out due to its unparalleled sampling intensity, its broad habitat coverage, and the integration of morphological and molecular identification methods. For example, Laroche and Ramananarivo [42], relying exclusively on morphological identification over a 7-month survey period, recorded only 35 fish families in Toliara Bay, less than half of the 74 reported here. Similarly, Jaonalison et al. [26] focused solely on larval and juvenile stages using light traps and mosquito net trawls. Although they successfully recorded 387 species from 66 families, their work captured only a portion of the local ichthyofauna. By contrast, this study revealed significantly greater taxonomic richness. The extended temporal scope allowed the detection of seasonal or rarely encountered species, consistent with findings by Laroche et al. [43], who documented temporal variation in fish assemblages in southwestern Madagascar. The use of diverse artisanal fishing methods further improved sampling coverage across ecological niches, including cryptic and reef-associated taxa. Broad-scale surveys may serve as an efficient way to rapidly increase the taxonomic coverage of DNA barcode libraries, as illustrated by Vences et al. [27]. They documented *COI* barcodes for 307 species across diverse locations in Madagascar. However, our findings demonstrate that high-frequency, site-specific sampling produces a more comprehensive and representative inventory of marine biodiversity.

Beyond contributing to the local biodiversity documentation, our findings also raise important concerns about the sustainability of current fishing practices. All specimens

analyzed in both Laroche & Ramananarivo [42] and the present study were collected from active fisheries. The marked increase in family-level richness observed may indicate a substantial rise in exploitation pressure over the intervening period. Notably, mosquito nets were not employed in 1995 [42], suggesting a shift toward more intensive or indiscriminate fishing methods. These results underscore the urgent need for systematic monitoring of fishery impacts and the integration of molecular tools into fisheries management strategies [44].

At a larger scale, such as that of the eastern Indian Ocean, our study represents a significant contribution to the genetic characterization of marine fish diversity. It notably led to the identification of 14 new BINs in the BOLD database. To date, substantial DNA barcoding efforts have been conducted in South Africa [45–47], Mozambique [48], the Red Sea [49], and the Persian Gulf [50,51]. Compared to the work carried out in Mozambique and South Africa, the sampling effort in Madagascar is more advanced than that of Mozambique. These two countries share 58 BINs, but each also harbors unique BINs: 444 BINs have been recorded exclusively in Madagascar, compared to 84 in Mozambique. When compared with the BINs recorded in South Africa, approximately half of those identified in Madagascar are also found there [47]. However, South Africa has a higher number of unique BINs, with 710 compared to 255 for Madagascar. Dissimilarities in biodiversity and taxonomic composition among these three countries can be explained by differences in sampling efforts and the biogeographic particularities of each region regarding ichthyological diversity. They also reflect that some species shared across multiple regions can vary significantly in local abundance. The relative abundance of a species in a given area directly influences its likelihood of being caught by fishers.

A second major contribution of this study is the improvement of taxonomic resolution through the integration of 953 new *COI* sequences. This addition to the existing dataset enabled the development of the first high-resolution DNA barcode reference library for marine fishes in southwestern Madagascar. Among the 502 species catalogued, 132 were associated with multiple BINs in BOLD, revealing previously undetected cryptic diversity. These cryptic taxa account for approximately 26% of the observed species richness. This proportion is consistent with patterns reported in other Indo-Pacific regions, where morphologically indistinguishable sister species are increasingly being uncovered through molecular approaches [36,45]. In our dataset, cryptic diversity was especially pronounced within the families Apogonidae, Gobiidae, and Labridae. These families are not only ecologically significant (often composed of small, reef-associated species with high habitat specificity) but also taxonomically complex and geographically widespread. The prevalence of cryptic diversity in these groups is unsurprising and can be attributed to a combination of factors: (1) They represent some of the most species-rich lineages of reef fishes. (2) Their diversity is frequently the result of recent radiations, producing closely related species with limited morphological divergence but clear genetic distinctiveness (e.g., Duchene et al. [52], Winterbottom [53]). (3) Subtle morphological differences among species render traditional identification methods ineffective [54]. (4) These taxa often exhibit fine-scale ecological partitioning, with species occupying different microhabitats or depth zones. Such ecological specialization can reduce interspecific competition while promoting reproductive isolation and genetic divergence [55–57]. Together, these findings highlight the limitations of morphology-based taxonomy in resolving biodiversity patterns in such groups. They emphasize the importance of incorporating molecular tools into routine biodiversity assessments. The widespread occurrence of cryptic lineages also lends support to the hypothesis that allopatric speciation, driven by historical geographic barriers and ecological isolation, plays a central role in the diversification of reef fishes in the Western Indo-Pacific [58,59]. In our dataset, 82% of cryptic lineages appeared to be

allopatric, reinforcing the idea that spatial fragmentation and ecological segregation are key contributors to hidden biodiversity in this region [60].

In the context of environmental DNA (eDNA) metabarcoding, the accuracy of species detection and identification is critically dependent on both the quality and taxonomic completeness of DNA reference libraries. High-quality libraries must include sequences derived from rigorously validated voucher specimens, as misidentified individuals can introduce systematic errors into biodiversity assessments [61,62]. Equally important is taxonomic completeness (i.e., the comprehensive representation of species likely to occur in a given ecosystem), to reduce false negatives and improve detection accuracy, especially in species-rich and poorly documented regions such as the Western Indo-Pacific [58,60]. The widespread presence of cryptic species complexes in marine taxa further complicates the development of effective reference libraries. These taxa are frequently misidentified or overlooked in global repositories, which reduces the reliability of species-level assignments in eDNA studies. In addition, global databases often contain sequences from geographically disparate populations, thereby capturing high levels of intraspecific genetic variation. This geographic variation can obscure species boundaries and lower the accuracy of automated taxonomic assignments. Li et al. [63] highlighted this issue by demonstrating that pairwise genetic distances among local barcode sequences were three to five times lower than those from non-local sources. These limitations underscore the need for locally curated reference libraries based on regionally collected and accurately identified specimens. Such resources not only minimize misidentifications and account for cryptic diversity, but also allow for the calibration of locally relevant genetic divergence thresholds. In line with this rationale, we assembled a complementary 12S reference library to enhance eDNA-based species detection in Madagascar's marine ecosystems.

Lastly, because eDNA-based biodiversity monitoring commonly relies on short fragments of the 12S *rRNA* gene, we conducted an *in silico* comparative assessment of five widely used primer sets (MiFish, Teleo1, AcMDB, Ac12S, and 12SF1/R1) to evaluate their taxonomic resolution. This analysis was performed using a curated dataset of full-length 12S sequences linked to COI-defined BINs. By comparing their performance across a range of similarity thresholds (90–99%), we were able to assess their discriminatory power and suitability for species-level identification in the context of Madagascar's marine biodiversity. No primer set performed optimally across all thresholds, but clear differences were observed. MiFish and Ac12S showed the lowest error rates at high thresholds, making them well-suited for high-resolution biodiversity assessments. In contrast, primers like 12SF1/R1, while offering broader taxonomic coverage, yielded higher error rates due to lower resolution among closely related species. These results illustrate the trade-off between universality and resolution in primer design [23]. In regions like Madagascar, where cryptic diversity is high, primers with high taxonomic discrimination (e.g., MiFish), coupled with localized reference databases, are essential for accurate eDNA-based biodiversity monitoring. The implementation of eDNA approaches in Madagascar's coastal waters will require careful primer selection, tailored to the ecological and taxonomic scope of each study. The reference frameworks and benchmarking results provided here offer essential guidance to ensure the accuracy and reliability of future biodiversity assessments.

5. Conclusions

This study represents a major step forward in documenting and understanding the marine fish biodiversity of southwestern Madagascar. By integrating extensive field sampling, morphological identification, and DNA barcoding (*COI* and 12S), it provides the most comprehensive molecular reference databases available for the region to date. The resulting catalog of 514 species across 84 families not only enhances our knowledge of local species richness

but also reveals substantial cryptic diversity and biogeographic structuring. Although this represents a substantial contribution to understanding the island's marine biodiversity, it still only covers about 30% of the total marine and transitional water fish species listed by [26], highlighting the need for continued sampling and molecular characterization.

Beyond its taxonomic contributions, this work underscores several critical methodological insights. First, it highlights the limitations of global genetic databases such as GenBank and BOLD for accurate species identification in eDNA metabarcoding studies, especially in regions with high cryptic diversity. Second, it demonstrates the necessity of building locally curated reference libraries, both to reflect regional genetic diversity and to support more precise species delimitation. Third, it confirms the importance of long-term, spatially diverse sampling strategies, particularly in ecosystems experiencing intense and unregulated fishing pressure.

The 12S reference library developed here, along with the in silico evaluation of commonly used primer sets, provides essential tools for the deployment of eDNA-based biodiversity monitoring in Madagascar. These molecular tools, when combined with traditional ecological knowledge and fisheries data, can greatly improve the accuracy of species detection, support sustainable management practices, and inform conservation strategies.

