# Phylogeography of Eleotris fusca (Teleostei: Gobioidei: Eleotridae) in the Indo-Pacific area reveals a cryptic species in the Indian Ocean

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# Phylogeography of *Eleotris fusca* (Teleostei: Gobioidei: Eleotridae) in the Indo-Pacific area reveals a cryptic species in the Indian Ocean

**Marion I. Mennesson[1] · Céline Bonillo[2] · Eric Feunteun[1] · Philippe Keith[1]**

**Abstract**
Indo-Pacific insular freshwater systems are mainly dominated by amphidromous species. *Eleotris fusca* is a widespread one, its life cycle is characterised by a marine pelagic larval phase allowing the species to disperse in the ocean and then to recruit to remote island rivers. In the present study, the population structure of *E. fusca* over its Indo-Pacific distribution range (Western Indian Ocean to French Polynesia, Pacific Ocean) was evaluated. We analysed a section of mitochondrial *COI* of 557 individuals sampled from 28 islands to visualise the population structure. Haplotypes diversity (Hd) was between 0.458 and 1 and, nucleotide diversity ($\pi$) was between 0.001 and 0.02. Two distinct genetic groups appeared, one in the Indian Ocean and the other in the Pacific Ocean ($F_{ST}$ mean = 0.901; 5.2% average divergence). Given these results, complete mitogenomes (mtDNA) were sequenced and combined with the nuclear Rhodopsin (Rh) gene for a subset of individuals. The two phylogenetic trees based on each analysis showed the same genetic pattern: two different groups belonging to the Indian and the Pacific oceans (6.6 and 1.6% of divergence for mtDNA and Rh gene respectively), which supported species level differentiation. These analyses revealed the presence of two sister species confounded until present under the name of *Eleotris fusca*. One of them is cryptic and endemic of the Indian Ocean and the other one is the true *E. fusca*, which keeps, nevertheless, its status of widespread species.

**Keywords** Freshwater fish · Amphidromous · Complete mitogenome · Nuclear gene

## Introduction

The Indo-Pacific is the largest biogeographic region in the world, which includes unnumbered islands (Ekman 1953; Briggs 1974; Spalding et al. 2007). Insular freshwater systems are known to be perilous habitats because they are subject to extreme climatic and hydrological seasonal variations such as drought or cyclonic flood events. To colonise these systems species have developed a specific life cycle: diadromy (Myers 1949). Diadromous species spend a part of their life cycle in the sea and a part in freshwater systems (McDowall 1992). Among diadromous species, amphidromous ones mainly dominate these habitats and are the biggest contributors to the diversity of the freshwater communities (Nelson et al. 1997; Watson et al. 2007; Keith and Lord 2011; Keith et al. 2015). Amphidromous species spawn in freshwater; after hatching, larvae drift downstream to the sea where they undergo a planktonic phase. After this marine phase, individuals return to rivers to grow and reproduce (McDowall et al. 1994; Keith et al. 2008). With the marine larval phase, amphidromous species are able to disperse and colonise new islands (Keith 2003).

The colonization of insular rivers is influenced by numerous factors such as species dispersal abilities, distances between islands, spatial and temporal characteristics of ocean currents and the presence of past or actual barriers (Lord et al. 2012; Pous et al. 2010; Feutry et al. 2013; Castelin et al. 2013; Taillebois et al. 2013). The Indo-Pacific

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area presents several such physical barriers that are the consequence of past geologic activity and they played a role in the limitation of dispersal (Gaither et al. 2011). These barriers emerged during various geologic periods, particularly in the Pleistocene. The repeated lowering of sea level during Pleistocene glacial cycles created a nearly complete barrier between the Indian and Pacific oceans, the Indo-Pacific Barrier (IPB) situated across the Sunda Shelf, and led to intraspecific genetic divergence in many taxa, and ultimately the formation of pairs of sister species on either side of the barrier (Barber et al. 2006; Gaither and Rocha 2013; Ludt and Rocha 2015). Currently, major phylogeographic breaks are observed between populations in the Pacific and the Indian oceans across the IPB (Briggs 1974; De Bruyn et al. 2004; De Bruyn and Mather 2007; Crandall et al. 2008) while other breaks are observed between some regions of the Central West Pacific across the Torres Strait (Mirams et al. 2011; Taillebois et al. 2013). Several studies have investigated population genetic and phylogeographic patterns in the Indo-Pacific region (Keyse et al. 2014), but mainly have focused on marine organisms and rarely including the entire geographic range of the species studied; furthermore, few exist on diadromous species (Lord et al. 2012; Castelin et al. 2013; Taillebois et al. 2013).

In amphidromous fishes, the *Eleotris* genus (Eleotridae), with the most widespread species *Eleotris fusca* Bloch and Schneider, 1801, is particularly interesting to such a large-scale phylogeographic study. This species is found in the Indo-Pacific area, from the Western Indian Ocean to New Caledonia, Vanuatu and French Polynesia (Keith et al. 2006, 2010, 2013; Mennesson et al. 2015). Its life cycle is characterised by a marine pelagic larval phase allowing the species to disperse in the ocean and then to recruit in remote island rivers (Mennesson et al. 2015). In these rivers, it is a common carnivorous species distributed primarily in freshwater (Maeda and Tachihara 2004, 2006). This species is is found in brackish waters, but mainly in the lower part of freshwater areas. It prefers to be close to the riverbank where the current is slow, or in the lentic zones (Keith et al. 2010). *Eleotris fusca* experiences pressures linked to the loss of the hydrological corridor and habitat degradation (Keith et al. 2013). No threats are clearly identify at the international scale due to a lack of investigations on this species. But due to the association of habitat loss and post-larvae fisheries in the Reunion Island (Mascarene), *E. fusca* was listed as an "Endangered" species on the French IUCN red List of Endangered Species in France (IUCN France et al. 2013). Because of its importance in the freshwater ecosystem and in local fisheries, it is important to understand the life cycle and the phylogeography of this species to help managers to implement conservation measures.

The aim of the present study was to explore the population structure of *E. fusca* within its entire distribution range, the Indo-Pacific region, using a phylogeographic approach. First, the population structure was explored through *COI* barcoding of 557 individuals sampled from 28 islands. Then, we used the results to select a subset of samples (32 individuals from 18 islands) that were sequenced for the complete mitogenome and the nuclear Rhodopsin (Rh) gene to assess *E. fusca* phylogeography in order to see if there was evidence of genetic structure across the IPB and/or other barriers.

