Sorting the wheat from the chaff: a review of BINs associated with groupers of Vietnam and the implications for species identification from DNA barcoding

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Jean-Dominique Durand[1], Manh Hung Pham[2], Thanh Thi Viet Tran[3], Duc Huy Hoang[2], Quang Van Vo[4]

The Epinephelidae is a marine fish family that has been the focus of worldwide taxonomical attention due to its economic value. Recent molecular-based phylogenies have improved understanding of species diversity within this family. Nonetheless, species misidentification or hybridization has led to a barcode index number (BIN) being associated with several species, which hampers a clear species identification within the BOLD system. In this study, grouper diversity in Vietnam was investigated to check the species inventory, provide a DNA barcode library for Vietnam, and reevaluate BINs associated with some species that are present in Vietnam. To this end, 157 specimens were barcoded corresponding to 30 species and 31 BINs. Nine species were new records, bringing to 49 the number of species inventoried for Vietnam. In BOLD, these species are associated with 75 BINs, 31 species being represented by more than one BIN. A careful review of these BINs, considering the present results from Vietnam, the species composition and frequency of each BIN in BOLD, and reference species sequences from phylogenetic studies, revealed those misidentified or hybrid specimens that artificially increased the number of BINs per species. After appropriate revision, 15 species still remained associated with more than one BIN. A phylogeographic analysis of these species demonstrated that most of the BINs derived from evolutionary lineages with geographic distributions that match well known biogeographic units in the Indo-Pacific. Beyond the species identification, these multiple BINs of each species can be used to track the biogeographic origin of each of the associated specimens.

Keywords Cytochrome oxidase I · BOLD · Misidentification · Biogeography · Epinephelidae

✉ Jean-Dominique Durand
jean-dominique.durand@ird.fr

[1] MARBEC, Univ Montpellier, IRD, Ifremer, CNRS, cc093, Place E. Bataillon, 34095 Montpellier Cedex 05, France

[2] Faculty of Biology and Biotechnology, Viet Nam National University Ho Chi Minh City-University of Science, 227 Nguyen Van Cu, District 5, Ho Chi Minh City, Vietnam

[3] Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, District Caugiay, Hanoi, Vietnam

[4] Department of Marine Vertebrate Resources, Institute of Oceanography, 01 Cauda, Nha Trang, Khanh Hoa, Vietnam

Introduction

DNA barcoding in fishes is now widely used for taxonomical investigations (Hubert and Hanner 2015), biodiversity evaluations (Galdino Brandao et al. 2016; Shen et al. 2016; Durand et al. 2017), and food control (Barcaccia et al. 2015; Brito et al. 2015). One of the main results of the broad scan of fish diversity across geographic areas, using cytochrome oxydase I (COI) polymorphisms, has been the revelation of numerous species complexes or cryptic species, leading to a profound change to our vision of biodiversity (Hubert et al. 2012, 2017; Jaafar et al. 2012; Winterbottom et al. 2014; Durand and Borsa 2015; Williams and Viviani 2016; Habib et al. 2018; Delrieu-Trottin et al. 2019). This important result justifies more barcoding investigations because gaps in taxonomical coverage limit accuracy of the species identification based on a DNA barcode. However, if the BOLD database is used as reference library and is continuously enriched with new barcodes, then there is also an increase in mis-labelled

sequences due to misidentification, which is a serious concern (Vilgalys 2003; Ward et al. 2009). By comparing sequences of a marine fish family from a reference library to those available in Genbank, an important source of barcodes for BOLD, Durand et al. (2017) demonstrated that misidentified species of a fish family were more frequent in barcoding studies than other types of studies like molecular phylogeny and phylogeography. This may be due to the difficulties of developing reliable systematic expertise for a wide range of fish taxa, for studies that do not target specific families.

As a consequence, while barcoding studies are still necessary to fill taxonomical knowledge gaps and solve some taxonomical issues, it is also necessary to review the DNA barcodes in BOLD, to better define species boundaries and track species misidentification. To reach this objective, new barcoding projects could be (1) more taxonomically restricted so as to limit potential species identification problems, (2) increase the number of specimens and sampling localities over the species distribution range so as to better estimate levels of intraspecific diversity, and (3) consider all DNA barcodes stored in the BOLD library, to track both mislabelled sequences and putative undescribed or cryptic species. While individual review of DNA barcodes in the BOLD library would be particularly tedious, the process could be facilitated by the barcode index number (BIN) provided by BOLD system that corresponds to an operational taxonomical unit (OTU). This OTU consists of barcodes that are identical or phylogenetically closely related and separated from others in the library by a barcode gap. OTUs are delimited in BOLD, using the Refined Single Linkage (RESL) algorithm (Ratnasingham and Hebert 2013). The BIN was created to circumvent taxonomical issues linked to identification problems, undescribed species or, more generally, to reveal cryptic diversity (Ratnasingham and Hebert 2013).

The grouper, Epinephelidae, is a marine fish family present in all temperate and tropical marine coastal waters. They comprise economically important species that are the target of fisheries and aquaculture. For this reason, the family has been the focus of a series of molecular phylogenetic and barcoding studies (Craig and Hastings 2007; Craig et al. 2011; Chu et al. 2011; Sachithanandam et al. 2012; Alcantara and Yambot 2016; Ma et al. 2016, 2018; Basheer et al. 2017). Among these studies, molecular phylogenetic or phylogeographic studies highlighted systematics issues such as the paraphyly of *Cephalopholis* (Bloch & Schneider, 1801) and *Epinephelus* (Bloch, 1793) genera, and the presence of cryptic species (Craig and Hastings 2007; Ma et al. 2016; Borsa et al. 2016; Ma and Craig 2018). In contrast, although all region-based barcoding studies provided new COI barcodes for groupers collected in countries such as India (Sachithanandam et al. 2012; Basheer et al. 2017), Malaysia (Chu et al. 2011), or the Philippines (Alcantara and Yambot 2016), none considered the barcodes in BOLD in order to review intraspecific diversity over a broader geographic scale than their barcoded species or the BIN diversity per species, which represent an alternative way to both track mis-labelling sequences or polyphyletic species.

Within global grouper diversity, 34 to 39 species may occur in Vietnamese waters (Heemstra and Randall 1993, 1999; Craig et al. 2011). Among these species, some are uncommon or have a limited distribution range. Due, however, to the difficulty in identifying some species by morphometric or phenotypic characters (Heemstra and Randall 1993; Guo et al. 2014; Alcantara and Yambot 2016) the species inventory should be considered with caution. Furthermore, new grouper species are still being described, such as *Epinephelus kupangensis* Tucker et al., 2016, *Epinephelus craigi* Frable et al., 2018 and *Epinephelus fuscomarginatus* Johnson & Worthington Wilmer, 2019. Other species have debateable taxonomic status, such as *Epinephelus moara* (Temminck & Schlegel, 1843) that is considered as valid by several authors (Guo et al. 2009; Liu et al. 2013; Guo et al. 2014) but a junior synonym of *Epinephelus bruneus* (Bloch, 1793) by others (Heemstra and Randall 1993; Craig et al. 2011; Fricke et al. 2019). For all these reasons, phylogenetic and barcoding studies are needed to clarify the species diversity present in Vietnam where barcoding analyses are rare (Thai et al. 2007; Tran et al. 2016; Ha et al. 2018; Huang et al. 2018) and missing for the Epinephelidae family.

In this context, this study aimed to create a reference barcoding library for Vietnam and test whether this approach can be used to update the species checklist and flag cryptic or undescribed species. Our ambition with this region-based barcoding study was also to review all BINs in BOLD for species present in Vietnam and determine significance of the presence of several BINs associated with a species. The last goal requires combining all available data to determine the congruency between species name associated with a BIN and some species' sequence considered as a reference (see hereafter), and investigate the phylogeographic structure of species associated with more than one BIN. Finally, this study represents a good opportunity to review the BOLD library and tracked misidentified specimens that can limit accuracy of species identification.