Table 2. List of species barcoded in the MADFI database on the BOLD platform. BIN: Barcode Index Number. COI: specimens barcoded with the COI gene. 12S: specimens barcoded with 12S gene. N COI in MADFI: Number of specimens barcoded with the COI gene in the MADFI database. N 12S in MADFI: Number of specimens barcoded with the 12S gene in the MADFI database. Nsp./BIN: number of species associated with each BIN in the BOLD. NBIN/Sp. In BOLD: number of BINs associated with each species (only if the species is linked to one BIN in the BOLD). New: new BINs in BOLD. NA: BINs that contain specimens not identified to the species level. Sp. complex: Y (Yes) indicates species complexes. Allo./Symp.: Cryptic species classified as allopatric (A) or sympatric (S).

Class		COI		12S	
Order					
Family					
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	N 12S in MADFI
Actinopterygii					
Acanthuriformes					
Acanthuridae					
<i>Acanthurus blochii</i>	BOLD:AAF0623	1	1	1	1
<i>Acanthurus dussumieri</i>	BOLD:AAE4046	26	1	>1	1
<i>Acanthurus leucosternon</i>	BOLD:AAB7142	1	>1		1
<i>Acanthurus lineatus</i>	BOLD:AAB3207	1	>1		1
<i>Acanthurus mata</i>	BOLD:AAE4025	6	1	>1	1
<i>Acanthurus nigricauda</i>	BOLD:AAB8729	14	1	1	1
<i>Acanthurus nigrofasciatus</i>	BOLD:AAB0201	3	>1		1
<i>Acanthurus tennentii</i>	BOLD:AAD2621	1	1	1	1
<i>Acanthurus triostegus</i>	BOLD:AAA9362	7	1	1	1
<i>Acanthurus xanthopterus</i>	BOLD:AAC6467	22	>1		1
<i>Ctenochaetus binotatus</i>	BOLD:AAB9166	2	1	1	1
<i>Ctenochaetus striatus</i>	BOLD:AAB9167	13	>1		2
<i>Naso annulatus</i>	BOLD:AEN2475	7	1	1	2
<i>Naso brevirostris</i>	BOLD:AAC1635	17	1	1	1
<i>Naso elegans</i>	BOLD:AAA8635	1	>1		1
<i>Naso unicornis</i>	BOLD:AAC8042	3	1	1	1
<i>Paracanthurus hepatus</i>	BOLD:AAC3227	1	1	>1	1

Table 2. Cont.

Class		COI					12S
Order							
Family							
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Zebrasoma cf. desjardini</i> [BOLD:AAF6311]	BOLD:AAF6311	2	1	>1	Y	S	1
<i>Zebrasoma cf. desjardini</i> [BOLD:ACV8450]	BOLD:ACV8450	1	>1		Y	S	1
<i>Zebrasoma cf. scopas</i> [BOLD:AAB3788]	BOLD:AAB3788	5	1	>1	Y	A	0
Zanclidae							
<i>Zanclus cornutus</i>	BOLD:ADI3027	1	1	1			1
Anguilliformes							
Congridae							
<i>Ariosoma cf. meeki</i> [BOLD:ADD3087]	BOLD:ADD3087	1	1	1	Y	A	0
<i>Ariosoma scheelei</i>	BOLD:AAJ1709	14	1	1			1
<i>Conger cinereus</i>	BOLD:AAL5915	6	1	>1	Y	A	1
<i>Uroconger</i> [BOLD:ACV7958]	BOLD:ACV7958	1	NA				0
Muraenidae							
<i>Echidna nebulosa</i>	BOLD:AAC5551	3	1	1			2
<i>Echidna polyzona</i>	BOLD:AAC9046	4	>1				3
<i>Gymnomuraena zebra</i>	BOLD:AAN1246	1	1	1			1
<i>Gymnothorax</i> [BOLD:ADB4048]	BOLD:ADB4048	1	NA				1
<i>Gymnothorax cf. chilospilus</i> [BOLD:AAC0198]	BOLD:AAC0198	4	1	>1	Y	A	2
<i>Gymnothorax cf. undulatus</i> [BOLD:AAC5500]	BOLD:AAC5500	4	>1		Y	S	3
<i>Gymnothorax cf. undulatus</i> [BOLD:AAC5502]	BOLD:AAC5502	1	>1		Y	S	1
<i>Gymnothorax elaineheemstrae</i>	BOLD:AAE6825	2	>1				3
<i>Gymnothorax favagineus</i>	BOLD:ADB3872	3	>1				1
<i>Gymnothorax flavimarginatus</i>	BOLD:AAE6819	1	>1				1
<i>Gymnothorax javanicus</i>	BOLD:AAE3588	1	1	1			1
<i>Gymnothorax pictus</i>	BOLD:AAE3580	1	1	>1			1
<i>Gymnothorax richardsonii</i>	BOLD:AAI6393	3	1	1			1
<i>Gymnothorax robinsi</i>	BOLD:AAI2654	1	1	1			1
<i>Gymnothorax rueppelliae</i>	BOLD:AAE3543	2	>1				1
<i>Scuticaria tigrina</i>	BOLD:AAH9730	1	1	1			0
Ophichthidae							
<i>Pisodonophis cancrivorus</i>		0					1
Atheriniformes							
Atherinidae							
<i>Atherinomorus cf. lacunosus</i> [BOLD:ACK7521]	BOLD:ACK7521	3	>1	Y	A		1
<i>Hypoatherina</i> [BOLD:AAL7563]	BOLD:AAL7563	1	>1				1
<i>Hypoatherina</i> [BOLD:ACV9758]	BOLD:ACV9758	5	>1				1
Aulopiformes							
Synodontidae							
<i>Saurida cf. gracilis</i> [BOLD:AAB1854]	BOLD:AAB1854	4	>1		Y	S	1
<i>Saurida cf. gracilis</i> [BOLD:AAE4190]	BOLD:AAE4190	2	>1		Y	S	2
<i>Saurida nebulosa</i>	BOLD:AAH0503	11	1	>1	Y	A	1
<i>Synodus</i> [BOLD:ACD1807]	BOLD:ACD1807	2	NA				2
<i>Synodus dermatogenys</i>		0					2
<i>Synodus rubromarmoratus</i>		0					1
<i>Synodus variegatus</i>	BOLD:AAB5069	4	>1				1
<i>Trachinocephalus</i> [BOLD:AAA9578]	BOLD:AAA9578	2	>1				0
<i>Trachinocephalus</i> [BOLD:ACY8623]	BOLD:ACY8623	1	1	>1			1
<i>Trachinocephalus cf. trachinus</i> [BOLD:ABX6347]	BOLD:ABX6347	4	>1		Y	A	0
Beloniformes							
Belonidae							
<i>Ablennes cf. hian</i> [BOLD:AAB9824]	BOLD:AAB9824	1	>1		Y	S	1
<i>Tylosurus cf. acus</i> [BOLD:AAH7713]	BOLD:AAH7713	1	1	>1	Y	S	1
<i>Tylosurus crocodilus</i>	BOLD:AAC4148	7	>1				1
Exocoetidae							
<i>Cheilopogon</i> [BOLD:ABZ7103]	BOLD:ABZ7103	1	>1				1
<i>Cheilopogon cyanopterus</i>	BOLD:ACK7919	1	>1				1
<i>Exocoetidae</i> [BOLD:AAK1099]	BOLD:AAK1099	1	>1				1
<i>Parexocoetus brachypterus</i>	BOLD:AAG2613	1	>1				1