## Materials and methods

### Sample collections

A total of 557 specimens of *E. fusca* were collected in the rivers of 28 islands included in the vast distribution range of the species and grouped in five biogeographic regions of the Indo-Pacific area as defined by Castelin et al. (2013) (Fig. 1; Table 1): (i) the Central Pacific Ocean, CPO (Society Islands, Austral Islands, Marquesas and Cook Islands); (ii) the Southwest Pacific Ocean, SWPO (Vanuatu, New Caledonia, Hoorn Islands, Fidji, Samoa and Solomon); (iii) the Northwest Pacific Ocean, NWPO (Indonesia, Philippines, Caroline Islands, Micronesia and Ryu-Kyu Islands); (iv) the Northwest Indian Ocean, NWIO (Seychelles Islands, Comoros Islands and Mayotte Island) and (v) the Southwest Indian Ocean, SWIO (Mascarene Islands and Madagascar). Individuals were sampled using a DEKA 3000 electrofishing system (Gerätebau, Marsberg, Germany). All the fish were sampled in the lower part of the freshwater streams, as defined by Keith et al. (2013), at 5–50 cm depth. Fish were put to sleep using clove essential oil (10%) and then a fin clip was taken, stored and preserved in 95% alcohol for molecular analysis. Less than 15% of the specimens were euthanised. Specimens and tissues samples were collected under permits of sampling and euthanasia No 60912-3120-2004/JJC, 60912-2320-2010/JJC, No 60912-120-2010/JJC, 6034-419/DENV/2007, 1224-08/PPS, Conv. 10C303 PS 11/10/2010, New Caledonia; Marine Research permit (Ministry of Natural Resources) No RE-11-06, Palau; Vanuatu Environment Unit No ENV 326/001/1/03/DK, No ENV 326/001/1/06/DK, No ENV 326/001/1/07/DK, No ENV 326/001/1/08/DK, and No ENV 326/1/10/DK, DEPC 326/001/10/14/DK; Office of Fisheries and aquaculture 7-9/3/2012, Pohnpei; Solomon Islands Governement No EX2014/117, No EX2016/156; Cook Islands Research Commitee No 101.1.1/4 and 03/10; Agreement of Ministry of environment and energy 30/11/2012, Seychelles; Consent approval of Ministry of natural resources & Environment 28/07/2008, Apia Samoa; Convention CNDRS No 1/11/2005, Comoros; IH-SM & SAVA conv. MNHN-IFB 2008–2010, Madagascar; Service de l'Environnement

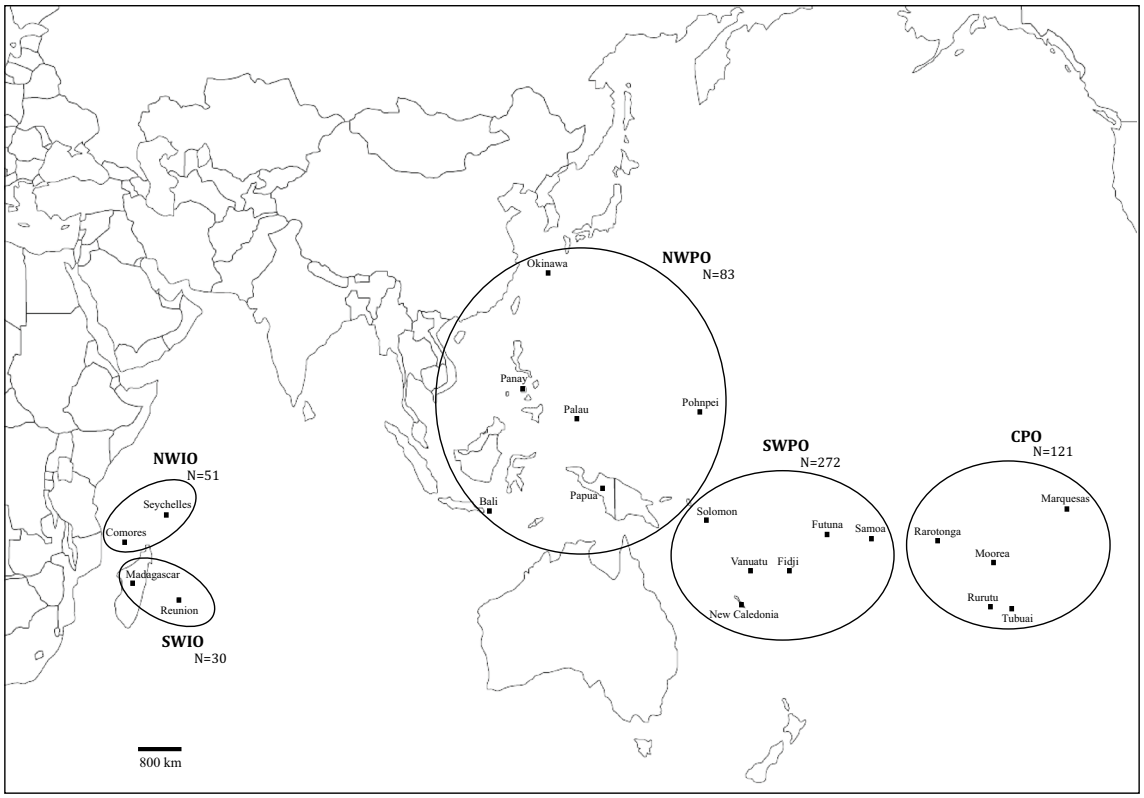


**Fig. 1** Map of the Indo-Pacific showing sampled localities

Conv. 5-23/10/2004, Futuna; Direction Départementale de l'Agriculture et de la Forêt Conv. ARDA/MNHN/DAF 05/2003 & 2006, Mayotte; DIREN Conv. Réunion-ARDA 2007 & 2012; Conv. Délégation à l'environnement-EPHE-MNHN, French Polynesia.

## DNA extraction and amplification

Pectoral fin tissue was used to extract total genomic DNA from the 557 individuals using the Macherey and Nagel NucleoSpin® Tissue kits following the manufacturer's instructions on an Eppendorf EpMotion 5075.

## COI gene

Genetic diversity and patterns of genetic connectivity were investigated using a 585 bp fragment of mtDNA cytochrome oxidase (*COI*). To amplify the partial *COI* gene specific fish primers TelF1 and TelR1 were used (Table 2). DNA amplification was performed by PCR in a final 20 µL volume containing 5% DMSO, 1 µL of dNTP 6.6 µM, 0.15 µL of Qiagen Taq DNA polymerase, using 2 µL of the buffer provided by the manufacturer, and 0.4 µL of each of the two primers at 10 pM; 1.2 µL of DNA extract was added. After denaturation for 2 min at 94 °C, the PCR was run for 55 cycles of (25 s, 94 °C; 25 s, 54 °C; 55 s, 72 °C) on a Bio-Rad C1000 Touch Thermal Cycler. Successful PCRs were selected on ethidium-bromide stained agarose gels. Sanger sequencing was performed in both directions by a commercial company (Eurofins; http://www.eurofins.fr) using the same primers as before.