Material and methods

Taxonomy and species checklist for Vietnam

The taxonomy and classification of groupers is debated (Ma and Craig 2018) because successive molecular phylogenetic studies have highlighted a number of inconsistencies, such as the non-monophyly of the Serranidae family and of some genera within the Epinephilinae subfamily (Maggio et al. 2005; Ding et al. 2006; Craig and Hastings 2007; Craig et al. 2011; Zhuang et al. 2013; Schoelinck et al. 2014; Ma

et al. 2016). To resolve this issue, some authors have proposed an updated taxonomy (Smith and Craig 2007; Ma and Craig 2018). Within this, the subfamily "Epinephelinae" (sensu Johnson 1983) was elevated to family status and the tribes Diploprionini, Epinephelini, Grammistini, and Liopropomini to subfamilies within Epinephelidae (sensu Smith and Craig 2007). Furthermore, the species classification of the Epinephelinae (sensu Smith and Craig 2007) was revised according to their molecular phylogeny (Ma et al. 2016).

In the current study, the taxonomy proposed by Smith and Craig (2007) has been adopted but we have conserved the "traditional" genus naming of species of the Epinephelinae, since this has not yet been adopted by Eschemeyer's Catalog of Fishes (Fricke et al. 2019) and thus not recognized by BOLD or GenBank. However, in Table 1, the second column has the revised taxonomic nomenclature of Ma and Craig (2018), involving the genus replacement of *Aethaloperca* by *Cephalopholis*, *Anyperodon*, and *Cromileptes* by *Epinephelus*; *Triso* by *Hyporthodus*; and *Epinephelus* by *Mycteroperca* for the following species: *E. epistictus*, *E. heniochus*, *E. morrhua*, and *E. poecilonotus*.

The species checklist of Epinephelidae present in Vietnam (Table 1) has been established on the basis of two references, Heemstra and Randall (1993) and Craig et al. (2011). One additional species have been added to this list, *Epinephelus craigi* because it was recently described in the South China Sea, probably including Vietnamese waters (Nha Trang) according to Frable et al. (2019).

Sampling

Grouper specimens were collected between 2015 and 2017 from major fish landings and markets at 18 locations belonging to 11 provinces in Vietnam (Fig. 1). We strived to obtain as many species as possible, to provide the broadest overview of diversity in Vietnam. When possible, several specimens from the same species were collected at different locations, to provide some information about intraspecific diversity and distribution ranges in Vietnam. A total of 157 groupers were collected, plus one specimen of *Diploprion bifasciatum* belonging to the *Diploprioniae* subfamily, used as outgroup (Table S1). An initial species identification was performed on site using the FAO identification sheet (Heemstra and Randall 1993) and Craig et al. (2011). Each specimen was photographed and a small section of the pectoral fin stored in ethanol 70° in a 1.5 ml Eppendorf, which were then stored at − 20 °C in the laboratory.

DNA extraction and barcoding

Genomic DNA was isolated from fins using the G-Spin Total DNA extraction mini kit (iNtRON biotechnology, Korea) following the manufacturer's recommendations. Approximately 655 base pairs (bp) of the cytochrome oxydase I (COI) were amplified using the reverse primer FishR1 and the two forward primers in cocktail: FishF1 and FishF2 (Ward et al. 2005). The 40 μl PCR reaction mixes included 20 μl of MyTaq PCR Mastermix (Bioline), 16 μl of ultrapure water, 0.8 μl of BSA (Euromedex), 0.6 μl of each primers (3 μM), and 2 μl of DNA template. Amplifications were performed in a PTC-100 BIORAD Thermal cycler. The thermal regime consisted of an initial step of 2 min at 92 °C followed by 35 cycles of 45 s at 92 °C, 45 s at 52 °C, and 1 min at 72 °C, followed in turn by 5 min at 72 °C and then held at 4 °C. PCR products were visualized on 1–2% agarose gels and the most intense products were selected for sequencing by Macrogen (https://dna.macrogen.com/).

Electropherograms were visually checked using 4Peaks by A. Griekspoor and Tom Groothuis, nucleobytes.com, and sequences aligned using MUSCLE implemented in MEGA7 (Kumar et al. 2016). DNA barcodes and specimens' data are available in the project file DS-SERV: Epinephelidae of Vietnam on BOLD systems site dx.doi.org/10.5883/DS-SERV). Each DNA barcode was assigned to a BIN by the BOLD system and deposited in GenBank under accession number MN708800 to MN708957 (Table S1). Sequence divergences (Table S2) were calculated using the Kimura two parameters (K2P) distance model (Kimura 1980). Neighbor-joining (NJ) trees of K2P distances were drawn to highlight pattern of divergence between species (Saitou and Nei 1987). A bootstrapping test was performed in MEGA3 (Kumar et al. 2004) with 500 replications, to test the relevance of the tree's nodes.

Species identification and species boundaries

The accuracy of the OTU delineation provided by BOLD using the RESL algorithm was first evaluated by comparing BINs to OTUs identified by the Automatic Barcode Gap Delineation (ABGD) algorithm (Puillandre et al. 2012). These two methods used DNA alignments as inputs, consisting of ABGD, of all DNA barcodes (with a core segment of a 502 bp) constituting BINs associated with species name listed in Table 1, plus all Vietnamese barcodes generated in this study (Table S3). The final fasta file contained 1648 sequences and was uploaded to the website that hosted the ABGD algorithm to run the analyses (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html).

The first phylogenetic analyse was performed on a dataset composed exclusively of DNA barcodes obtained in this study, i.e., the groupers sampled in Vietnam. The tree was rooted with *Diploprion bifasciatum*. The species name recovered using the morphology and the BIN provided by BOLD was mapped onto this tree, to flag polyphyletic species and to reveal intraspecific and interspecific diversity within the Epinephelidae.

Table 1 Epinephilidae species inventoried in Vietnam according Heemstra and Randall (1993), Craig et al. (2011), and this study. In bold, species name that represent new records for Vietnam. For each species, the associated BINs in BOLD, in Ma et al. (2016) and in this study, are listed. In the BIN's BOLD column, number of public members present in the BIN/number of members labeled with the species name are mentioned in brackets, probable species misidentifications are in italics (see Table S3 for the rationale)