Table 2. *Cont.*

Class	COI	12S
Order		
Family		
Species	BIN	N 12S in MADFI
Hemiramphidae		
<i>Hemiramphus</i> cf. <i>far</i> [BOLD:AAC0565]	BOLD:AAC0565	1
<i>Hemiramphus lutkei</i>	BOLD:ACK7284	1
<i>Hyporhamphus affinis</i>	BOLD:AAD0579	1
Blenniiformes		
Blenniidae		
<i>Aspidontus dussumieri</i>	BOLD:AAJ3001	0
<i>Omobranchus</i> [BOLD:ACY8418]	BOLD:ACY8418	1
<i>Omobranchus elongatus</i>	BOLD:AFC2971	1
<i>Petroscirtes</i> cf. <i>mitratus</i> [BOLD:AAE6131]	BOLD:AAE6131	1
<i>Petroscirtes</i> cf. <i>mitratus</i> [BOLD:AAE6132]	BOLD:AAE6132	1
<i>Plagiotremus tapeinosoma</i>	BOLD:AAD0784	0
<i>Salarias</i> [BOLD:ADN2100]	BOLD:ADN2100	1
<i>Salarias</i> cf. <i>fasciatus</i> [BOLD:ACV7965]	BOLD:ACV7965	1
Tripterygiidae		
<i>Enneapterygius</i> [BOLD:ACV9383]	BOLD:ACV9383	1
<i>Helcogramma</i> [BOLD:AAT9889]	BOLD:AAT9889	1
Carangiformes		
Carangidae		
<i>Alectis ciliaris</i>	BOLD:AAB7827	1
<i>Atropus hedlandensis</i>	BOLD:AAD6117	1
<i>Atropus mentalis</i>	BOLD:AAD6118	1
<i>Atule mate</i>	BOLD:AAB3382	1
<i>Carangichthys dinema</i>	BOLD:AAO5886	1
<i>Caranx ignobilis</i>	BOLD:AAB0587	3
<i>Caranx melampygus</i>	BOLD:AAB0585	1
<i>Caranx papuensis</i>	BOLD:ACF4541	1
<i>Caranx sexfasciatus</i>	BOLD:AAB0584	1
<i>Caranx tille</i>	BOLD:ACS0288	0
<i>Elagatis bipinnulata</i>	BOLD:AAB3730	0
<i>Ferdauia ferdau</i>	BOLD:AAE7640	1
<i>Gnathanodon speciosus</i>	BOLD:AAB7462	1
<i>Parastromateus niger</i>	BOLD:AAB3884	1
<i>Platycaranx</i> [BOLD:AAB4362]	BOLD:AAB4362	0
<i>Platycaranx chrysophrys</i>	BOLD:AAB2977	1
<i>Scomberoides lysan</i>	BOLD:AAB0512	0
<i>Selar</i> cf. <i>crumenophthalmus</i> [BOLD:AAB0871]	BOLD:AAB0871	1
<i>Seriolina</i> [BOLD:AAB8503]	BOLD:AAB8503	1
<i>Trachinotus blochii</i>	BOLD:ACF4014	1
<i>Turrun</i> cf. <i>coeruleopinnatum</i> [BOLD:AAD2297]	BOLD:AAD2297	2
<i>Turrun fulvoguttatum</i>	BOLD:AAC2745	2
Coryphaenidae		
<i>Coryphaena hippurus</i>	BOLD:AAA5277	1
Echeneidae		
<i>Echeneis naucrates</i>	BOLD:AAB6121	1
Sphyraenidae		
<i>Sphyraena</i> [BOLD:AAF8783]	BOLD:AAF8783	1
<i>Sphyraena</i> [BOLD:ACV9716]	BOLD:ACV9716	1
<i>Sphyraena barracuda</i>	BOLD:AAA6100	1
<i>Sphyraena chrysotaenia</i>	BOLD:AAD0400	1
<i>Sphyraena flavicauda</i>	BOLD:AAF8900	1
<i>Sphyraena putnamae</i>	BOLD:AAB2694	1
<i>Sphyraena qenie</i>	BOLD:AAD8414	1
Clupeiformes		
Chirocentridae		
<i>Chirocentrus</i> cf. <i>dorab</i> [BOLD:AAC2273]	BOLD:AAC2273	1
Clupeidae		

Table 2. Cont.

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp/BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Herklotsichthys</i> [BOLD:AEQ8868]	BOLD:AEQ8868	1	new				1
<i>Dussumieria</i> cf. <i>elopoides</i> [BOLD:AEP5161]	BOLD:AEP5161	2	new		Y	A	2
<i>Herklotsichthys quadrimaculatus</i>	BOLD:AAC2887	8	>1				3
<i>Spratelloides</i> cf. <i>delicatus</i> [BOLD:ACV7999]	BOLD:ACV7999	6	1	>1	Y	A	1
<i>Spratelloides</i> cf. <i>gracilis</i>		0					1
Engraulidae							
<i>Stolephorus belaeius</i>	BOLD:AAG4825	5	1	>1			1
<i>Thryssa baelama</i>	BOLD:ABU9831	1	>1				1
Elopiformes							
Megalopidae							
<i>Megalops cyprinoides</i>	BOLD:AAC4501	1	1	>1			1
Gobiiformes							
Eleotridae							
<i>Eleotris klunzingerii</i>	BOLD:ACV7471	1	1	1			1
Gobiidae							
<i>Amblygobius nocturnus</i>		0					1
<i>Amblygobius semicinctus</i>	BOLD:AAB8727	10	>1				1
<i>Amblygobius sphynx</i>	BOLD:AAJ2321	5	1	>1			1
<i>Asterropteryx</i> [BOLD:AEU6891]	BOLD:AEU6891	1	new				1
<i>Asterropteryx</i> cf. <i>semipunctata</i> [BOLD:AAC0108]	BOLD:AAC0108	12	1	>1	Y	A	1
<i>Bathygobius</i> [BOLD:AAF8787]	BOLD:AAF8787	1	NA				1
<i>Bathygobius cyclopterus</i>	BOLD:AAB9729	1	>1				1
<i>Callogobius flavobrunneus</i>	BOLD:ACV9382	2	1	1			1
<i>Cryptocentrus</i> [BOLD:ADM8879]	BOLD:ADM8879	1	NA				1
<i>Cryptocentrus</i> [BOLD:AEP3749]	BOLD:AEP3749	4	new				1
<i>Cryptocentrus</i> [BOLD:AFC5965]	BOLD:AFC5965	1	new				1
<i>Cryptocentrus cryptocentrus</i>	BOLD:AAM4607	1	1	1			1
<i>Drombus key</i>	BOLD:AEF3691	1	new				0
<i>Favonigobius</i> cf. <i>melanobranchus</i> [BOLD:AAL8921]	BOLD:AAL8921	1	>1		Y	A	1
<i>Favonigobius reichei</i>	BOLD:AAJ4455	1	1	1			1
<i>Gunnellichthys copleyi</i>	BOLD:ADM8209	1	1	1			0
<i>Istigobius</i> cf. <i>decoratus</i> [BOLD:ADM7236]	BOLD:ADM7236	3	NA		Y	A	1
<i>Istigobius</i> cf. <i>ornatus</i> [BOLD:AAD7940]	BOLD:AAD7940	1	1	>1	Y	A	1
<i>Oplopomus</i> [BOLD:AEX8266]	BOLD:AEX8266	1	NA				1
<i>Oplopomus</i> cf. <i>oplopomus</i> [BOLD:AAI3352]	BOLD:AAI3352	13	1	>1	Y	A	2
<i>Palutrus reticularis</i>	BOLD:AAL7906	1	>1				1
<i>Psammogobius biocellatus</i>	BOLD:AAC2888	8	>1				1
<i>Ptereleotris evides</i>	BOLD:AAD9105	1	1	1			1
<i>Valenciennea</i> cf. <i>puellaris</i> [BOLD:AAC4124]	BOLD:AAC4124	1	>1		Y	A	1
<i>Valenciennea helsdingenii</i>	BOLD:AAD4704	1	1	>1	Y	A	1
<i>Valenciennea sexguttata</i>	BOLD:AAC5611	6	1	1			1
<i>Vanderhorstia ornatissima</i>	BOLD:AAF0288	4	1	>1	Y	A	1
<i>Yongeichthys audax</i>	BOLD:AAJ2911	1	1	>1	Y	A	0
<i>Yongeichthys nebulosus</i>	BOLD:AAC3655	2	>1				0
<i>Yongeichthys signatus</i>	BOLD:ACX9631	3	NA				1
Oxudercidae							
<i>Gnatholepis</i> cf. <i>anjerensis</i> [BOLD:AAI5395]	BOLD:AAI5395	10	>1		Y	A	1
<i>Oxyurichthys papuensis</i>	BOLD:ACM4179	3	1	1			2
Holocentriiformes							
Holocentridae							
<i>Myripristis</i> [BOLD:AAA9764]	BOLD:AAA9764	1	>1				0
<i>Myripristis berndti</i>	BOLD:AAA9763	2	>1				1
<i>Myripristis kuntee</i>	BOLD:AAA9765	2	>1				1
<i>Neoniphon sammara</i>	BOLD:AAC8278	7	1	>1	Y	A	2
<i>Sargocentron caudimaculatum</i>	BOLD:AEZ5902	2	>1				1
<i>Sargocentron diadema</i>	BOLD:AAB3424	9	1	>1	Y	A	1
<i>Sargocentron praslin</i>	BOLD:AAC4647	3	>1				1