## Complete mitogenome and Rh gene

The population structure observed with the mtDNA *COI* data was tested using the complete mitogenome (NGS), with a subset of 29 *E. fusca* individuals: 21 for the Pacific Ocean and 8 for the Indian Ocean, and 3 outgroups (*E. acanthopoma, E. melanosoma* and *E. bosetoi*), following the protocol established by Hinsinger et al. (2015) and using specific fish primers listed in Table 2. Hinsinger et al. (2015) developed a specific framework for the sequencing and multiplexing of mitogenomes on NGS platforms following 3 steps: (i) a universal long-range PCR-based amplification technique; (ii) a two-level multiplexing approach and (iii) a dedicated demultiplexing assembling script from an Ion Torrent sequencing platform. With this method, obtaining complete or almost complete mitogenome sequences is now easier and low cost. Moreover, having an extensive dataset for each specimen (i.e. 13 coding genes, 22 tRNA, 2 rRNA

**Table 1** Details of tissue samples of *Eleotris fusca* specimens. Samples size of each island (N) and abbreviated names of each analysed population

| Regions | Countries | Islands | N | Populations |
|---|---|---|---|---|
| Central Pacific Ocean (CPO) | Society Islands | Moorea | 42 | SOC |
| | Austral Islands | Rurutu | 54 | AUS |
| | – | Tubuai | 2 | |
| | Marquesas | Ua huka | 4 | MAR |
| | Cook Islands | Rarotonga | 19 | COO |
| Southwest Pacific Ocean (SWPO) | Vanuatu | Maewo | 11 | VAN |
| | – | Santo | 37 | |
| | – | Malakula | 1 | |
| | – | Epi | 7 | |
| | – | Gaua | 8 | |
| | New Caledonia | Grande Terre | 71 | NCA |
| | Hoorn Islands | Futuna | 4 | HOO |
| | Fidji | Taveuni | 4 | FID |
| | Samoa | Upolu | 14 | SAM |
| | Solomon | Choiseul | 20 | SOL |
| | – | Kolobangara | 95 | |
| Northwest Pacific Ocean (NWPO) | Indonesia | Bali | 17 | I-BA |
| | – | Papua | 33 | I-PA |
| | Philippines | Panay | 1 | – |
| | Caroline Islands | Palau | 3 | CAR |
| | Micronesia | Pohnpei | 2 | – |
| | Ryū-Kyū Islands | Okinawa | 27 | RKI |
| Northwest Indian Ocean (NWIO) | Seychelles Island | Silhouette | 2 | – |
| | Comoros Islands | Anjouan | 2 | COM |
| | – | Moheli | 11 | |
| | Mayotte Island | Mayotte | 36 | MAY |
| Southwest Indian Ocean (SWIO) | Mascarene Islands | Reunion | 25 | MAS |
| | Madagascar | Madagascar | 5 | MAD |
| | | Total | 557 | |

Specimens from Philippines and Micronesia were not used for the AMOVA because of their small sample size

**Table 2** List of the primers used in this study for short and long PCR

| Gene | Primer name | 5′-3′ sequence | Publication |
|---|---|---|---|
| *COI* | Tel F1 | TCGACTAATCAYAAAGAYATYGGCAC | Dettai et al. (2011) |
| *COI* | Tel R1 | ACTTCTGGGTGNCCAAARAATCARAA | Dettai et al. (2011) |
| Complete mitogenome | 12S-L1091R | AAACTGGGATTAGATACCCCACTAT | Kocher et al. (1989) |
| Complete mitogenome | MtH7061 | GGGTTATGTGGCTGGCTTGAAAC | Hinsinger et al. (2015) |
| Complete mitogenome | MtL5231 | TAGATGGGAAGGCTTCGATCCTACA | Hinsinger et al. (2015) |
| Complete mitogenome | MtH11944 | CATAGCTTTTACTTGGATTTGCACCA | Hinsinger et al. 2015 |
| Complete mitogenome | MtL11910 | CAGCTCATCCATTGGTCTTAGGAAC | Hinsinger et al. (2015) |
| Complete mitogenome | 12S-H1478 | TGACTGCAGAGGGTGACGGGCGGTGTGT | Hinsinger et al. 2015 |
| *Rh* | F193 | CARTGGTGCTACCTSTGCGA | Chen et al. (2003) |
| *Rh* | R1039 | CGTGGTCYTTCCKGAAGCG | Chen et al. (2003) |

and the control region; namely, around 17,000 bp) allows (i) to easily compare the data obtained with those available in GenBank, (ii) to have the best precision of the species genetic history by targeting the most variable genes.

We amplified the mitogenome with 3 overlapping fragments (primers used are in Table 2). A HotStart LongAmp® Taq DNA Polymerase (New England Biolabs, Ipswich MA) modified protocol was used. The 3 fragments amplification were performed by PCR in a final 18 µL volume including 5X LongAmp Taq Reaction Buffer, 0.4 ng/µL Bovine Serum Albumin, 3.5% DMSO, 300 nM of each primer, 300 µM of dNTPs, and 1 unit of LongAmp Taq polymerase. After an initial denaturation of 30 s at 94 °C, the DNA was amplified through 45 cycles of 20 s at 94 °C, 30 s at 62.5 °C, and 15 min at 65 °C, with a terminal elongation for 15 min at 65 °C (Hinsinger et al. 2015) on a Bio-Rad C1000 Touch Thermal Cycler. Successful PCRs were selected on ethidium-bromide stained agarose gels.

The nuclear rhodopsin gene (*Rh*) was used to add support to the population structure observed with the mtDNA data. We used F193 and R1039 as primers (Chen et al. 2003) (Table 2), DNA amplification, from 37 specimens, by PCR was the same as *COI* and then, PCR were sequenced with the Ion Torrent following the protocol of Hinsinger et al. (2015).

## COI analysis

For mtDNA *COI* gene (585 bp), data processing and sequence assembly were done in Geneious 9.0.5 (Kearse et al. 2012), all sequences were aligned with Muscle Alignment (implemented in Geneious) and compared (*p*-distances) (Geneious). Arlequin v.3.11 (Excoffier et al. 2005) was used to measure the genetic diversity in the population of *E. fusca* in the Indo-Pacific area. Different indices, such as number of haplotypes (h), haplotype diversity (Hd) and mean nucleotide diversity (π) were calculated.

A median-joining network was built from the *COI* dataset using Network v.5 (Bandelt et al. 1999) with equal weight for variable. We applied a maximum of parsimony algorithm and the criterion "frequency > 1" to simplify the complex branching pattern and to generate a network representing all the most parsimonious intraspecific phylogenies.

To study the partitioning of genetic variance within and among populations, analyses of molecular variance (AMOVA, Excoffier et al. 1992) were performed using Arlequin v.3.11. Samples were first partitioned into 18 countries (Table 3); Philippines, Micronesia and Seychelles samples weren't added to the analysis because of their small size. Secondly, to have a large view, samples were assembled into the five defined regions (CPO, SWPO, NWPO, SWIO and NWIO) (Table 4).

Pairwise $F_{ST}$ values were calculated between each pair of populations to assess to the genetic differentiation (Arlequin v.3.11) (Tables 5, 6). Significance of *F*-statistics was calculated from 10,000 replicate analyses based on the samples drawn randomly and alpha value of 0.05. To test for isolation by distance, the Mantel test implemented in *XL*STAT (1000 permutations) was performed between genetic ($F_{ST}$) and geographic distances (km, calculated with Google Earth v7.3.0.3832).