Classification "Catalog of Fishes"	Classification according Ma and Craig (2018)		Heemstra and Randall 1993 (FAO)	Craig et al. 2011	This study	BIN's BOLD	Ma et al. (2016)	BIN present in Vietnam (this study)	Multi-BIN species	Phylogeographic pattern
Aethaloperca rogaa	*Cephalopholis rogaa*	Forsskål, 1775	X	X		BOLD:AAD9665 [16/15], BOLD:ACC0322 [3/3]	BOLD:AAD9665	NA	Y?	N
Anyperodon leucogrammic-us	*Epinephelus leucogrammicus*	Valenciennes in Cuvier & Valenciennes, 1828	X	X		BOLD:AAC8328 [7/6], BOLD:AAC8327 [5/5]	BOLD:AAC8328	NA	Y	Y
Cephalopholis argus	–	Bloch & Schneider, 1801	X	X		BOLD:AAC4474 [26/24], BOLD:AAC0217 [21/1] , BOLD:AAD4737 [26/1]	BOLD:AAC4474	NA	N	N
Cephalopholis boenak	–	Bloch, 1790	X	X	X	BOLD:ACT7478 [32/31], BOLD:AAB3684 [7/7], BOLD:ACT7479 [1/1]	BOLD:ACT7478	BOLD:ACT7478	Y	Y
Cephalopholis cyanostigma	–	Valenciennes in Cuvier & Valenciennes, 1828	X	X	X	BOLD:AAC0217 [21/15]	BOLD:AAC0217	BOLD:AAC0217	N	N
Cephalopholis formosa	–	Shaw & Nodder, 1812	X	X	X	BOLD:AAF0621 [29/27]	BOLD:AAF0621	BOLD:AAF0621	N	N
Cephalopholis leopardus	–	Lacepède, 1802		X		BOLD:AAD9032 [7/7]	BOLD:AAD9032	NA	N	–
Cephalopholis microprion	–	Bleeker, 1852	X	X		BOLD:AAC0217 [21/4] , BOLD:AAF0629 [4/4]	*BOLD:AAC0217*	NA	N	–
Cephalopholis miniata	–	Forsskål, 1775	X	X	X	BOLD:AAC0216 [28/28], BOLD:AAC0217 [21/1]	BOLD:AAC0216	BOLD:AAC0216	N	N
Cephalopholis sonnerati	–	Valenciennes in Cuvier & Valenciennes, 1828	X	X	X	BOLD:AAD4737 [26/25], BOLD:AAB5431 [15/14], BOLD:ACC0647 [5/5], BOLD:AAB5432 [6/2]	BOLD:AAD4737	BOLD:AAD4737	Y	Y
Cephalopholis urodeta	–	Forster in Bloch & Schneider, 1801	X	X	X	BOLD:AAC4202 [234/93]	BOLD:AAC4202	BOLD:AAC4202	N	N
Cromileptes altivelis	*Epinephelus altivelis*	Valenciennes, 1828	X	X		BOLD:AAC1876 [16/16]	BOLD:AAC1876	NA	N	–
Epinephelus akaara	–	Temminck & Schlegel, 1842	X	X		BOLD:AAF1053 [83/81], BOLD:AAC5362 [50/1]	BOLD:AAF1053	NA	N	–
Epinephelus amblycephalus	–	Bleeker, 1857	X	X	X	BOLD:AAB1345 [67/48] , BOLD:AAB1346 [3/3], BOLD:ADA9689 [1/1]	*BOLD:AAB1345*	BOLD:AAB1346	N	N
Epinephelus areolatus	–	Forsskål, 1775	X	X	X	BOLD:AAA9821 [37/36], BOLD:AAA9822 [25/23], BOLD:ACC0344 [3/3]	BOLD:AAA9821	BOLD:AAA9821, BOLD:AA-A9822	Y	Y
Epinephelus awoara	–	Temminck & Schlegel, 1842	X	X	X	BOLD:AAF0829 [8/7], BOLD:AAB8391 [113/2]	BOLD:AAF0829	BOLD:AAF0829	N	-

Table 1 (continued)

Classification "Catalog of Fishes"	Classification according Ma and Craig (2018)		Heemstra and Randall 1993 (FAO)	Craig et al. 2011	This study	BIN's BOLD	Ma et al. (2016)	BIN present in Vietnam (this study)	Multi-BIN species	Phylogeographic pattern
Epinephelus bleekeri	–	Vaillant, 1878	X	X	X	BOLD:AAC5362 [50/48]	BOLD:AAC5362	BOLD:AAC5362	N	N
Epinephelus bruneus	–	Bloch, 1793			X	BOLD:AAJ0588 [6/6] , BOLD:ACS0266 [3/3]	*BOLD:AAJ0588*	BOLD:ACS0266	N	N
Epinephelus chlorostigma	–	Cuvier & Valenciennes, 1828	X	X	X	BOLD:AAC5363 [19/14] , BOLD:ABA7404 [5/5], BOLD:ADE3544 [1/1]	*BOLD:AAA9822*	BOLD:AAC3591	Y	–
Epinephelus coioides	–	Hamilton, 1822	X	X	X	BOLD:AAB8391 [113/76], BOLD:AAB8389 [35/1] , BOLD:AAC2802 [19/1]	BOLD:AAB8391	BOLD:AAB8391	N	N
Epinephelus corallicola	–	Valenciennes in Cuvier & Valenciennes, 1828		X	X	BOLD:AAD8682 [9/9]	BOLD:AAD8682	BOLD:AAD8682	N	–
Epinephelus craigi	–	Frable, Tucker & Walker, 2019				The name "craigi" is not in BOLD but the sequence match BOLD:AAB1345	NA	NA	–	–
Epinephelus cyanopodus	–	Richardson, 1846	X	X		BOLD:AAD1767 [16/3]	BOLD:AAD1767	NA	N	–
Epinephelus epistictus	*Mycteroperca epistictus*	Temminck & Schlegel, 1842	X	X		BOLD:ADB3169 [13/13], BOLD:AAF1066 [4/4]	BOLD:AAF1066	NA	Y	Y
Epinephelus erythrurus	–	Valenciennes in Cuvier & Valenciennes, 1828	X	X	X	BOLD:ACC0501 [15/11]	NA	BOLD:ACC0501	N	N
Epinephelus fasciatomaculosus	–	Peters, 1866	X	X		BOLD:AAC5392 [11/2], BOLD:AAF0829 [8/1] , BOLD:AAF1053 [83/2]	BOLD:AAC5392	NA	N	–
Epinephelus fasciatus	–	Forsskål, 1775	X	X	X	BOLD:AAB1332 [30/29], BOLD:AAB1334 [23/18], BOLD:AAB1333 [4/3], BOLD:AAB1335 [1/1]	BOLD:AAB1333	BOLD:AAB1332	Y	Y
Epinephelus fuscoguttatus	–	Forsskål, 1775			X	BOLD:AAD8872 [23/18], BOLD:AAD8873 [6/3]	BOLD:AAD8872	BOLD:AAD8872	N	Y
Epinephelus heniochus	*Mycteroperca heniochus*	Fowler, 1904	X	X		BOLD:AAD3855 [5/5]	NA	NA	–	–
Epinephelus hexagonatus	–	Forster in Bloch & Schneider, 1801			X	BOLD:AAD3458 [11/10]	BOLD:AAD3458	BOLD:AAD3458	N	Y
Epinephelus lanceolatus	–	Bloch, 1790	X	X		BOLD:AAD8914 [6/6], BOLD:AAD8872 [23/1]	BOLD:AAD8914	NA	N	–
Epinephelus latifasciatus	–	Temminck & Schlegel, 1842	X	X		BOLD:AAD8911 [9/9], BOLD:ADM3791 [5/5]	BOLD:ADM3791	NA	Y	Y
Epinephelus macrospilos	–	Bleeker, 1855		X		BOLD:AAE1882 [11/9], BOLD:AAB8386 [42/4]	BOLD:AAE1882	NA	N	-
Epinephelus malabaricus	–	Bloch & Schneider, 1801		X	X	BOLD:AAB8389 [35/27], BOLD:AAB8391 [113/1]	BOLD:AAB8389	BOLD:AAB8389	N	Y

Table 1 (continued)