Table 2. *Cont.*

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. in BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
Kurtiiformes							
Apogonidae							
<i>Apogon</i> [BOLD:AAF8427]	BOLD:AAF8427	5	>1				1
<i>Apogon</i> [BOLD:AAJ8751]	BOLD:AAJ8751	4	1	>1			2
<i>Apogon</i> [BOLD:ACY1702]	BOLD:ACY1702	3	NA				1
<i>Apogon</i> cf. <i>crassiceps</i> [BOLD:ACW9154]	BOLD:ACW9154	3	1	>1	Y	A	1
<i>Apogon erythrosoma</i>	BOLD:ACC5690	6	>1				1
<i>Apogon semiornatus</i>	BOLD:AAD2206	4	1	>1			1
<i>Apogonichthyoides timorensis</i>	BOLD:ACV6948	19	1	>1			1
<i>Apogonichthys</i> [BOLD:ACW8182]	BOLD:ACW8182	2	NA				1
<i>Apogonichthys</i> cf. <i>ocellatus</i> [BOLD:AAL6796]	BOLD:AAL6796	1	1	>1	Y	A	0
<i>Cheilodipterus</i> cf. <i>quiquelineatus</i> [BOLD:ABU8169]	BOLD:ABU8169	5	1	>1	Y	A	1
<i>Cheilodipterus macrodon</i>	BOLD:AAB7874	1	1	>1	Y	A	1
<i>Foa</i> cf. <i>fo</i> [BOLD:ABU8857]	BOLD:ABU8857	20	1	>1	Y	A	1
<i>Foa fo</i>	BOLD:ABU8856	6	1	1			1
<i>Fowleria</i> cf. <i>marmorata</i> [BOLD:AAU0944]	BOLD:AAU0944	1	1	>1	Y	A	0
<i>Fowleria</i> cf. <i>vaiulae</i> [BOLD:AAD1017]	BOLD:AAD1017	1	1	>1	Y	A	1
<i>Fowleria variegata</i>	BOLD:AAD8726	15	1	>1	Y	A	2
<i>Jaydia novaeguineae</i>	BOLD:ACM4616	1	1	1			1
<i>Neamia octospina</i>	BOLD:ACY8539	1	>1				1
<i>Nectamia</i> cf. <i>fusca</i> [BOLD:AAL9262]	BOLD:AAL9262	10	1	>1	Y	A	1
<i>Nectamia</i> cf. <i>savayensis</i> [BOLD:AAD9453]	BOLD:AAD9453	3	>1		Y	A	1
<i>Ostorhinchus</i> [BOLD:AAJ1260]	BOLD:AAJ1260	4	1	>1			1
<i>Ostorhinchus</i> [BOLD:ADI1552]	BOLD:ADI1552	3	NA				1
<i>Ostorhinchus aureus</i>	BOLD:ACE9301	1	1	>1	Y	A	1
<i>Ostorhinchus</i> cf. <i>angustatus</i> [BOLD:AAD5116]	BOLD:AAD5116	2	1	>1	Y	A	2
<i>Ostorhinchus</i> cf. <i>apogonoides</i> [BOLD:AAD5125]	BOLD:AAD5125	1	>1		Y	A	1
<i>Ostorhinchus</i> cf. <i>cooki</i> [BOLD:AAC2084]	BOLD:AAC2084	16	1	>1	Y	A	1
<i>Ostorhinchus</i> cf. <i>cyanosoma</i> [BOLD:ACV9601]	BOLD:ACV9601	14	NA		Y	A	3
<i>Ostorhinchus</i> cf. <i>fasciatus</i> [BOLD:AAC1243]	BOLD:AAC1243	1	1	>1	Y	A	1
<i>Ostorhinchus</i> cf. <i>gularis</i> [BOLD:ACS5956]	BOLD:ACS5956	2	1	>1	Y	A	1
<i>Ostorhinchus</i> cf. <i>taeniophorus</i> [BOLD:AAD8453]	BOLD:AAD8453	1	>1		Y	S	1
<i>Ostorhinchus</i> cf. <i>taeniophorus</i> [BOLD:AAD8454]	BOLD:AAD8454	7	1	>1	Y	S	1
<i>Ostorhinchus flagelliferus</i>	BOLD:AAJ1254	2	1	1			1
<i>Ostorhinchus fleurieu</i>	BOLD:AAD5600	5	>1				1
<i>Pristiapogon</i> cf. <i>fraenatus</i> [BOLD:AAJ1264]	BOLD:AAJ1264	3	1	>1	Y	A	1
<i>Pristiapogon kallopterus</i>	BOLD:AAB4082	5	1	>1	Y	A	1
<i>Pseudamia</i> [BOLD:AFB6766]	BOLD:AFB6766	3	new				2
<i>Siphamia</i> [BOLD:ADO4802]	BOLD:ADO4802	1	NA				0
<i>Siphamia mossambica</i>	BOLD:AEF3435	1	NA				0
<i>Taeniamia</i> cf. <i>fucata</i> [BOLD:AAB8394]	BOLD:AAB8394	10	>1		Y	A	1
<i>Taeniamia flavofasciata</i>	BOLD:AAU1539	5	1	1			1
<i>Zoramia</i> cf. <i>leptacanthus</i> [BOLD:AAU1535]	BOLD:AAU1535	4	>1		Y	A	1
Lophiiformes							
Antennariidae							
<i>Antennariidae</i>		0					1
<i>Antennarius</i> cf. <i>striatus</i> [BOLD:AAO6018]	BOLD:AAO6018	3	1	>1	Y	A	0
Moroniiformes							
Ephippidae							
<i>Platax orbicularis</i>	BOLD:AAC6496	6	1	1			1
<i>Platax teira</i>	BOLD:AAC5812	1	1	>1			1
Mugiliformes							
Mugilidae							
<i>Crenimugil buehanani</i>	BOLD:AAE3561	1	>1				0
<i>Moolgarda crenilabis</i>	BOLD:AAG6597	3	>1				2
<i>Mugil</i> cf. <i>cephalus</i> [BOLD:AAA7833]	BOLD:AAA7833	8	>1		Y	A	1
Ovalentaria							
Ambassidae							

Table 2. Cont.

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp/BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Ambassis dussumieri</i>	BOLD:AAJ2348	2	>1				1
Plesiopidae							
<i>Plesiops coeruleolineatus</i>	BOLD:AAE4785	5	1	>1			1
Pomacentridae							
<i>Abudefduf sparoides</i>	BOLD:AAD7433	2	1	1			1
<i>Abudefduf vaigiensis</i>	BOLD:ACK8109	6	>1				1
<i>Amblyglyphidodon</i> [BOLD:ACF1984]	BOLD:ACF1984	2	1	>1			1
<i>Amblypomacentrus annulatus</i>	BOLD:AAF2723	12	1	1			1
<i>Amphiprion allardi</i>	BOLD:AAC4980	2	1	1			1
<i>Chromis</i> [BOLD:ACF0042]	BOLD:ACF0042	4	>1				1
<i>Chromis atripectoralis</i>	BOLD:AAB9018	1	1	1			0
<i>Chromis</i> cf. <i>ternatensis</i> [BOLD:AAC6972]	BOLD:AAC6972	1	1	>1	Y	A	0
<i>Chromis fieldi</i>	BOLD:AAC0458	1	>1				1
<i>Chromis ternatensis</i>	BOLD:AAF3462	1	1	>1	Y	A	1
<i>Chromis viridis</i>	BOLD:AAB4985	7	1	1			1
<i>Chrysiptera</i> cf. <i>brownriggii</i> [BOLD:AAB6234]	BOLD:AAB6234	2	>1		Y	A	0
<i>Dascyllus abudafur</i>	BOLD:AAB4091	6	>1				1
<i>Dascyllus carneus</i>	BOLD:AAD7114	5	1	>1	Y	A	0
<i>Dascyllus trimaculatus</i>	BOLD:AAB2685	13	>1				1
<i>Neoglyphidodon melas</i>	BOLD:AAC5328	1	1	1			1
<i>Neopomacentrus cyanomos</i>	BOLD:ABX6100	1	1	>1	Y	A	1
<i>Neopomacentrus fuliginosus</i>	BOLD:AAI3160	9	>1				0
<i>Neopomacentrus sororius</i>	BOLD:AAC8318	6	>1				1
<i>Plectroglyphidodon</i> cf. <i>lacrymatus</i> [BOLD:AAB6988]	BOLD:AAB6988	8	1	>1	Y	A	1
<i>Plectroglyphidodon dickii</i>	BOLD:ACC1132	1	1	1			1
<i>Pomacentrus aquilus</i>	BOLD:AAJ3550	33	>1				1
<i>Pomacentrus baenschi</i>	BOLD:AAC9672	1	1	1			0
<i>Pomacentrus caeruleus</i>	BOLD:AAB9539	5	>1				1
<i>Pomacentrus sulfureus</i>	BOLD:AAD2952	2	1	1			0
<i>Pycnchromis nigrurus</i>	BOLD:AAD5022	1	1	1			1
Pseudochromidae							
<i>Halidesmus</i> [BOLD:AEU2441]	BOLD:AEU2441	1	new				1
<i>Halidesmus</i> [BOLD:AAU1500]	BOLD:AAU1500	1	NA				0
<i>Pseudochromis</i> cf. <i>kristinae</i> [BOLD:ADI5215]	BOLD:ADI5215	11	NA		Y	A	2
<i>Pseudochromis madagascariensis</i>	BOLD:AAU3299	3	1				1
Perciformes							
Caesionidae							
<i>Caesio</i> cf. <i>caeruleaurea</i> [BOLD:AAB4823]	BOLD:AAB4823	12	>1		Y	S	2
<i>Caesio</i> cf. <i>caeruleaurea</i> [BOLD:AAB4822]	BOLD:AAB4822	2	>1		Y	S	1
<i>Caesio lunaris</i>	BOLD:AAJ8622	1	1	1			0
<i>Caesio xanthonota</i>	BOLD:AAE8330	1	1	1			0
<i>Dipterygonotus balteatus</i>	BOLD:AAD3666	1	1	1			1
<i>Pterocaesio chrysozona</i>	BOLD:AAE8474	1	>1				0
<i>Pterocaesio marri</i>	BOLD:AAC2204	3	>1				1
<i>Pterocaesio trilineata</i>	BOLD:AAE8473	1	>1				1
Chaetodontidae							
<i>Chaetodon auriga</i>	BOLD:AAB1540	7	1	1			2
<i>Chaetodon blackburnii</i>	BOLD:AAE1178	3	1	1			1
<i>Chaetodon</i> cf. <i>bennetti</i> [BOLD:ACE8647]	BOLD:ACE8647	1	1	>1	Y	A	1
<i>Chaetodon guttatissimus</i>	BOLD:AAD3872	1	>1				1
<i>Chaetodon kleinii</i>	BOLD:AAC2841	3	>1				1
<i>Chaetodon lineolatus</i>	BOLD:ABZ0931	1	1	1			1
<i>Chaetodon lunula</i>	BOLD:AAB6339	2	>1				1
<i>Chaetodon madagaskariensis</i>	BOLD:AAC2634	2	>1				1
<i>Chaetodon melanotus</i>	BOLD:AAC2848	5	>1				1
<i>Chaetodon trifasciatus</i>	BOLD:AAB7102	5	>1				2