The demographic history of *E. fusca* was analysed with Arlequin v.3.11 using the Fu's *F*s-statistic (Fu 1997); genetic

**Table 3** Molecular diversity indices for mtDNA *COI* sequences for each analysed population

| Regions | Countries | Population | N | Hd | h | π | Fs |
|---|---|---|---|---|---|---|---|
| Central Pacific Ocean (CPO) | Society Islands | SOC | 42 | 0.558 | 5 | 0.001 | −0.79 |
| | Austral Islands | AUS | 56 | 0.458 | 7 | 0.001 | **−4.18** |
| | Marquesas | MAR | 4 | – | 1 | – | – |
| | Cook Islands | COO | 19 | 0.725 | 6 | 0.002 | **−2.26** |
| Southwest Pacific Ocean (SWPO) | Vanuatu | VAN | 64 | 0.875 | 27 | 0.004 | **−22.84** |
| | New Caledonia | NCA | 68 | 0.914 | 28 | 0.004 | **−25.47** |
| | Hoorn Islands | HOO | 4 | 0.5 | 2 | 0.002 | 1.1 |
| | Fidji | FID | 4 | 1 | 4 | 0.004 | **−1.51** |
| | Samoa | SAM | 14 | 0.912 | 9 | 0.002 | **−3.77** |
| | Solomon | SOL | 118 | 0.864 | 51 | 0.02 | **−14.37** |
| Northwest Pacific Ocean (NWPO) | Indonesia—Bali | I-BA | 17 | 0.868 | 9 | 0.004 | −3.46 |
| | Indonesia—Papua | I-PA | 33 | 0.934 | 11 | 0.004 | **−18.64** |
| | Caroline Islands | CAR | 3 | 0.667 | 2 | 0.001 | 0.2 |
| | Ryū-Kyū Islands | RKI | 27 | 0.915 | 18 | 0.004 | **−15.27** |
| Northwest Indian Ocean (NWIO) | Comoros Islands | COM | 13 | 0.897 | 8 | 0.003 | **−4.5** |
| | Mayotte Island | MAY | 36 | 0.844 | 18 | 0.006 | **−8.1** |
| Southwest Indian Ocean (SWIO) | Mascarene Islands | MAS | 25 | 0.76 | 9 | 0.01 | 1.17 |
| | Madagascar | MAD | 5 | 0.9 | 4 | 0.002 | **−1.65** |

Sample size (N), haplotype diversity (Hd), number of haplotypes (h), nucleotide diversity (π), Fu's *F*s-statistics. *F*s values Significant (p value ≤ 0.05) are in bold

**Table 4** Molecular diversity indices for mtDNA *COI* sequences for each analysed population grouped into 5 regions

| Regions | Population | N | Hd | h | π | Fs |
|---|---|---|---|---|---|---|
| Central Pacific Ocean | CPO | 121 | 0.573 | 14 | 0.002 | **−7.12** |
| Southwest Pacific Ocean | SWPO | 271 | 0.886 | 87 | 0.011 | **−24.61** |
| Northwest Pacific Ocean | NWPO | 85 | 0.884 | 35 | 0.004 | **−27.15** |
| Northwest Indian Ocean | NWIO | 30 | 0.805 | 12 | 0.009 | −0.84 |
| Southwest Indian Ocean | SWIO | 53 | 0.820 | 23 | 0.005 | **−14.97** |

Sample size (N), haplotype diversity (Hd), number of haplotypes (h), nucleotide diversity (π), Fu's *F*s-statistics. *F*s values Significant ($p$-value ≤ 0.05) are in bold

pattern expected under demographic expansion are indicated with significant negative values of Fu's *F*s.

### Complete mtDNA and Rh gene analyses

To reconstruct the mitochondrial genome of each individual, we used as a starting reference the *Eleotris acanthopoma* mtDNA (Miya et al. 2003) available on MitoFish (Mitochondrial Genome Database of Fish; Iwasaki et al. 2013). The consensus of each mitogenome was primarily checked manually (assembly success, coverage assessment, comparison to avaible *COI* sequences for the same specimen, BLAST searches; Altschul et al. 1997) in Geneious 9.0.5 (Kearse et al. 2012). Then the consensus sequence was annotated using MitoAnnotator (Iwasaki et al. 2013) and each gene was quality checked for coding sequences, stop codons, position of the SNPs. After checking the content and the order of each gene, mitogenomes were aligned with MAFFT 7.309 (implemented in Geneious).

In the present study, we decided to use the 13 protein-coding genes and not the complete mitogenome (11 516 vs. 16 510 bp) because the 22 tRNA, the 2 rRNA and the control region were not informative enough; percentages of divergence were under 2% while for protein-coding genes they were higher than 2% (except for ATPase 8) and one mitogenome presented an incomplete tRNA-Asn. However, we also performed an analysis in the complete mitogenome dataset to check whether the results were the same.

A phylogenetic tree based on the thirteen concatenated genes was performed using Bayesian inference (MrBayes v.3.2; Ronquist et al. 2012). The best-fitting models of evolution were computed in PartitionFinder (Lanfear et al. 2012). The analysis was realised using the three-codon positions for each genes as partition (Table 7) and was run for 10 million generations, sampling every 200 generations with two independent runs to access convergence.

For *Rh* gene (803 bp), data processing and sequence assembly were done in Geneious 9.0.5 (Kearse et al. 2012) and all sequences were aligned with Muscle Alignment (implemented in Geneious). A phylogenetic tree was performed using Bayesian inference (MrBayes v.3.2; Ronquist et al. 2012). Three models computed in PartitionFinder (Lanfear et al. 2012) (1st position, JC model; 2nd position, HKY model; 3rd position, HKY + I model) were run for 10 million generations, sampling every 100 generations with two independent runs to access convergence.

For each analysis, run convergence was checked using TRACER v.1.6.0 (Rambaut and Drummond 2007). Trees were summarised using the 50% majority rule method after discarding the first 25% of the sample as burnin and visualised using FigTree v.1.4.2 (Rambaut 2007). The percentage of differences between sequences and the number of bases, which are not identical were calculated on Geneious 9.0.5 (Tables 8, 9 in Supplementary material).

For each bayesian tree, three other species of *Eleotris* were used as outgroups: *E. acanthopoma* Bleeker, 1853, *E. melanosoma* Bleeker, 1852 and *E. bosetoi* Mennesson et al. 2016. The corresponding mtDNA and *Rh* sequences were generated for this study.

## Results

### Gene diversity for mtDNA COI

A total of 585 bp of mtDNA *COI* from 557 individuals were obtained from *E. fusca* and deposited in GenBank (Accession Numbers: MH497864–MH498420). The number of individuals sequenced (N), haplotypes diversity (Hd), number of haplotypes (h) and nucleotides diversity (π) for each country and each region are provided in Tables 3 and 4.