Classification "Catalog of Fishes"	Classification according Ma and Craig (2018)		Heemstra and Randall 1993 (FAO)	Craig et al. 2011	This study	BIN's BOLD	Ma et al. (2016)	BIN present in Vietnam (this study)	Multi-BIN species	Phylogeographic pattern
Epinephelus merra	–	Bloch, 1793	X	X		BOLD:AAB8386 [42/38], BOLD:AAB8387 [13/4]	BOLD:AAB8386	NA	Y	Y
Epinephelus morrhua	*Mycteroperca morrhua*	Valenciennes in Cuvier & Valenciennes, 1833	X	X	X	BOLD:ACE6821 [15/14], BOLD:ADL0994 [13/4] , BOLD:AAC6284 [2/2], BOLD:ADC2027 [1/1]	BOLD:AAC6284	BOLD:ACE6821	Y	N
Epinephelus poecilonotus	*Mycteroperca poecilonotus*	Temminck and Schlegel, 1842	X	X		BOLD:AAD3854 [30/28], BOLD:AAD3855 [5/1]	BOLD:AAD3854	NA	N	–
Epinephelus polyphekadion	–	Bleeker, 1849			X	BOLD:AAD8873 [29/13], BOLD:ABX5597 [9/9]	BOLD:AAD8873	BOLD:AAD8873	Y	Y
Epinephelus quoyanus	–	Valenciennes in Cuvier & Valenciennes, 1830	X	X	X	BOLD:AAC2809 [18/18], BOLD:AAC5362 [50/1]	BOLD:AAC2809	BOLD:AAC2809	N	Y
Epinephelus rivulatus	–	Cuvier and Valenciennes, 1830	X	X	X	BOLD:ACZ9919 [22/17], BOLD:AAF0433 [3/1]	BOLD:ACZ9919	BOLD:AAF0433	Y	Y
Epinephelus sexfasciatus	–	Valenciennes in Cuvier & Valenciennes, 1828	X	X	X	BOLD:AAC5392 [11/9], BOLD:ACM3827 [13/13]	*BOLD:AAC5392*	BOLD:ACM3827	N	N
Epinephelus stictus	–	Randall and Allen, 1987	X	X		BOLD:AAB1345 [67/18]	NA	NA	–	–
Epinephelus trimaculatus	–	Cuvier & Valenciennes, 1828		X		BOLD:AAJ0586 [6/4]	BOLD:AAJ0586	NA	N	–
Plectropomus leopardus	–	Lacepède, 1802	X	X	X	BOLD:AAC9348 [86/54]	BOLD:AAC9348	BOLD:AAC9348	N	Y
Plectropomus maculatus	–	Bloch, 1790			X	BOLD:ACK7859 [16/16], BOLD:AAC9348 [86/9] , BOLD:ADL8508 [4/4]	BOLD:ACK7859	BOLD:ACK7859	Y	Y
Plectropomus oligacanthus	–	Bleeker, 1855			X	BOLD:AAL4434 [12/12]	BOLD:AAL4434	BOLD:AAL4434	N	–
Triso dermopterus	*Hyporthodus dermopterus*	Temminck and Schlegel, 1842			X	BOLD:AAD9769 [6/6]	BOLD:AAD9769	BOLD:AAD9769	N	–
Variola albimarginata	–	de Baissac, 1953			X	BOLD:AAC9675 [22/20]	BOLD:AAC9675	BOLD:AAC9675	N	Y
Variola louti	–	Forsskål, 1775			X	BOLD:AAC5719 [30/25], BOLD:AAC5720 [3/3]	BOLD:AAC5720	BOLD:AAC5719	Y	Y

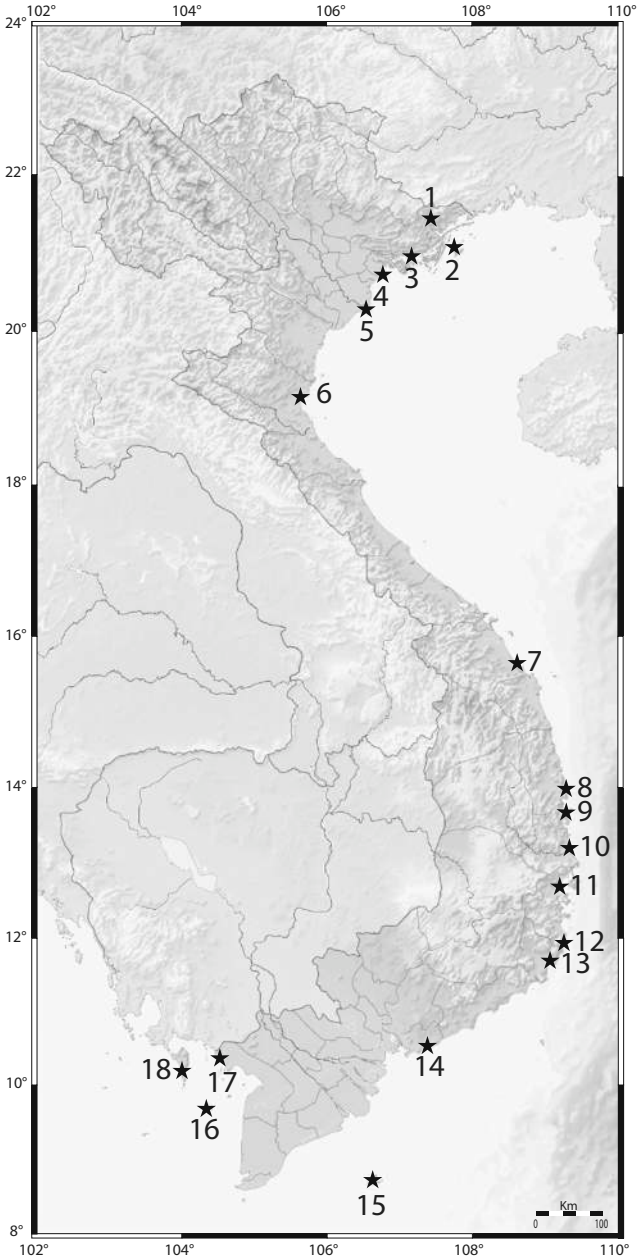


Fig. 1 Sampling locations in Vietnam for the DNA barcoding of the Epinephelidae subfamily. 1 Tien Yen, Quang Ninh; 2 Co To Island, Quang Ninh; 3 Ha Long bay, Quang Ninh; 4 Do Son, Hai Phong; 5 Xuan Thuy, Nam Dinh; 6 Hau Loc, Thanh Hoa; 7 Nui Thanh, Quang Nam; 8 Quy Nhon, Binh Dinh; 9 Cu Mong, Phu Yen; 10 Thuy Hoa, Phu Yen; 11 Nha Trang, Khanh Hoa; 12 Binh Ba Island, Khanh Hoa; 13 Ninh Thuan, Ninh Thuan; 14 Loc An, Ba Ria Vung Tau; 15 Con Dao, Ba Ria Vung Tau; 16 Nam Du, Kien Giang; 17 Ha Tien, Kien Giang; 18 Phu Quoc, Kien Giang

The second phylogenetic analysis was performed on a large dataset that comprised:

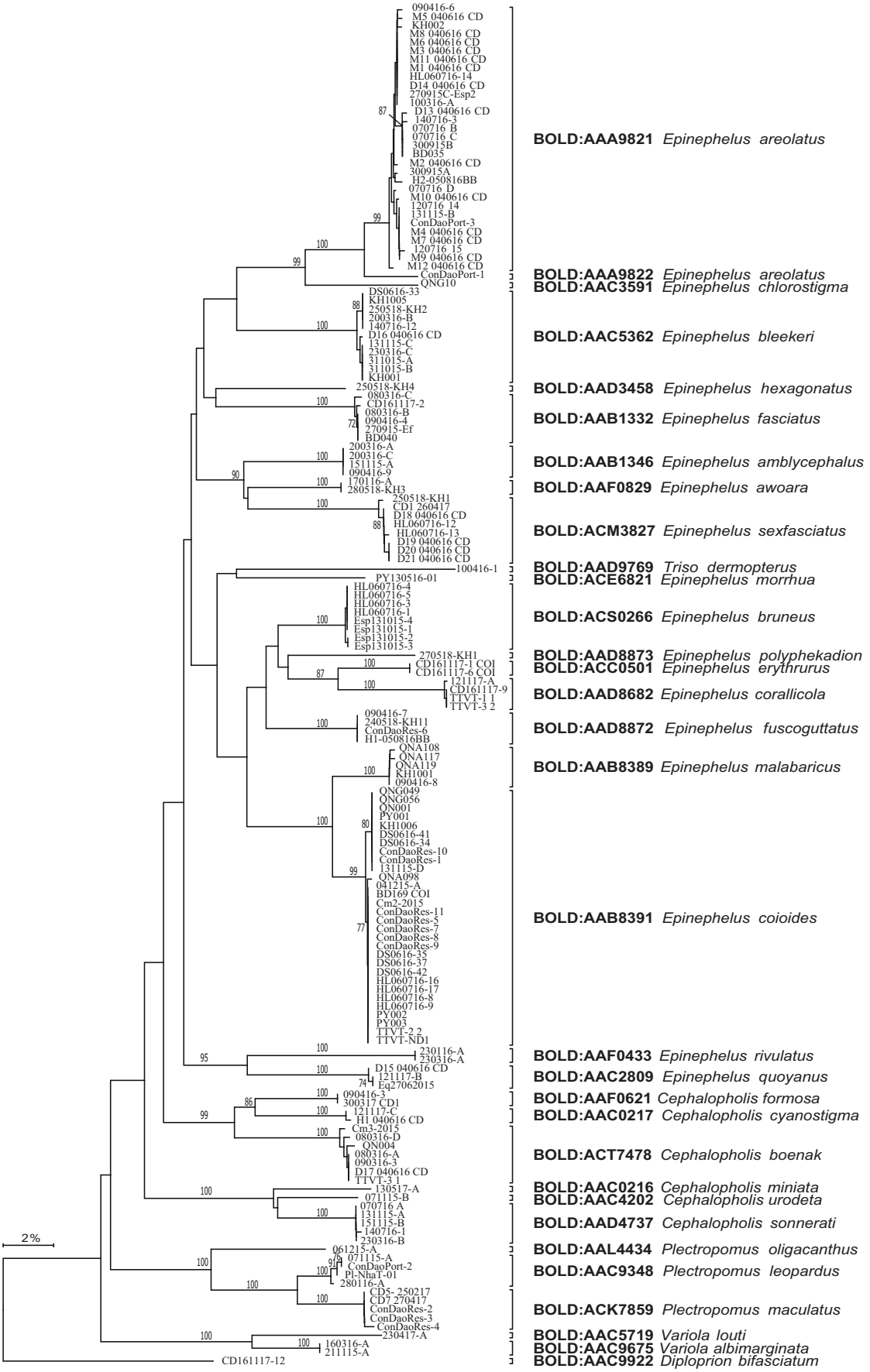
- One sequence representative of each species and lineage (BIN) observed in this study.
- All sequences published by Ma et al. (2016) that represent 87% of the known species diversity in the Epinephelidae and that may be considered as the reference.
- The COI sequences of *Epinephelus moara* resurrected by Liu et al. (2013), *Epinephelus craigi* described by Frable et al. (2019), and *Epinephelus fuscomarginatus* + *E. magniscuttis* described and redescribed respectively by Johnson and Worthington Wilmer (2019).
- One COI barcode of each BIN associated with a species name in BOLD, i.e., those present in the updated species checklist for Vietnam. BINs have been identified through the public data portal of BOLD (consultation 29/11/2018).

The aim of this second phylogenetic tree was to check concordance between species identification based on morphological criteria (present data) and DNA barcode (BOLD), to reveal misidentification or introgression in BINs associated with grouper species present in Vietnam and, finally, to highlight species that are polyphyletic due to the presence of several BINs. Species misidentification or introgression is responsible for more than one species name within a BIN. However, it is possible to determine specimens in a BIN that were misidentified or a hybrid, by considering:

- the number and origin (laboratory and geography) of specimens of different species within a BIN: the more barcodes were similarly identified by independent laboratories from various locations, the more the species identification may be considered reliable,
- the phenotype of the specimen when a picture is available in BOLD,
- the phylogeny, by considering the phylogenetic position of reference species sequence and BINs.

Following this rationale, we reassigned BINs to a probable species.

A phylogeographic analysis was used to further investigate processes responsible for intraspecific diversity in the groupers. A special attention was paid to polyphyletic species, those that present several BINs that form a clade in the phylogenetic tree, in order to determine the evolutionary significance of these BINs. All public COI barcodes belonging to BINs associated with a species name were uploaded and added to "Vietnamese" grouper data (Table S4). Haplotypic networks were estimated using the TCS method of Clement et al. (2002) implemented in PopART (French et al. 2014; http://popart.otago.ac.nz), and the geography and BIN were mapped


090416-6
M5 040616 CD
KH002
M8 040616 CD
M6 040616 CD
M3 040616 CD
M11 040616 CD
M1 040616 CD
HL060716-14
D14 040616 CD
270915C-Esp2
100316-A
D13 040616 CD
140716-3
070716 B
070716 C
300915B
BD035
M2 040616 CD
300915A
H2-050816BB
070716 D
M10 040616 CD
120716 14
131115-B
ConDaoPort-3
M4 040616 CD
M7 040616 CD
120716 15
M9 040616 CD
M12 040616 CD
ConDaoPort-1
QNG10
DS0616-33
KH1005
250518-KH2
200316-B
140716-12
D16 040616 CD
131115-C
230316-C
311015-A
311015-B
KH001
250518-KH4
080316-C
CD161117-2
080316-B
090416-4
270915-Ef
BD040
200316-A
200316-C
151115-A
090416-9
170116-A
280518-KH3
250518-KH1
CD1 260417
D18 040616 CD
HL060716-12
HL060716-13
D19 040616 CD
D20 040616 CD
D21 040616 CD
100416-1
PY130516-01
HL060716-4
HL060716-5
HL060716-3
HL060716-1
Esp131015-4
Esp131015-1
Esp131015-2
Esp131015-3
270518-KH1
CD161117-1 COI
CD161117-6 COI
121117-A
CD161117-9
TTVT-1 1
TTVT-3 2
090416-7
240518-KH11
ConDaoRes-6
H1-050816BB
QNA108
QNA117
QNA119
KH1001
090416-8
QNG049
QNG056
QN001
PY001
KH1006
DS0616-41
DS0616-34
ConDaoRes-10
ConDaoRes-1
131115-D
QNA098
041215-A
BD169 COI
Cm2-2015
ConDaoRes-11
ConDaoRes-5
ConDaoRes-7
ConDaoRes-8
ConDaoRes-9
DS0616-35
DS0616-37
DS0616-42
HL060716-16
HL060716-17
HL060716-8
HL060716-9
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PY003
TTVT-2 2
TTVT-ND1
230116-A
230316-A
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121117-B
Eq27062015
090416-3
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121117-C
H1 040616 CD
Cm3-2015
080316-D
QN004
080316-A
090316-3
D17 040616 CD
TTVT-3 1
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071115-B
070716 A
131115-A
151115-B
140716-1
230316-B
061215-A
071115-A
ConDaoPort-2
PI-NhaT-01
280116-A
CD5- 250217
CD7 270417
ConDaoRes-2
ConDaoRes-3
ConDaoRes-4
230417-A
160316-A
211115-A
CD161117-12
2%
BOLD:AAA9821 Epinephelus areolatus
BOLD:AAA9822 Epinephelus areolatus
BOLD:AAC3591 Epinephelus chlorostigma
BOLD:AAC5362 Epinephelus bleekeri
BOLD:AAD3458 Epinephelus hexagonatus
BOLD:AAB1332 Epinephelus fasciatus
BOLD:AAB1346 Epinephelus amblycephalus
BOLD:AAF0829 Epinephelus awoara
BOLD:ACM3827 Epinephelus sexfasciatus
BOLD:AAD9769 Triso dermopterus
BOLD:ACE6821 Epinephelus morrhua
BOLD:ACS0266 Epinephelus bruneus
BOLD:AAD8873 Epinephelus polyphekadion
BOLD:ACC0501 Epinephelus erythrurus
BOLD:AAD8682 Epinephelus corallicola
BOLD:AAD8872 Epinephelus fuscoguttatus
BOLD:AAB8389 Epinephelus malabaricus
BOLD:AAB8391 Epinephelus coioides
BOLD:AAF0433 Epinephelus rivulatus
BOLD:AAC2809 Epinephelus quoyanus
BOLD:AAF0621 Cephalopholis formosa
BOLD:AAC0217 Cephalopholis cyanostigma
BOLD:ACT7478 Cephalopholis boenak
BOLD:AAC0216 Cephalopholis miniata
BOLD:AAC4202 Cephalopholis urodeta
BOLD:AAD4737 Cephalopholis sonnerati
BOLD:AAL4434 Plectropomus oligacanthus
BOLD:AAC9348 Plectropomus leopardus
BOLD:ACK7859 Plectropomus maculatus
BOLD:AAC5719 Variola louti
BOLD:AAC9675 Variola albimarginata
BOLD:AAC9922 Diploprion bifasciatum