Table 2. Cont.

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Chaetodon ulietensis</i>	BOLD:AAC8636	1	>1				1
<i>Chaetodon vagabundus</i>	BOLD:AAB3198	8	1	1			2
<i>Chaetodon xanthocephalus</i>	BOLD:AAE1213	5	1	1			1
<i>Chaetodon zanzibarensis</i>	BOLD:AAE9260	1	1	1			1
<i>Heniochus</i> cf. <i>acuminatus</i> [BOLD:AAB5716]	BOLD:AAB5716	4	>1		Y	A	1
<i>Heniochus diphreutes</i>	BOLD:AAB5717	1	>1				1
<i>Heniochus monoceros</i>	BOLD:AAC7417	1	1	1			1
Cirrhitidae							
<i>Cirrhitus</i> cf. <i>pinnulatus</i> [BOLD:AAC5875]	BOLD:AAC5875	2	>1		Y	A	2
<i>Cyprinocirrhites</i> cf. <i>polyactis</i> [BOLD:AAF8813]	BOLD:AAF8813	1	1	>1	Y	A	1
<i>Paracirrhites arcatus</i>	BOLD:AAC6007	2	1	1			1
<i>Paracirrhites forsteri</i>	BOLD:AAC5873	1	>1				1
Gerreidae							
<i>Gerres</i> cf. <i>filamentosus</i> [BOLD:AAC0377]	BOLD:AAC0377	3	>1		Y	A	0
<i>Gerres filamentosus</i>	BOLD:AAC0380	2	>1				1
<i>Gerres longirostris</i>	BOLD:AAE6359	8	1	>1	Y	A	3
<i>Gerres oblongus</i>	BOLD:AAE6346	2	1	1			1
<i>Gerres oyena</i>	BOLD:AAC1291	2	1	>1	Y	A	0
Haemulidae							
<i>Diagramma</i> [BOLD:AAD4477]	BOLD:AAD4477	12	>1				2
<i>Plectorhinchus</i> cf. <i>vittatus</i> [BOLD:AAE4709]	BOLD:AAE4709	1	1	>1	Y	A	1
<i>Plectorhinchus</i> [BOLD:AAF8797]	BOLD:AAF8797	1	>1				1
<i>Plectorhinchus flavomaculatus</i>	BOLD:AAC4020	10	1	1			1
<i>Plectorhinchus gaterinus</i>	BOLD:AAH9156	12	1	1			1
<i>Plectorhinchus plagiodesmus</i>	BOLD:ACC0439	1	1	1			0
<i>Pomadasyd</i> cf. <i>furcatus</i> [BOLD:AAD1382]	BOLD:AAD1382	1	1	>1	Y	A	1
Kyphosidae							
<i>Kyphosus cinerascens</i>	BOLD:ABX5727	1	>1				1
<i>Kyphosus vaigiensis</i>	BOLD:AAC3456	2	>1				1
Labridae							
<i>Anampses caeruleopunctatus</i>	BOLD:AAB9969	1	>1				0
<i>Bodianus</i> cf. <i>perditio</i> [BOLD:AAC7631]	BOLD:AAC7631	1	1	>1	Y	A	1
<i>Cheilinus</i> cf. <i>chlorourus</i> [BOLD:AAB4186]	BOLD:AAB4186	8	>1		Y	A	1
<i>Cheilinus oxycephalus</i>	BOLD:AAB4187	3	>1				1
<i>Cheilinus trilobatus</i>	BOLD:AAB4188	5	>1				1
<i>Cheilinus undulatus</i>	BOLD:AAF3078	1	1	1			1
<i>Cheilio inermis</i>	BOLD:AAA6101	5	1	1			3
<i>Coris caudimacula</i>	BOLD:AAC2101	4	1	1			1
<i>Cymolutes</i> cf. <i>torquatus</i> [BOLD:ADM8951]	BOLD:ADM8951	1	1	>1	Y	A	1
<i>Cymolutes praetextatus</i>	BOLD:AAF7751	7	1	>1			1
<i>Cymolutes torquatus</i>	BOLD:AAI9445	3	>1				1
<i>Epibulus</i> cf. <i>insidiator</i> [BOLD:AAB8858]	BOLD:AAB8858	8	1	>1	Y	S	1
<i>Epibulus</i> cf. <i>insidiator</i> [BOLD:ADU6797]	BOLD:ADU6797	1	1	>1	Y	S	1
<i>Gomphosus caeruleus</i>	BOLD:AAD5965	2	1	1			2
<i>Halichoeres</i> [BOLD:AAT9960]	BOLD:AAT9960	1	>1				1
<i>Halichoeres</i> cf. <i>hortulanus</i> [BOLD:AAB3085]	BOLD:AAB3085	2	1	>1	Y	A	1
<i>Halichoeres</i> cf. <i>nebulosus</i> [BOLD:AAC7896]	BOLD:AAC7896	3	1	>1	Y	A	1
<i>Halichoeres</i> cf. <i>zeylonicus</i> [BOLD:AAF7654]	BOLD:AAF7654	5	1	>1	Y	A	1
<i>Halichoeres lapillus</i>	BOLD:AAE3021	1	1	1			1
<i>Halichoeres scapularis</i>	BOLD:AAC0139	6	1	>1	Y	A	1
<i>Halichoeres timorensis</i>	BOLD:AAU1265	1	>1				1
<i>Hemigymnus</i> cf. <i>fasciatus</i> [BOLD:ACE5917]	BOLD:ACE5917	1	1	>1	Y	A	0
<i>Hemigymnus melapterus</i>	BOLD:AAB5264	1	1	1			1
<i>Hologymnosus doliatus</i>	BOLD:AAD1381	1	>1				1
<i>Iniistius bimaculatus</i>	BOLD:ACT0048	2	1	1			1
<i>Iniistius naevus</i>	BOLD:ACY5613	1	new				0
<i>Iniistius pavo</i>	BOLD:AAD8586	1	1	>1			0

Table 2. Cont.