Haplotypes diversity (Hd) in the 18 populations was between 0.458 and 1 (Table 3) and, nucleotide diversity (π) was between 0.001 and 0.02. Same results were observed when the analysis was performed for the populations grouped by regions (Table 4) i.e., the lowest haplotype and nucleotide diversities were found for the CPO (Hd = 0.573; π = 0.002) while all the others—SWPO, NWPO, NWIO, SWIO—have an haplotype diversity around 0.8 and a nucleotide diversity between 0.004 and 0.011.

**Table 5** Pairwise $F_{ST}$ values for the mtDNA *COI* haplotypes of the 18 populations (below diagonal) and associated *p* values (above diagonal; significant *p* values are indicated with the symbol "+")

| | MAD | MAY | MAS | COM | I-BA | RKI | CAR | I-PA | VAN | NCA | FID | HOO | SAM | SOL | COO | AUS | SOC | MAR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MAD | | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MAY | −0.042 | | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MAS | 0.103 | 0.010 | | − | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| COM | 0.039 | −0.019 | **0.015** | | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| I-BA | **0.935** | **0.900** | **0.849** | **0.937** | | − | − | − | − | − | − | − | − | − | + | + | + | + |
| RKI | **0.929** | **0.901** | **0.858** | **0.931** | −0.020 | | − | − | − | − | − | − | − | − | + | + | + | + |
| CAR | **0.963** | **0.892** | **0.816** | **0.952** | 0.043 | −0.038 | | − | + | + | − | − | − | − | + | + | + | + |
| I-PA | **0.926** | **0.902** | **0.862** | **0.928** | −0.004 | 0.001 | −0.116 | | − | − | − | − | − | − | + | + | + | + |
| VAN | **0.927** | **0.909** | **0.881** | **0.928** | 0.005 | **0.024** | −0.149 | 0.000 | | − | − | − | − | − | + | + | + | + |
| NCA | **0.932** | **0.913** | **0.887** | **0.933** | 0.010 | **0.023** | −0.121 | −0.009 | 0.002 | | − | − | − | + | + | + | + | + |
| FID | **0.941** | **0.889** | **0.816** | **0.942** | −0.061 | −0.058 | −0.055 | −0.070 | −0.043 | −0.058 | | − | − | + | + | + | + | + |
| HOO | **0.960** | **0.892** | **0.820** | **0.951** | −0.001 | −0.026 | 0.268 | −0.009 | 0.008 | 0.031 | 0.000 | | − | − | + | + | + | + |
| SAM | **0.928** | **0.894** | **0.838** | **0.932** | −0.019 | 0.005 | −0.065 | 0.008 | 0.007 | 0.023 | −0.092 | −0.032 | | − | + | + | + | + |
| SOL | **0.705** | **0.728** | **0.683** | **0.716** | −0.001 | 0.010 | −0.144 | 0.013 | **0.027** | **0.028** | −0.095 | −0.084 | −0.002 | | + | + | + | + |
| COO | **0.968** | **0.920** | **0.881** | **0.963** | **0.736** | **0.704** | **0.823** | **0.688** | **0.673** | **0.697** | **0.763** | **0.808** | **0.712** | **0.293** | | + | + | + |
| AUS | **0.982** | **0.948** | **0.930** | **0.978** | **0.836** | **0.802** | **0.900** | **0.785** | **0.748** | **0.765** | **0.869** | **0.891** | **0.826** | **0.364** | 0.048 | | + | + |
| SOC | **0.974** | **0.937** | **0.913** | **0.970** | **0.779** | **0.746** | **0.845** | **0.729** | **0.700** | **0.721** | **0.804** | **0.833** | **0.763** | **0.324** | −0.015 | 0.030 | | + |
| MAR | **0.975** | **0.901** | **0.835** | **0.960** | **0.723** | **0.685** | **0.953** | **0.673** | **0.679** | **0.700** | **0.800** | **0.909** | **0.699** | **0.248** | **0.891** | **0.936** | **0.902** | |

Significant pairwise $F_{ST}$ values are given bold ($p$ value $\leq 0.05$)

**Table 6** Pairwise $F_{ST}$ values for the mtDNA *COI* haplotypes of the 5 biogeographic regions (below diagonal) and associated *p* values (above diagonal; significant *p* values are indicated with the symbol "+")

| | CPO | SWPO | NWPO | SWIO | NWIO |
|---|---|---|---|---|---|
| CPO | | + | + | + | + |
| SWPO | **0.461** | | – | + | + |
| NWPO | **0.723** | 0.005 | | + | + |
| SWIO | **0.936** | **0.794** | **0.895** | | – |
| NWIO | **0.949** | **0.823** | **0.921** | 0.013 | |

Significant pairwise $F_{ST}$ values are given in bold ($p$ value $\leq 0.05$)

## Phylogeographic analysis for mtDNA COI

The haplotype network from mtDNA *COI* showed two main distinct haplogroups separated by 25 nucleotide substitutions (5.2% average divergence, *p*-distance) plus 3 median vectors (Fig. 2). The individuals sampled in the Indian Ocean (NWIO and SWIO) were included in the first haplogroup, which exhibited a star-like topology with no obvious geographic patterns (Fig. 2). The second haplogroup was composed of samples from the Pacific Ocean (NWPO, SWPO and CPO) and a geographical structuration is obvious;

**Table 7** Details of BIC models used for the three-codon positions for each gene

| BIC model | Gene_codon positions |
|---|---|
| HKY + I | ATP6_1, ATP6_2, ATP8_1, ATP8_2, Cytb_1, Cytb_2,_ND1_1, ND1_2, ND2_1, ND2_2, ND3_1, ND3_2, ND4L_1, ND4L_2, ND4_1, ND4_2, ND5_1, ND5_2, COIII_2, COII_2, COI_2 |
| GTR + I + G | ATP6_3, ATP8_3, COII_3, COII_3, Cytb_3, ND1_3, ND3_3, ND4L_3, ND4_3, ND5_3 |
| GTR + I | ND2_1, ND2_3 |
| K80 + I | COIII_1, COII_1, COI_1 |
| GTR + G | ND6_1 |
| HKY + I + G | ND6_2, ND6_3 |