◀ **Fig. 2** Phylogenetic relationships among cytochrome oxydase I barcodes and BINs recovered among Vietnamese groupers. The pattern of divergence between species is represented by a neighbor-joining (NJ) tree based on K2P distances. The relevance of tree nodes are highlighted by bootstrap values (only values > 70% are indicated). The tree is rooted using *Diploprion bifasciatum*

onto these TCS networks to determine a phylogeographic structure.

Results

Known diversity of groupers in Vietnam and their associated BINs

By compiling knowledge from two major grouper species checklists for the West Pacific or worldwide (Heemstra and Randall 1993; Craig et al. 2011) and reviewing all recent grouper species description or redescription (Liu et al. 2013; Tucker et al. 2016; Frable et al. 2019), 40 species were recorded for Vietnam (Table 1). These species belong to either six or four different genera, according Eschmeyer's Catalog of Fishes (Fricke et al. 2019) or Ma and Craig (2018), respectively (Table 1). With the exception of *Epinephelus craigi* described recently (Frable et al. 2019) and still not referenced in BOLD, all these species names were associated with one or several BINs (up to 4, Table 1). Only 1/3 of the species were associated with only a single BIN.

Species diversity and BINs recovered in Vietnam

A total of 158 DNA barcodes (with a core segment of a 603 bp) were uploaded to the DS-SERV project (dx.doi.org/10.5883/DS-SERV). These DNA barcodes correspond to 31 species matching 32 BINs (Fig. 2), only one species *Epinephelus areolatus* presented haplotypes that belonged to two BINs (Tables 1 and 2). With the exception of *Epinephelus areolatus* that presented 3.5% of divergence between the two BINs, the intraspecies diversity (similar to the intra BIN) was extremely low and ranged from 0% for several species sampled more than one time to 0.4% for *Cephalopholis boenak* (Table S2). Interspecies divergence ranged from 3.8% between *Epinephelus malabaricus* and *Epinephelus coioides* to 24.3% between *Epinephelus malabaricus* and *Diploprion bifasciatum* (Table S2)

Among the species identified, nine were new records for Vietnam: *Epinephelus bruneus*, *Epinephelus fuscoguttatus*, *Epinephelus hexagonatus*, *Epinephelus polyphekadion*, *Plectropomus maculatus*, *Plectropomus oligacanthus*, *Triso dermopterus*, *Variola albimarginata*, and *Variola louti*. The grouper checklist for Vietnam therefore now includes 49 species. These species were associated with 76 BINs. The ABGD algorithm identified 77 OTUs that broadly matched the RESL delineation (i.e., BINs) (Table S3). Actually, if we exclude three cases, there are no differences between OTUs delineated by the ABGD or RESL algorithms. The OTU6 included sequences belonging to two BINs BOLD:AAD4737 and BOLD:AAB5431 while one sequence of this last BIN is highlighted as a distinct OTU: OTU7 (Table S3). All sequences belonging to OTU6, OTU7, BOLD:AAD4737, and BOLD:AAB5431 come from specimens identified as *Cephalopholis sonnerati*. The last discrepancy concerned a unique sequence that belonged to the BIN BOLD:AAC5719 but was assigned by ABGD to a specific OTU (OTU72). Because the RESL algorithm appeared more conservative by comparison to ABGD, which was able to create a specific OTU for only one sequence, we hereafter only considered BINs for further analyses.

Multi-BIN species may correspond to polyphyletic species, but specimens that were misidentified or hybridized would also artificially increase the number of BIN per species. For these latter cases, the careful reexamination revised the species associated with them (Table 2). For 23 BINs, it was possible to sort hybrids and misidentified specimens, and thus limit these BINs to one species name (see Table S5 for rationale). For one BIN (BOLD:ADE3544) originally assigned to *E. chlorostigma*, it was not possible to check the species identification because no picture was provided for any BIN members while its phylogenetic position did not show any close relationships with either *E. chlorostigma* lineages or the species barcode reference.

In the phylogenetic tree (Fig. 3) we also noticed some issues for a number of barcodes published by Ma et al. (2016). The COI sequence corresponding to *E. chlorostigma* in Ma et al. (2016) is grouped with sequences identified as *E. areolatus* in BOLD and the present study (Fig. 3 (5)). The Ma et al. (2016) COI sequence of *E. bruneus* matched the sequence of the resurrected species *E. moara* published by Liu et al. (2013) but not the *E. bruneus* sampled in Vietnam that belonged to the BIN BOLD:ACS0266 that was also associated with *E. bruneus*. (Fig. 3 (3)). Their sequence labelled as *E. sexfasciatus* was not different to their sequence labelled as *E. fasciatomaculosus* while, in this study, we identified a specimen as *E. sexfasciatus* that presented a different sequence that belonged to the BIN BOLD:ACM3827 that only comprised *E. sexfasciatus* members (Fig. 3 (6)). Their sequence of *E. amblycephalus* corresponded to the DNA barcode later described as *E. craigi* (Frable et al. 2019) (Fig. 3 (6)). Their COI sequence corresponding to *E. irroratus* was phylogenetically extremely close to *E. fasciatus* that comprised 4 BINs. Similarly, no genetic divergence was observed between their sequence of *Cephalopholis nigripinnis* and *C. urodeta* (Fig. 3 (7)), or between *C. cyanostigma* and *C. microprion* (Fig. 3 (8)). Considering this revision, several conditions can be highlighted:

Table 2 List of BINs present in the Table 1 and associated species name after revision (see rationale in Table S5) or in BOLD