Class	COI					12S
Order						
Family						
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp. N 12S in MADFI
<i>Novaculichthys taeniourus</i>	BOLD:AAB3259	3	1	1		1
<i>Novaculoides macrolepidotus</i>	BOLD:AAD8577	15	1	1		1
<i>Oxycheilinus</i> cf. <i>bimaculatus</i> [BOLD:AAC3195]	BOLD:AAC3195	26	>1		Y	A
<i>Oxycheilinus digramma</i>	BOLD:AAC0639	1	1	1		1
<i>Pseudojuloides argyreogaster</i>	BOLD:ADI3805	6	1	1		1
<i>Pteragogus flagellifer</i>	BOLD:AAZ4093	11	>1			1
<i>Pteragogus taeniops</i>	BOLD:AAV2736	1	1	1		1
<i>Pteragogus trispilus</i>	BOLD:ADI3844	2	1	1		1
<i>Stethojulis albovittata</i>	BOLD:AAD4824	8	1	1		1
<i>Stethojulis interrupta</i>	BOLD:AAC2336	7	>1			1
<i>Stethojulis strigiventer</i>	BOLD:AAE2186	12	1	1		3
<i>Stethojulis</i>		0				1
<i>Thalassoma amblycephalum</i>	BOLD:ADI4249	1	>1			1
<i>Thalassoma hardwicke</i>	BOLD:AAB0061	2	1	1		1
<i>Thalassoma hebraicum</i>	BOLD:AAC2788	2	1	1		1
<i>Thalassoma lunare</i>	BOLD:AAB0673	4	1	1		2
<i>Thalassoma trilobatum</i>	BOLD:AAC2785	1	>1			1
Leiognathidae						
<i>Aurigequula fasciata</i>	BOLD:AAB2489	1	>1			0
<i>Deveximentum</i> cf. <i>insidiator</i> [BOLD:ACF0053]	BOLD:ACF0053	1	1	>1	Y	A
<i>Equulites klunzingeri</i>	BOLD:AAC0238	1	>1			0
<i>Equulites</i> [BOLD:AAX8534]	BOLD:AAX8534	1	NA			0
<i>Gazza</i> [BOLD:AAB7096]	BOLD:AAB7096	2	>1			0
Lutjanidae						
<i>Aprion virescens</i>	BOLD:AAB8692	1	1	1		1
<i>Lutjanus argentimaculatus</i>	BOLD:AAB2440	3	1	1		1
<i>Lutjanus bengalensis</i>	BOLD:AAB7901	1	>1			1
<i>Lutjanus bohar</i>	BOLD:AAB4501	2	>1			0
<i>Lutjanus</i> cf. <i>fulvus</i> [BOLD:AAB7015]	BOLD:AAB7015	2	1	>1	Y	A
<i>Lutjanus</i> cf. <i>kasmira</i> [BOLD:ACC0930]	BOLD:ACC0930	4	>1		Y	A
<i>Lutjanus</i> cf. <i>lutjanus</i> [BOLD:AAA8168]	BOLD:AAA8168	4	>1		Y	A
<i>Lutjanus fulviflamma</i>	BOLD:ADF5681	18	>1			2
<i>Lutjanus gibbus</i>	BOLD:AAB3276	10	>1			1
<i>Lutjanus monostigma</i>	BOLD:AAB2907	1	>1			1
<i>Lutjanus notatus</i>	BOLD:AAF7731	6	>1			1
<i>Lutjanus rivulatus</i>	BOLD:AAB7684	1	1	>1		1
<i>Lutjanus sebae</i>	BOLD:AAB5388	1	1	1		1
Monodactylidae						
<i>Monodactylus argenteus</i>	BOLD:AAA9698	3	1	>1	Y	A
Mullidae						
<i>Mulloidichthys flavolineatus</i>	BOLD:AAB2592	3	>1			2
<i>Parupeneus</i> [BOLD:AAB2590]	BOLD:AAB2590	15	>1			1
<i>Parupeneus</i> [BOLD:ADM7122]	BOLD:ADM7122	2	NA			1
<i>Parupeneus</i> cf. <i>barberinus</i> [BOLD:AAB5978]	BOLD:AAB5978	7	>1		Y	S
<i>Parupeneus</i> cf. <i>barberinus</i> [BOLD:AAB5980]	BOLD:AAB5980	6	>1		Y	S
<i>Parupeneus cyclostomus</i>	BOLD:AAD1433	2	>1			0
<i>Parupeneus fraserorum</i>	BOLD:AAF8776	2	1	1		1
<i>Parupeneus indicus</i>	BOLD:AAB0334	5	1	1		1
<i>Parupeneus macronemus</i>	BOLD:ACF0223	7	>1			1
<i>Parupeneus pleurostigma</i>	BOLD:AAD6266	6	1	1		1
<i>Parupeneus rubescens</i>	BOLD:AAC1405	6	1	1		1
<i>Parupeneus trifasciatus</i>	BOLD:AAI4266	2	1	1		1
<i>Upeneus</i> cf. <i>margarethae</i> [BOLD:AAB9714]	BOLD:AAB9714	3	>1		Y	A
<i>Upeneus guttatus</i>	BOLD:AAH7551	1	>1			1
<i>Upeneus moluccensis</i>	BOLD:AAB6469	4	>1			1
<i>Upeneus pori</i>	BOLD:AAC1406	4	>1			1
<i>Upeneus supravittatus</i>	BOLD:ABZ7416	3	>1			1

Table 2. Cont.

Class	COI					12S
Order						
Family						
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	N 12S in MADFI
<i>Upeneus tragula</i>	BOLD:AAB9327	1	>1			1
<i>Upeneus vittatus</i>	BOLD:ACV4665	3	>1			1
Pempheridae						
<i>Parapriacanthus</i> [BOLD:AAC7599]	BOLD:AAC7599	3	>1			1
<i>Pempheris connelli</i>	BOLD:AAC6084	1	1	1		1
<i>Pempheris ibo</i>	BOLD:AAF8820	3	>1			0
<i>Pempheris mangula</i>	BOLD:AAD1777	3	>1			1
Polynemidae						
<i>Polydactylus plebeius</i>	BOLD:AAC6244	4	1	>1		0
<i>Polydactylus sextarius</i>	BOLD:AAB7311	1	>1			0
Pomacanthidae						
<i>Centropyge multispinis</i>	BOLD:AAD3135	2	1	1		1
<i>Pomacanthus chrysurus</i>	BOLD:AAL9941	1	1	1		1
<i>Pomacanthus semicirculatus</i>	BOLD:ACK8181	5	1	1		1
Priacanthidae						
<i>Heteropriacanthus carolinus</i>	BOLD:AAB1874	2	>1			1
<i>Priacanthus</i> cf. <i>arenatus</i> [BOLD:AAB1642]	BOLD:AAB1642	2	>1		Y	1
<i>Priacanthus hamrur</i>	BOLD:AAB1643	10	>1			1
Scaridae						
<i>Calotomus carolinus</i>	BOLD:ADI4703	9	>1			1
<i>Calotomus spinidens</i>	BOLD:AAD4765	23	1	1		1
<i>Chlorurus atrilunula</i>	BOLD:AAE8961	2	>1			1
<i>Chlorurus cyanescens</i>	BOLD:AEW3945	2	1	1		1
<i>Leptoscarus vaigiensis</i>		0				3
<i>Scarus</i> [BOLD:AAD0849]	BOLD:AAD0849	18	>1			2
<i>Scarus</i> cf. <i>ghobban</i> [BOLD:ABY4451]	BOLD:ABY4451	12	1	>1	Y	2
<i>Scarus psittacus</i>	BOLD:AAB8901	6	1	1		1
<i>Scarus scaber</i>	BOLD:AAE9524	1	>1			1
Serranidae						
<i>Aethaloperca rogaa</i>	BOLD:AAD9665	3	1	>1	Y	1
<i>Cephalopholis argus</i>	BOLD:AAC4474	1	1	>1		1
<i>Cephalopholis</i> cf. <i>sonnerati</i> [BOLD:AAB5431]	BOLD:AAB5431	1	1	>1	Y	1
<i>Cephalopholis miniata</i>	BOLD:AAC0216	2	1	1		1
<i>Cephalopholis nigripinnis</i>	BOLD:AAC4202	1	>1			1
<i>Epinephelus areolatus</i>	BOLD:AAA9822	2	>1			1
<i>Epinephelus coeruleopunctatus</i>	BOLD:ADL0994	2	>1			1
<i>Epinephelus fasciatus</i>	BOLD:AAB1334	1	1	>1	Y	1
<i>Epinephelus flavocaeruleus</i>	BOLD:AAD1767	2	>1			1
<i>Epinephelus longispinis</i>	BOLD:AAD8800	1	>1			1
<i>Epinephelus macrospilos</i>	BOLD:AAE1882	2	1	>1	Y	1
<i>Epinephelus merra</i>	BOLD:AAB8387	1	>1			1
<i>Epinephelus rivulatus</i>	BOLD:ACZ9919	5	1	>1	Y	1
<i>Epinephelus spilotoceps</i>	BOLD:AAC3590	1	1	>1		1
<i>Plectropomus punctatus</i>	BOLD:AAN4283	3	1	1		1
<i>Variola louti</i>	BOLD:AAC5719	1	1	>1	Y	1
Siganidae						
<i>Siganus argenteus</i>	BOLD:AAC6461	2	1	>1		2
<i>Siganus luridus</i>	BOLD:AAL9467	2	>1			0
<i>Siganus stellatus</i>	BOLD:AAB2341	4	>1			1
<i>Siganus sutor</i>	BOLD:AAB6556	20	>1			1
Terapontidae						
<i>Pelates quadrilineatus</i>	BOLD:AAA9700	2	>1			1
<i>Terapon</i> [BOLD:ACV8977]	BOLD:ACV8977	1	>1			1
<i>Terapon jarbua</i>	BOLD:AAA9351	1	1	>1	Y	1
Pleuronectiformes						
Bothidae						
<i>Bothus</i> cf. <i>pantherinus</i> [BOLD:AAC9155]	BOLD:AAC9155	13	>1		Y	1
<i>Crossorhombus valderostratus</i>	BOLD:AAF8808	1	1	1		1

Table 2. Cont.