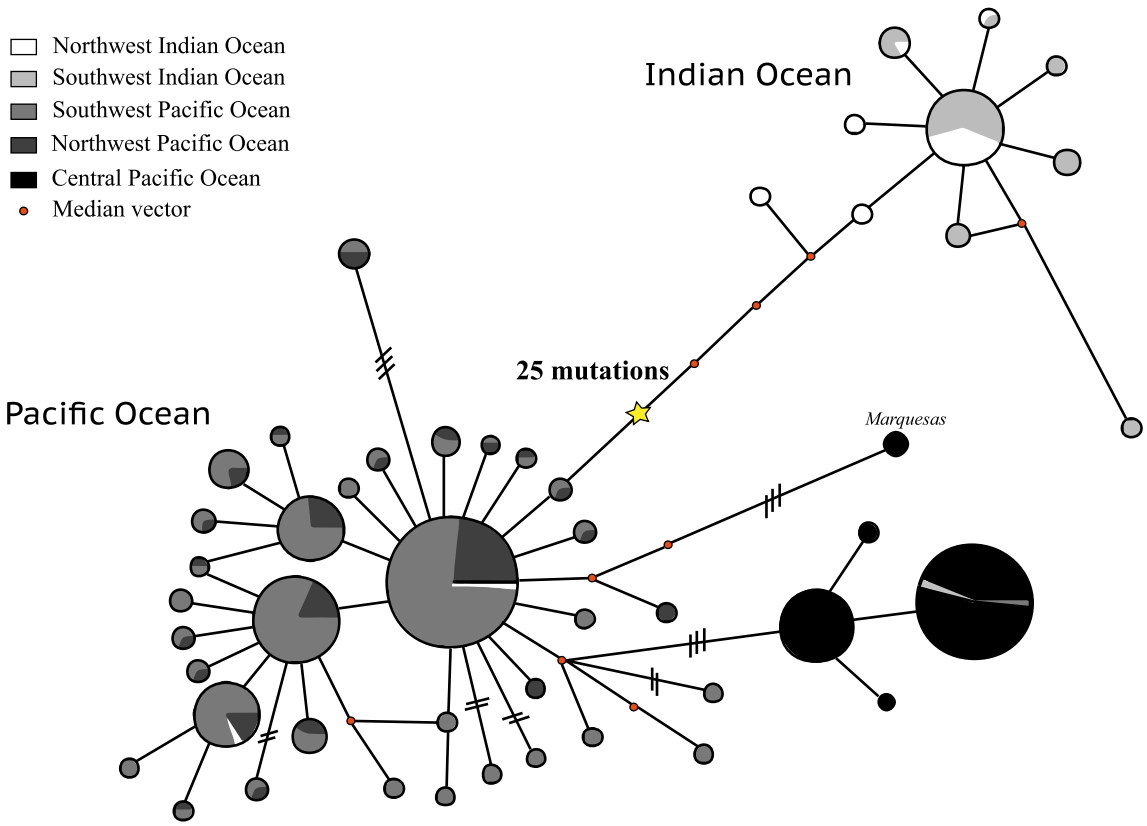


**Fig. 2** Median-joining network for *E. fusca* from the mtDNA *COI*. Circles represent individual haplotypes and are color-coded on geographical locations. Circles are sized proportionally to the haplotype frequencies. All haplotypes are separated by one mutational step; cross-bars on branches indicate when mutation steps are higher than one nucleotide. Each little red circle represents a median vector

the NWPO and SWPO are pooled in a sub-group, and the CPO is in fringe of it (Fig. 2). Two individuals sampled in the Indian Ocean (both from Reunion Island) and one from Moorea (French Polynesia) were included in the NWPO–SWPO sub-group. One specimen from Mayotte Island and one from Vanuatu were found in the CPO sub-group, Marquesas formed an isolated group and shared no haplotypes with any other location (Fig. 2).

The AMOVA permitted to calculate $F_{ST}$ values. The observation of significant $F_{ST}$ values ($p \leq 0.05$) revealed different genetic divergence patterns across among the 18 countries (Table 6). No significant genetic differentiation ($p > 0.05$) was observed within the Indian Ocean countries. The comparison of Indian and Pacific oceans countries indicated a strong and significant genetic differentiation ($F_{ST}$ mean = 0.901; 5.2% average divergence). Within the Pacific Ocean countries, we observed that few $F_{ST}$ were significant between NWPO and SWPO, those that were significant presented a comparatively lower genetic differentiation levels ($F_{ST}$ VAN–RKI = 0.024; $F_{ST}$ NCA–RKI = 0.023; $F_{ST}$ VAN–SOL = 0.027; $F_{ST}$ NCA–SOL = 0.028). A high level of population structure between CPO and the sub-group NWPO–SWPO was found ($F_{ST}$ mean = 0.725; 0.5% average divergence); however this structuration level was less high when we compared CPO to Solomon Islands ($F_{ST}$ mean = 0.307). Within the CPO, Marquesas presented high genetic structure ($F_{ST}$ mean = 0.910; 1.4% average divergence) with the other CPO islands (i.e. Rarotonga [Cook Islands], Rurutu, Tubuai and Moorea [French Polynesia]). When countries were grouped by region, we still observe a strong significant genetic structure between the Indian and Pacific Ocean ($0.794 \leq F_{ST} \leq 0.949$) (Table 6) and that CPO region is less genetically differentiated from SWPO ($F_{ST} = 0.461$) than NWPO ($F_{ST} = 0.723$). The majority of Fu's *F*s values were significantly negative (Table 3) that could be evidence for recent demographic expansion for some of the 18 countries. For the 5 regions of the Indo-Pacific, all Fu's *F*s values were significantly negative except for the NWIO (Table 4).

The Mantel test showed a positive and significant correlation between genetic and geographic distances when we focused on the 5 regions (r = 0.83; *p* value = 0.002); and geography explained 68% of the genetic structuration. However, when the test was based on the 18 countries (r = 0.36; *p* value $< 10^{-4}$) geography explained only 13% of the genetic structuration among populations.

### Phylogenetic analyses: complete mtDNA and Rh gene

Thirty-two complete mtDNA were obtained and deposited in GenBank (Accession Numbers: MH540156–MH540192). The phylogenetic tree-based on the 13 concatenated coding genes (11,546 bp) showed the same topology of the tree obtained only with the *COI* gene (not shown) (Fig. 3). Indeed, we observed two sub-clades separated by a mean divergence of 6.6% between sequences, namely 756 nucleotides of difference (Table 8 in Supplementary material). The sub-clade $A_2$ is composed with specimens sampled only in the Indian Ocean, and the other one ($A_1$) is composed with Pacific Ocean specimens plus two sampled in Reunion Island. These two specimens were the two of the three ones we observed in the *COI* haplotype network (Fig. 2). When we looked at the intra-population variation, the two clades presented low divergence (0.47 and 0.89% between sequences and 52 and 102 nucleotides of difference for clade $A_2$ and clade $A_1$ respectively). Within the CPO group (Moorea, Rarotonga and Rurutu specimens) the divergence between sequences is lower (difference of 36 bases and 0.3%) than when they were compared to the NWPO and SWPO groups (154 bases and 1.3%). As seen in the *COI* haplotype network, the CPO islands differs from the others Pacific Ocean islands. The three outgroups (clade B and C), *E. melanosoma, E. bosetoi* and *E. acanthopoma* presented a high divergence with the clade $A_1$ and $A_2$, the difference of number bases varied around 1383 to 1872 namely 12–16.3% of divergence between sequences (Table 8 in Supplementary material).