BIN	According to this review	In BOLD			
	Species name	Species 1	Species 2	Species 3	Species 4
BOLD:AAB1332	*Epinephelus fasciatus*	*Epinephelus fasciatus*			
BOLD:AAB1333	*Epinephelus fasciatus*	*Epinephelus fasciatus*	*Epinephelus irroratus*		
BOLD:AAB1334	*Epinephelus fasciatus*	*Epinephelus fasciatus*			
BOLD:AAB1335	*Epinephelus fasciatus*	*Epinephelus fasciatus*			
BOLD:AAB3684	*Cephalopholis boenak*	*Cephalopholis boenak*			
BOLD:ACT7478	*Cephalopholis boenak*	*Cephalopholis boenak*	*Cephalopholis igarashiensis*		
BOLD:ACT7479	*Cephalopholis boenak*	*Cephalopholis boenak*			
BOLD:AAB5431	*Cephalopholis sonnerati*	*Cephalopholis sonnerati*			
BOLD:AAD4737	*Cephalopholis sonnerati*	*Cephalopholis sonnerati*	*Cephalopholis argus*		
BOLD:ACC0647	*Cephalopholis sonnerati*	*Cephalopholis sonnerati*			
BOLD:AAA9821	*Epinephelus areolatus*	*Epinephelus areolatus*			
BOLD:AAA9822	*Epinephelus areolatus*	*Epinephelus areolatus*			
BOLD:ACC0344	*Epinephelus areolatus*	*Epinephelus areolatus*			
BOLD:AAC6284	*Epinephelus morrhua*	*Epinephelus morrhua*			
BOLD:ACE6821	*Epinephelus morrhua*	*Epinephelus morrhua*			
BOLD:ADC2027	*Epinephelus morrhua*	*Epinephelus morrhua*			
BOLD:AAD9665	*Aethaloperca rogaa*	*Aethaloperca rogaa*			
BOLD:ACC0322	*Aethaloperca rogaa*	*Aethaloperca rogaa*			
BOLD:AAC8327	*Anyperodon leucogrammicus*	*Anyperodon leucogrammicus*			
BOLD:AAC8328	*Anyperodon leucogrammicus*	*Anyperodon leucogrammicus*			
BOLD:AAC3591	*Epinephelus chlorostigma*	*Epinephelus spilotoceps*			
BOLD:ABA7404	*Epinephelus chlorostigma*	*Epinephelus chlorostigma*			
BOLD:AAF1066	*Epinephelus epistictus*	*Epinephelus epistictus*			
BOLD:ADB3169	*Epinephelus epistictus*	*Epinephelus epistictus*			
BOLD:AAD8911	*Epinephelus latifasciatus*	*Epinephelus latifasciatus*			
BOLD:ADM3791	*Epinephelus latifasciatus*	*Epinephelus latifasciatus*			
BOLD:AAB8386	*Epinephelus merra*	*Epinephelus merra*	*Epinephelus macrospilos*		
BOLD:AAB8387	*Epinephelus merra*	*Epinephelus merra*	*Epinephelus faveatus*		
BOLD:AAD8873	*Epinephelus polyphekadion*	*Epinephelus polyphekadion*	*Epinephelus fuscoguttatus*		
BOLD:ABX5597	*Epinephelus polyphekadion*	*Epinephelus polyphekadion*			
BOLD:AAF0433	*Epinephelus rivulatus*	*Epinephelus rivulatus*			
BOLD:ACZ9919	*Epinephelus rivulatus*	*Epinephelus rivulatus*			
BOLD:ACK7859	*Plectropomus maculatus*	*Plectropomus maculatus*			
BOLD:ADL8508	*Plectropomus maculatus*	*Plectropomus maculatus*			
BOLD:AAC5719	*Variola louti*	*Variola louti*			
BOLD:AAC5720	*Variola louti*	*Variola louti*			
BOLD:AAB1346	*Epinephelus amblycephalus*	*Epinephelus amblycephalus*			
BOLD:AAJ0588	*Epinephelus moara*	*Epinephelus bruneus*			

Table 2 (continued)

BIN	According to this review	In BOLD			
	Species name	Species 1	Species 2	Species 3	Species 4
BOLD:ACS0266	*Epinephelus bruneus*	*Epinephelus bruneus*			
BOLD:AAC4474	*Cephalopholis argus*	*Cephalopholis argus*			
BOLD:AAB5432	*Cephalopholis aurantia*	*Cephalopholis aurantia*	*Cephalopholis sonnerati*	*Cephalopholis spiloparaea*	
BOLD:AAC0217	*Cephalopholis cyanostigma*	*Cephalopholis cyanostigma*	*Cephalopholis microprion*	*Cephalopholis miniata*	*Cephalopholis argus*
BOLD:AAF0621	*Cephalopholis formosa*	*Cephalopholis formosa*			
BOLD:AAD9032	*Cephalopholis leopardus*	*Cephalopholis leopardus*			
BOLD:AAF0629	*Cephalopholis microprion*	*Cephalopholis microprion*			
BOLD:AAC0216	*Cephalopholis miniata*	*Cephalopholis miniata*			
BOLD:AAC1876	*Cromileptes altivelis*	*Cromileptes altivelis*			
BOLD:AAF1053	*Epinephelus akaara*	*Epinephelus akaara*	*Epinephelus fasciatomaculosus*		
BOLD:AAF0829	*Epinephelus awoara*	*Epinephelus awoara*	*Epinephelus fasciatomaculosus*		
BOLD:AAC5362	*Epinephelus bleekeri*	*Epinephelus bleekeri*	*Epinephelus quoyanus*	*Epinephelus akaara*	
BOLD:ADL0994	*Epinephelus coeruleopunctatus*	*Epinephelus coeruleopunctatus*	*Epinephelus morrhua*		
BOLD:AAB8391	*Epinephelus coioides*	*Epinephelus coioides*	*Epinephelus tauvina*	*Epinephelus awoara*	*Epinephelus malabaricus*
BOLD:AAD8682	*Epinephelus corallicola*	*Epinephelus corallicola*			
BOLD:AAB1345	*Epinephelus craigi*	*Epinephelus amblycephalus*	*Epinephelus stictus*		
BOLD:ACC0501	*Epinephelus erythrurus*	*Epinephelus erythrurus*	*Epinephelus coeruleopunctatus*		
BOLD:AAC5392	*Epinephelus fasciatomaculosus*	*Epinephelus sexfasciatus*	*Epinephelus fasciatomaculosus*		
BOLD:AAD8872	*Epinephelus fuscoguttatus*	*Epinephelus fuscoguttatus*	*Epinephelus lanceolatus*		
BOLD:AAD3855	*Epinephelus heniochus*	*Epinephelus heniochus*	*Epinephelus poecilonotus*		
BOLD:AAD3458	*Epinephelus hexagonatus*	*Epinephelus hexagonatus*			
BOLD:AAD8914	*Epinephelus lanceolatus*	*Epinephelus lanceolatus*			
BOLD:AAE1882	*Epinephelus macrospilos*	*Epinephelus macrospilos*			
BOLD:AAB8389	*Epinephelus malabaricus*	*Epinephelus malabaricus*	*Epinephelus coioides*		
BOLD:AAC2802	*Epinephelus ongus*	*Epinephelus ongus*	*Epinephelus coioides*		
BOLD:AAD3854	*Epinephelus poecilonotus*	*Epinephelus poecilonotus*			
BOLD:AAC5363	*Epinephelus polylepis*	*Epinephelus chlorostigma*	*Epinephelus polylepis*		
BOLD:AAC2809	*Epinephelus quoyanus*	*Epinephelus quoyanus*			
BOLD:ACM3827	*Epinephelus sexfasciatus*	*Epinephelus sexfasciatus*			
BOLD:ADA9689	*Epinephelus stictus*	*Epinephelus amblycephalus*			
BOLD:ADE3544	*Epinephelus* sp. 1	*Epinephelus chlorostigma*			
BOLD:AAJ0586	*Epinephelus trimaculatus*	*Epinephelus trimaculatus*	*Epinephelus fario*		
BOLD:AAC9348	*Plectropomus leopardus*	*Plectropomus leopardus*	*Plectropomus areolatus*	*Plectropomus maculatus*	*Plectropomus pessuliferus*

Table 2 (continued)

BIN	According to this review	In BOLD			
	Species name	Species 1	Species 2	Species 3	Species 4
BOLD:AAL4434	*Plectropomus oligacanthus*	*Plectropomus oligacanthus*			
BOLD:AAD9769	*Triso dermopterus*	*Triso dermopterus*			
BOLD:AAC9675	*Variola albimarginata*	*Variola albimarginata*			
BOLD:AAC4202	*Cephalopholis urodeta/C. nigripinnis*	*Cephalopholis urodeta*	*Cephalopholis nigripinnis*		
BOLD:AAD1767	*Epinephelus cyanopodus/E. flavocaeruleus*	*Epinephelus flavocaeruleus*	*Epinephelus cyanopodus*		