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Engyprosopon</i> [BOLD:AD1616]	BOLD:ADI1616	5	NA				1
<i>Engyprosopon</i> [BOLD:ADM7915]	BOLD:ADM7915	3	NA				1
<i>Engyprosopon</i> [BOLD:AFI7142]	BOLD:AFI7142	1	new				0
Cynoglossidae							
<i>Cynoglossus</i> [BOLD:AFB7676]	BOLD:AFB7676	1	new				1
Soleidae							
<i>Pardachirus marmoratus</i>	BOLD:AAI5984	1	1	1			0
Scombriformes							
Nomeidae							
<i>Psenes cyanophrys</i>	BOLD:AAE0701	1	>1				1
Scombridae							
<i>Auxis thazard</i>	BOLD:AAB0928	1	>1				1
<i>Rastrelliger kanagurta</i>	BOLD:AAA9666	1	>1				1
Trichiuridae							
<i>Trichiurus</i> cf. <i>lepturus</i> [BOLD:AAB0165]	BOLD:AAB0165	2	>1		Y	A	2
Scorpaeniformes							
Platycephalidae							
<i>Papilloculiceps longiceps</i>	BOLD:AAI6160	20	1	>1			2
<i>Platycephalus</i> [BOLD:AAB2371]	BOLD:AAB2371	5	1	>1			1
<i>Rogadius</i> cf. <i>pristiger</i> [BOLD:ACY6212]	BOLD:ACY6212	10	1	>1	Y	A	1
<i>Sunagocia otaitensis</i>	BOLD:AAD2134	1	1	1			1
Scorpaenidae							
<i>Ablabys binotatus</i>	BOLD:AAF8834	2	1	1			0
<i>Dendrochirus</i> cf. <i>brachypterus</i> [BOLD:AAC9564]	BOLD:AAC9564	6	1	>1	Y	A	1
<i>Parascorpaena</i> [BOLD:AAU1214]	BOLD:AAU1214	7	NA				1
<i>Pterois miles</i>	BOLD:AAB8146	5	>1				2
<i>Scorpaenopsis</i> cf. <i>poss</i> [BOLD:AAD4142]	BOLD:AAD4142	1	1	>1	Y	A	1
<i>Scorpaenopsis diabolus</i>	BOLD:AAE1598	3	1	1			1
<i>Scorpaenopsis longispina</i>	BOLD:AAD7048	2	>1				1
<i>Scorpaenopsis venosa</i>	BOLD:AAD9168	3	1	1			1
<i>Sebastapistes</i> cf. <i>strongia</i> [BOLD:AAC4542]	BOLD:AAC4542	14	1	>1	Y	S	1
<i>Sebastapistes</i> cf. <i>strongia</i> [BOLD:AAC4543]	BOLD:AAC4543	41	1	>1	Y	S	1
<i>Synanceia verrucosa</i>	BOLD:AAE2821	3	>1				1
Siluriformes							
Plotosidae							
<i>Plotosus</i> cf. <i>lineatus</i> [BOLD:ACF3921]	BOLD:ACF3921	8	1	>1	Y	A	2
Spariformes							
Lethrinidae							
<i>Lethrinus</i>		0					1
<i>Gnathodentex aureolineatus</i>	BOLD:AAC8927	1	1	1			1
<i>Gymnocranius</i> cf. <i>microdon</i> [BOLD:AAB5193]	BOLD:AAB5193	1	1	>1	Y	A	1
<i>Gymnocranius elongatus</i>	BOLD:AAB5194	3	>1				1
<i>Lethrinus</i> [BOLD:AAC1547]	BOLD:AAC1547	5	NA				2
<i>Lethrinus</i> [BOLD:AAC8078]	BOLD:AAC8078	19	>1				1
<i>Lethrinus borbonicus</i>	BOLD:AAB0511	50	>1				3
<i>Lethrinus</i> cf. <i>lentjan</i> [BOLD:ABZ0131]	BOLD:ABZ0131	11	>1		Y	A	1
<i>Lethrinus</i> cf. <i>nebulosus</i> [BOLD:ABY6363]	BOLD:ABY6363	12	1	>1	Y	A	0
<i>Lethrinus harak</i>	BOLD:AAC1521	34	1	>1	Y	A	2
<i>Lethrinus mahsena</i>	BOLD:AAB6438	24	>1				1
<i>Lethrinus obsoletus</i>	BOLD:AAC1522	1	>1				1
<i>Lethrinus rubrioperculatus</i>	BOLD:AAB6439	2	>1				1
<i>Lethrinus xanthochilus</i>	BOLD:AAC1546	4	1	>1			1
<i>Monotaxis grandoculis</i>	BOLD:ABZ0166	1	1	>1	Y	A	1
Lobotidae							
<i>Lobotes surinamensis</i>	BOLD:AAC1878	1	1	1			0
Nemipteridae							
<i>Nemipterus bipunctatus</i>	BOLD:AAF2507	1	>1				1

Table 2. Cont.

Class	COI					12S	
Order							
Family							
Species	BIN	N COI in MADFI	Nsp/BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Nemipterus elaine</i>	BOLD:AEH3406	2	NA				1
<i>Nemipterus zysron</i>	BOLD:AAD1868	1	>1				0
<i>Scolopsis</i> [BOLD:AAC3574]	BOLD:AAC3574	1	>1				0
<i>Scolopsis bimaculata</i>	BOLD:AAD6249	4	1	1			0
<i>Scolopsis ghanam</i>	BOLD:AAC4767	5	1	1			0
Sillaginidae							
<i>Sillago</i> cf. <i>sihama</i> [BOLD:AAA7598]	BOLD:AAA7598	1	1	>1	Y	A	1
Sparidae							
<i>Argyrops spinifer</i>	BOLD:AAB3720	1	1	>1			1
<i>Crenidens</i> cf. <i>crenidens</i> [BOLD:AAE4408]	BOLD:AAE4408	4	1	>1	Y	A	1
Syngnathiiformes							
Aulostomidae							
<i>Aulostomus chinensis</i>	BOLD:AAB9194	1	1	1			0
Callionymidae							
<i>Callionymus</i> [BOLD:AAI6959]	BOLD:AAI6959	2	>1				1
<i>Callionymus</i> [BOLD:ADN1799]	BOLD:ADN1799	2	NA				1
<i>Callionymus</i> cf. <i>filamentosus</i> [BOLD:ACZ1215]	BOLD:ACZ1215	4	1	>1	Y	A	1
<i>Callionymus</i>		0					2
<i>Diplogrammus infulatus</i>	BOLD:AAN1422	4	1	1			2
Centriscidae							
<i>Aeoliscus punctulatus</i>	BOLD:AAD3585	4	1	1			1
Dactylopteridae							
<i>Dactyloptena orientalis</i>	BOLD:AAB5966	9	>1				1
Fistulariidae							
<i>Fistularia commersonii</i>	BOLD:AAB5992	7	1	1			1
Pegasidae							
<i>Eurypegasus draconis</i>		0					1
Solenostomidae							
<i>Solenostomus</i> cf. <i>cyanopterus</i> [BOLD:ACG8874]	BOLD:ACG8874	4	1	>1	Y	A	1
Syngnathidae							
<i>Acentronura</i> [BOLD:ADI1740]	BOLD:ADI1740	7	NA				1
<i>Corythoichthys</i> cf. <i>conspicillatus</i> [BOLD:AAI8860]	BOLD:AAI8860	7	>1		Y	A	1
<i>Corythoichthys haematopterus</i>	BOLD:AAF0628	8	>1				2
<i>Hippichthys</i> [BOLD:AAE5342]	BOLD:AAE5342	5	>1				2
<i>Hippichthys</i> cf. <i>cyanospilos</i> [BOLD:AAK6079]	BOLD:AAK6079	11	1	>1	Y	A	3
<i>Hippocampus</i> [BOLD:ACE6993]	BOLD:ACE6993	8	>1				1
<i>Hippocampus camelopardalis</i>	BOLD:AAX0875	20	1	1			3
<i>Hippocampus</i> cf. <i>histris</i> [BOLD:AAE5356]	BOLD:AAE5356	1	1	>1	Y	A	1
<i>Syngnathidae</i> [BOLD:AEF4074]	BOLD:AEF4074	1	new				0
<i>Syngnathoides biaculeatus</i>	BOLD:AAD7359	2	>1				3
<i>Trachyrhamphus</i> cf. <i>bicoarctatus</i> [BOLD:ADG5410]	BOLD:ADG5410	2	NA		Y	A	1
Tetraodontiformes							
Balistidae							
<i>Balistapus undulatus</i>	BOLD:AAC2755	2	1	1			2
<i>Balistoides viridescens</i>	BOLD:AAD0474	8	>1				1
<i>Pseudobalistes fuscus</i>	BOLD:ACZ6203	5	1	1			1
<i>Rhinecanthus aculeatus</i>	BOLD:AAB6992	2	1	1			1
<i>Rhinecanthus rectangularis</i>	BOLD:AAD6918	1	1	1			1
<i>Sufflamen chrysopteron</i>	BOLD:AAB1339	3	>1				1
Diodontidae							
<i>Lophodiodon calori</i>	BOLD:AEW6267	1	new	NA			0
Monacanthidae							
<i>Amanses</i> cf. <i>scopas</i> [BOLD:ADX8121]	BOLD:ADX8121	1	1	>1	Y	A	1
<i>Cantherhines pardalis</i>	BOLD:AAB9564	4	>1				1
<i>Paraluteres prionurus</i>	BOLD:AAC3185	1	1				1
<i>Paramonacanthus frenatus</i>	BOLD:AAI6357	14	1	1			1