The phylogenetic tree based on the *Rh* dataset was similar to the complete mtDNA tree. Two distinct clades were observed, the clade A included all the specimens sampled in the Pacific Ocean plus two from Reunion Island (the same observed in the haplotype network and the complete mtDNA tree); the clade B included those sampled in the Indian Ocean and clade C consisted of the outgroups (Fig. 4). The intra-population variation within each clade was about one or two nucleotides of difference between sequences namely 0.1 or 0.2% of divergence for clade B and clade A respectively (Table 9 in Supplementary material). When we compared these two clades 13 nucleotides were different with 1.6% of divergence. *E. bosetoi* and *E. melanosoma* (outgroups) presented a nucleotide difference of 23 and 34 (on average) and, 3 and 4% of divergence with clade A and B respectively. Between the two outgroups, 1.9% of divergence and 15 nucleotides of difference were observed.

## Discussion

### An endemic cryptic species

Our mtDNA *COI* analysis reveals two distinct genetic groups in the Indo-Pacific area (separated by at least 25 nucleotide substitutions i.e., 5.2% of divergence), one group corresponding to the Indian Ocean and the other to the Pacific Ocean. This kind of result was found in several

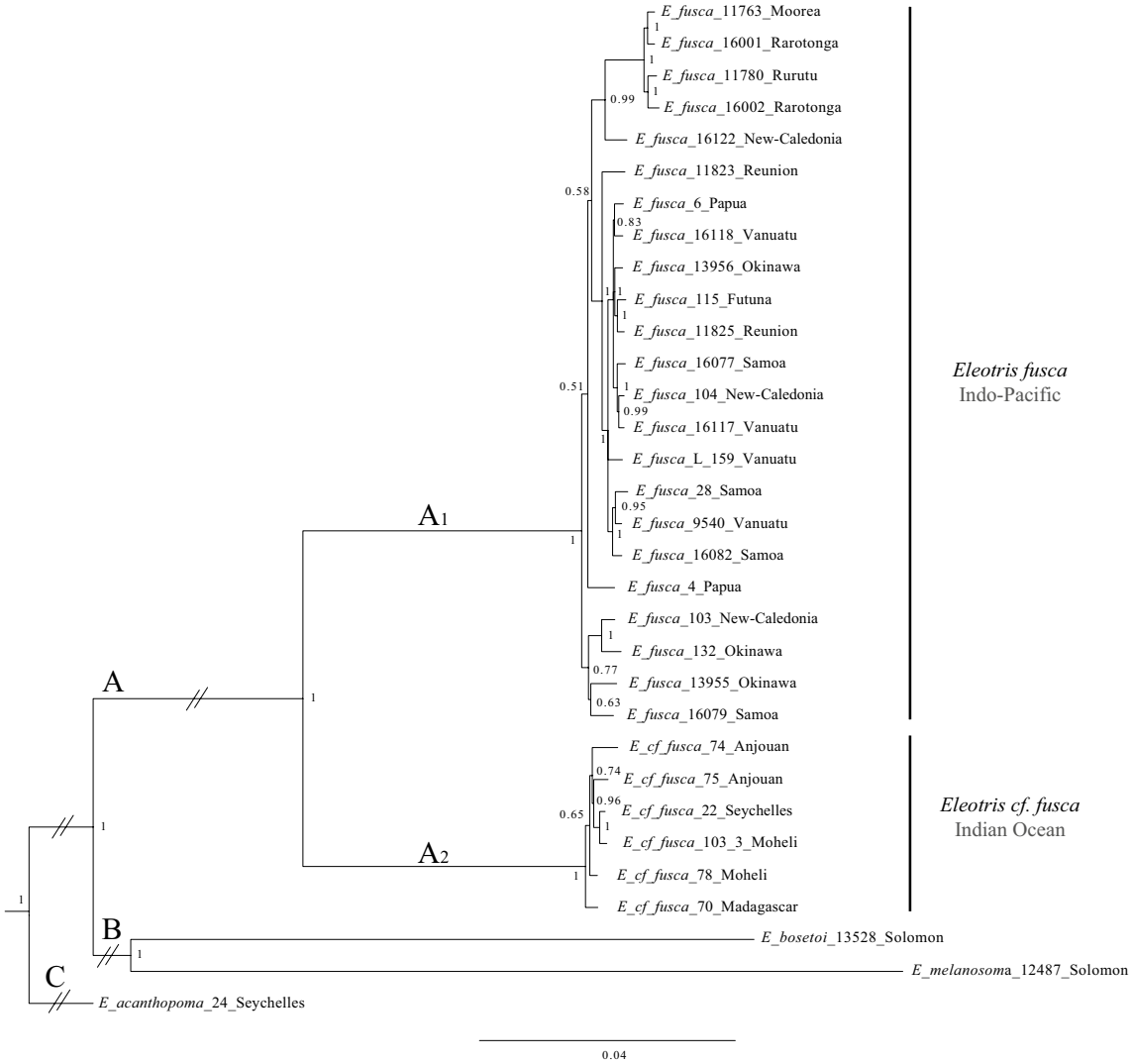


**Fig. 3** Bayesian tree of the complete mtDNA for sequences specimens of *E. fusca*. Numbers on the nodes represent posterior probabilities

marine and diadromous widespread species as *Nerita albicilla* Linnaeus, 1758 (Crandall et al. 2008) a gasteropod and, three fish species, *Kuhlia rupestris* Lacepède, 1802 (Feutry et al. 2013), *Sicyopterus lagocephalus* Pallas, 1770 (an amphidromous goby) (Lord et al. 2012) and *Cephalopholis argus* Schneider, 1801 (Gaither et al. 2011). However, in these studies, according to their results and low percentages of divergence, the authors concluded to the structuration in two different populations, one in the Indian Ocean and the other in the Pacific Ocean. mtDNA *COI* is commonly used for Barcoding and molecular taxonomy and it is generally accepted that 3% of divergence between two groups, is enough to assume two different species (Dettai et al. 2011). Especially as the species level differentiation is also supported by morpho-meritics analyses with a difference in scales series (Mennesson and Keith 2017). In our study, the complete mtDNA and the *Rh* gene also supported species level differentiation i.e., they supported the same groups including the same specimens with respectively 756 and 13 nucleotide substitutions (6.6 and 1.6% of mean divergence; Table 8 in Supplementary material). One lineage is composed by the real *E. fusca* [type locality CPO, French Polynesia], specimens of Pacific Ocean and two sampled in Reunion Island [clade $A_1$ (Fig. 3) and clade A (Fig. 4)] and the other lineage is composed by a cryptic species present only in the Indian Ocean and named here *E.* cf. *fusca* [clade A2 (Fig. 3) and clade B (Fig. 4)].

Few studies on widespread Pacific species found also cryptic species such as within the fish *Acanthurus nigroris* Valenciennes, 1835 (Dibattista et al. 2011) (25 mutations and 4.1% of divergence) between Hawaii specimens and the remaining of the Pacific Ocean or, within the shrimp *Parupeneus multifasciatus* Quoy and Gaimard,


**Fig. 4** Bayesian tree of the Rhodopsin gene (*Rh*) for sequenced specimens of *E. fusca*. Numbers on the nodes represent posterior probabilities

1825 (Szabó et al. 2014) (25 nucleotide substitutions ($F_{ST} = 0.9$) and a genetic difference of 4.12%) separating the specimens from Marquesas Islands from the other Pacific islands.

The star-like pattern of the Indian Ocean group in the *COI* haplotype network (Fig. 2) is typically associated to a scenario of past colonisation events followed by demographic expansions, which could be due to past bottlenecks after population depletions caused by climatic events [e.g. during Pleistocene with the repeated lowering sea levels (Sathiamurthy and Voris 2006)]. This star-like pattern has been observed in some studies with *Lutjanus fulvus* Forster, 1801, a reef fish, (Gaither et al. 2010), or *S. lagocephalus* (Lord et al. 2012) or *Sicyopus zosterophorum* Bleeker, 1856 (Taillebois et al. 2013), two amphidromous fishes.

## *E. fusca*, a widespread species in the Indo-Pacific

In the Pacific group, the CPO populations (Polynesia) of *E. fusca* are genetically differentiated from the NWPO and the SWPO populations (see *COI* and mtDNA results). Castelin et al. (2013) observed the same isolation of the CPO for *Macrobrachium lar* Fabricius, 1798, a widespread and amphidromous crustacean species, as Lord et al. (2012) and Coleman et al. (2016) for *S. lagocephalus* and *Pygoplites diacanthus* Boddaert, 1772 (a reef fish), respectively. This isolation of the CPO from the remaining of the Pacific might be explained by the use of different currents by the post-larvae when they disperse in the ocean (Abdou et al. 2015). Indeed, the South Equatorial Current (SEC), the South Equatorial Counter Current (SECC) and the Marquesas

Counter Current (MCC) disperse the organisms in different way; the SEC might disperse sporadically post-larvae from the CPO to the other Pacific Islands and the SECC and the MCC might keep them in the CPO (Gaither et al. 2010). Another fact that could induce the differentiation between NWPO/SWPO and CPO is the high ocean depth between Samoa and French Polynesia, which could act as a barrier for post-larvae (Planes 1993; Planes and Fauvelot 2002).

Another interesting result is the isolation of the Marquesas Islands within the CPO and the fact that they present less differentiation from the NWPO and SWPO than from the CPO ($F_{ST}$ of 0.707 vs. 0.910). The biogeographic genetic break for Marquesas Islands has commonly been observed for reef fishes such as *Acanthurus triostegus* Linnaeus, 1758 (Planes 1993), *Lutjanus kasmira* Forsskål, 1775 and *L. fulvus* Forster, 1801 (Gaither et al. 2010) or *Parupeneus multifasciatus* (Szabó et al. 2014). Planes (1993) indicated that the opposition between the MCC and the SEC may constitute a barrier for larvae and may explain the strong divergence of the Marquesas population. Ocean currents may have a strong influence on the population genetic flow (Gaither et al. 2010) depending on the post-larvae behaviour (active or passive swimming in the current, depth of swimming …) (Keith 2003).

Figures 3 and 4 show in the Indo-Pacific clade the exactly same two individuals (11823 and 11825) from La Reunion Island. This demonstrates that there might still be a low gene flow between the two oceans. The Indo-Pacific Barrier (IPB) with the lowering and rising sea levels during Pleistocene (Briggs 1974; Voris 2000) played probably a great role in the ability of *Eleotris* post-larvae to disperse. Moreover, the larval dispersal could be subject to numerous other physical barriers (Grant and Bowen 1998) such as the depth, the salinity variation, the gyres and the current patterns (Borkin 1991; Hare and Cowen 1996; Murphy and Cowan 2007). Our results indicate that post-larvae might pass or have been passing from the Pacific Ocean to the Indian Ocean, but very sporadically with the SEC (Figs. 2, 3, 4). On the contrary, the post-larval dispersion from the Indian Ocean to the Pacific Ocean was probably not possible, the SEC which links the two oceans is one-way from Pacific to Indian Ocean and, we have no specimens sampled in the Pacific Ocean showing an Indian Ocean haplotype. This type of gene flow results has been found in other amphidromous species as *S. lagocephalus* (Lord et al. 2012), *M. lar* (Castelin et al. 2013) and, *Neritina stumpffi* Boettger, 1890 (Abdou 2016). In these species, gene flow appears to be unidirectional, from the Pacific Ocean into the Indian Ocean. A study on a reef fish species, *P. diacanthus* (Coleman et al. 2016) showed the same results but included samples from Christmas Island (eastern Indian Ocean, close to Indonesia) and Diego Garcia (in the middle of the Indian Ocean). The authors demonstrated that these two islands are keys areas for the stepping-stone model of dispersal for *P. diacanthus* as they shared both Pacific and Indian haplotypes. For this species, the IPB seems to be not the main barrier to dispersal. There are few spots of freshwater where amphidromous species, as *E. fusca*, could recruit in these islands (Diego Garcia is an atoll) but it could be interesting to investigate these regions to know if *Eleotris* (and what species) is present or not.

### Phylogeographic results vs. pelagic larval duration

Mennesson et al. (2015) showed that *E. fusca* presented a high variability in the pelagic larval duration (PLD) according to sampling localities, which illustrates a high plasticity in the PLD of the species. Indeed, significant differences were found in the Indian Ocean; PLD varied by means of two between the studied islands, Mayotte [63.4 ± 5.8 days; (38–78 days)] and Reunion Island [114.8 ± 21.8 days; (82.5–141 days)], although ranges of standard length are similar. On the contrary, both PLD and standard length ranges are similar for Vanuatu [90 ± 19.2 days; (57–130.5 days)] and French Polynesia [96.2 ± 17.5 days; (62–131.5 days)] in the Pacific Ocean. These authors suggested that their results could be due to a genetic differentiation between populations inducing a variable capacity to disperse far away. Thus, our phylogeographic study was an opportunity to compare the PLD obtained by Mennesson et al. (2015) and the genetic structuration of *E. fusca* in the Indo-Pacific. Nevertheless, our study shows a significant high level of genetic structure across the Indo-Pacific; Indian and Pacific oceans represent two structured lineages, but no genetic break is observed in the Indian Ocean (although differences in the PLD between Mayotte and Reunion were observed by Mennesson et al. (2015)), and two potential populations might exist in the Pacific Ocean (NWPO-SWPO and CPO, Polynesia). So there is no link between the PLD observed by Mennesson et al. (2015) and the genetic break observed in our study.

## Conclusion

The use of mtDNA *COI*, complete mtDNA and *Rh* gene revealed the presence of two sister species confounded under the name of *Eleotris fusca*. One of them is cryptic and endemic of the Indian Ocean and is called here *E.* cf. *fusca* and the other one is *E. fusca*, which keeps its status of a widespread species. As *E. fusca* was classified as "Endangered" on the red List of Endangered Species in France (Reunion Island) (IUCN France et al. 2013), our results show that this status should be revised as it included in fact two sister species. We hope that our study will help to implement conservation and management measures, such as life traits studies, to protect the endemic species, *E.* cf. *fusca*,

in the Indian Ocean and also the widespread one, *E. fusca*, which seems rare in this ocean.

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