Table 2. Cont.

Class		COI					12S
Order							
Family							
Species	BIN	N COI in MADFI	Nsp./BIN in BOLD	NBIN/Sp. In BOLD	Sp. Complex	Allo./Symp.	N 12S in MADFI
<i>Paramonacanthus pusillus</i>	BOLD:AAV6227	2	1	>1	Y	A	1
<i>Pervagor melanocephalus</i>	BOLD:AAD6159	2	>1				1
<i>Pseudalutarius cf. nasicornis</i> [BOLD:AAI4531]	BOLD:AAI4531	9	>1		Y	A	0
Ostraciidae							
<i>Lactoria cornuta</i>	BOLD:AAB2988	8	1	1			1
<i>Lactoria fornasini</i>	BOLD:AAF2668	2	>1				1
<i>Ostracion cubicus</i>	BOLD:AAC2246	5	>1				1
<i>Tetrosomus concatenatus</i>	BOLD:AAD3999	1	>1				1
Tetraodontidae							
<i>Arothron hispidus</i>	BOLD:AAB9202	18	>1				3
<i>Arothron immaculatus</i>	BOLD:AAD2857	2	>1				1
<i>Arothron stellatus</i>	BOLD:AAC8066	2	1	1			1
<i>Canthigaster bennetti</i>	BOLD:AAC6218	3	1	1			2
<i>Canthigaster petersii</i>	BOLD:AAD2222	7	>1				1
<i>Canthigaster punctatissima</i>	BOLD:ACC1190	2	>1				1
<i>Canthigaster valentini</i>	BOLD:AAC9721	1	1	1			0
<i>Lagocephalus</i> [BOLD:AAC5565]	BOLD:AAC5565	1	>1				1
<i>Torquigener flavimaculosus</i>	BOLD:AAG3690	5	>1				1
<i>Torquigener hypselogeneion</i>	BOLD:ADC5749	1	1	1			0
Trachiniformes							
Pinguipedidae							
<i>Parapercis hexophthalma</i>	BOLD:AAD6753	3	1	1			1
<i>Parapercis maculata</i>	BOLD:AAE3727	3	1	>1			2
Trichonotidae							
<i>Trichonotus</i> [BOLD:ACG8296]	BOLD:ACG8296	1	NA				0
Uranoscopidae							
<i>Uranoscopus guttatus</i>	BOLD:ACX9882	1	NA				1
Elasmobranchii							
Myliobatiformes							
Dasyatidae							
<i>Neotrygon indica</i>	BOLD:AAA5611	2	>1				1
Pristiiformes							
Rhinobatidae							
<i>Acroteriobatus andysabini</i>	BOLD:AAG4398	4	>1				2
Torpediniformes							
Torpedinidae							
<i>Torpedo</i> [BOLD:AAU1234]	BOLD:AAU1234	3	1	>1			1
Total		2146					524

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d17070495/s1>, Table S1: Detailed information on all fish BINs from Madagascar in the MADFI database on the BOLD system. Information presented: the names associated with each BIN in BOLD, the number of BINs linked to a single species name (in cases where BINs are assigned to only one species name). The type localities of the BINs according to the Catalog of Fishes, the hypothetical species name assigned after morphological identification, the final species name attributed to each BIN, as well as comments regarding each BIN and its level of identification. The numbers in brackets in column E indicate the number of specimens available in BOLD for each species associated with each BIN listed in the MADFI database. The numbers in brackets in column F indicate the number of BINs corresponding to the species name when only one species name is in column E. Table S2: Details of the main characteristics of the 424 fish species validated by WoRMS and the 502 BINs recorded in the MADFI container on BOLD. The information presented includes: taxonomic novelty, biogeographic distribution of the recorded species,

species status according to the IUCN Red List, and the distribution of species based on their habitats and ecological lifestyles, according to WoRMS (World Register of Marine Species) data.

Author Contributions: Project leader: J.-D.D.; Conceptualization, J.J.A.V., J.-D.D., D.R. and D.P.; Development of the data collection protocol: J.J.A.V., J.-D.D., D.P., M.L., D.R., A.C., H.J., F.B., S.R. and A.E.M.F.; Data collection: J.J.A.V., A.C., H.J., F.B., R.M.R., S.R., J.M. (Jovial Mbonny) and A.E.M.F.; Preparation of sample export permits: J.J.A.V., J.-D.D., D.P., A.C., H.J., S.R., A.E.M.F., J.M. (Jamal Mahafina) and G.T.; Molecular laboratory analyses: J.J.A.V., J.-D.D., F.R., H.J. and A.C.; Data analyses: J.J.A.V., J.-D.D. and E.R. Writing—original draft preparation: J.J.A.V. and J.-D.D.; Writing—review and editing: J.J.A.V., J.-D.D., E.R., D.P., A.H., M.L., H.J., A.C. and S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded through the JEAI-ACOM/IH.SM/IRD grant and the International Laboratory (LMI) MIKAROKA, funded by Institut de Recherche pour le Développement (IRD), France. Additional support came from the Belmont Forum by the ARMSRestore project in Madagascar via the National Science Foundation (RISE-2022717 to AH), the South African National Research Foundation (BF-CRA 12854 to GT), Svenska FORMAS (2019-02394), the Rose Service Learning Fellowship at Harvard University, Good Planet Foundation. The University of Montpellier through the MARBEC laboratory and the Regional Cooperation Fund (CALMA project) also contributed financial support. The COMAD, RFSIO, and IFBIO projects were funded by the Critical Ecosystem Partnership Fund (grant MG 66341), the Rufford Foundation (grant 2ac29a-1), the Western Indian Ocean Marine Science Association (WIOMSA, MARG II contract 1/2016), and the European Union (grant POE 2.10 POCT FED-FEDER “Biodiversity of the Indian Ocean”).

Institutional Review Board Statement: The specimens analyzed in this study were fin clips from dead fish recovered from artisanal fisheries’ catches (in the areas of Toliara Bay, Ranobe, and the Anakao reef), in accordance with the international Nagoya Protocol, under the following approval codes: ABSCH-CPC-FR-264975-1 and ABSCH-CPC-FR-265446-1.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the BOLD (Barcode of Life Data System) database and will be submitted to GenBank.

Acknowledgments: This study was carried out under a doctoral fellowship from the ARTS program (IRD), within the framework of a research partnership between IH.SM, the MIKAROKA international laboratory, and the MARBEC laboratory at the University of Montpellier (France). We gratefully acknowledge the Belmont Forum for its financial support, which facilitated tissue sample collection and the preparation of export permits as part of the ARMSRestore project. Special thanks go to the administrative staff of IH.SM (Institut Halieutique et des Sciences Marines) for their assistance with export documentation, as well as to the staff of Madagascar’s DREDD (Direction Régionale de l’Environnement et du Développement Durable) and DAPRNE (Direction des Aires Protégées, des Ressources Naturelles Renouvelables et des Écosystèmes) for their collaboration. We are especially grateful to Léa Jeanine RAVAOARISOA, former principal secretary of IH.SM, for her invaluable support in preparing these documents. We warmly thank all the technicians from IH.SM and the NGO Reef Doctor for their dedicated assistance in the field, in the laboratory, and during data collection. Our sincere thanks also go to the local authorities of each village involved in the study, and to the local assistants who facilitated communication and cooperation with the fishing communities. Finally, we express our deepest gratitude to the fishers for their generous welcome, availability, and active participation, without whom this research would not have been possible.

Conflicts of Interest: The authors declare no conflicts of interest.

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