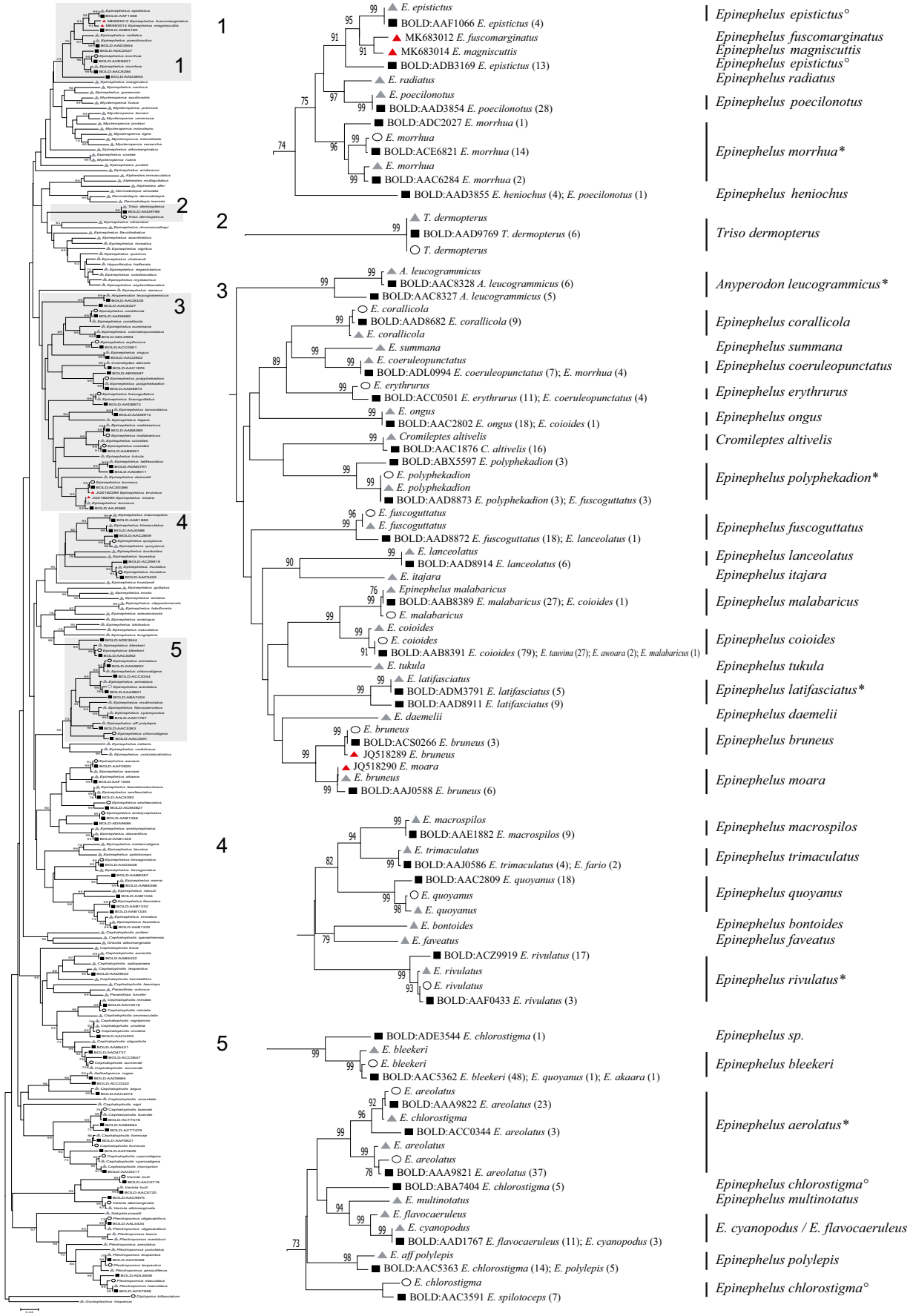
- One BIN–several species: Only two BINs corresponded to this situation where there was no apparent misidentification: BOLD:AAD1767 composed of members either identified as *E. cyanopodus* or *E. flavocaeruleus*, and BOLD:AAC4202 composed of members that can either corresponded to *Cephalopholis urodeta* or *C. nigripinnis*.
- One BIN–one species: This concerned 37 species listed in Table 2. *Chromileptes altivelis*, *Cephalopholis argus*, *C. aurantia*, *C. cyanostigma*, *C. formosa*, *C. leopardus*, *C. microprion*, *C. miniata*, *Epinephelus akaraa*, *E. amblycephalus*, *E. awoara*, *E. bleekeri*, *E. bruneus*, *E. coioides*, *E. corallicola*, *E. craigii*, *E. heniochus*, *E. coeruleopunctatus*, *E. erythrurus*, *E. hexagagonatus*, *E. fasciatomaculosus*, *E. fuscoguttatus*, *E. lanceolatus*, *E. malabaricus*, *E. macrospilos*, *E. moara*, *E. ongus*, *E. poecilonotus*, *E. polylepis*, *E. quoyanus*, *E. sexfasciatus*, *E. stictus*, *E. trimaculatus*, *Plectropomus leopardus*, *P. oligacanthus*, *Triso dermopterus*, *Variola albimarginata.*
- One species–several BINs: This concerned 15 species: *Aethaloperca rogaa*, *Anyperodon leucogrammicus*, *Cephalopholis boenak*, *C. sonnerati*, *Epinephelus areolatus*, *E. chlorostigma*, *E. epistictus*, *E. fasciatus*, *E. latifasciatus*, *E. merra*, *E. morruha*, *E. polyphekadion*, *E. rivulatus*, *Plectropomus maculatus*, *Variola louti.*

Phylogeographic structure of polyphyletic species

The TCS networks built for species that remained polyphyletic (several BINs for a unique species name) after BIN/species reassignment showed various phylogeographic patterns.

- A geographic overlap of BINs/clades associated with a species name. This was only observed for *Epinephelus morrhua*. However, it is noticeable that, among the three clades corresponding to 3 BINs, two are limited to one geographic location (East Australia for BOLD:ADC2027 and the South China Sea for BOLD:AAC6284) while the third was widespread and present in the whole Indo-Pacific Ocean (Fig. 4l).
- A strict allopatric/parapatric distribution for at least one BIN associated with a species. This was observed for: *Aethaloperca rogaa*, *Epinephelus areolatus* and *Plectropomus maculatus* with the BINs BOLD:ACC0322, BOLD:ACC0344, and BOLD:ADL8508 respectively, restricted to the Andaman Islands (Fig. 4a, f, o); *Cephalopholis sonnerati* with BINs BOLD:AAB5431 and BOLD:ACC0647 restricted to SW Indian Ocean and the Andaman Islands, respectively (Fig. 4d); *Epinephelus epistictus* with the BIN BOLD:ADB3169 and the BIN BOLD:AAF1066 restricted to the Indian Ocean and the South China Sea, respectively (Fig. 4h); *E. fasciatus* and *E. rivulatus* with their BINs (BOLD:AAB1334 and BOLD:ACZ9919, respectively) restricted to the West Indian Ocean and the Red Sea (Fig. 4i, k); *E. polyphekadion* for which the distribution of the two BINs showed a clear geographic barrier located in the central part of the Indian Ocean (Fig. 4m), and *Variola louti* with the BIN BOLD:AAC5720 restricted to the Central Pacific Ocean: (Fig. 4p).
- Partial parapatry corresponded to species that presented several BINs at one location that, elsewhere, were in parapatry. This was the case for: *Anyperodon leucogrammicus* with two BINs present in Taiwan whereas BOLD:AAC8328 presented a distribution usually

Fig. 3 Phylogenetic relationships among all BINs recovered in Vietnam (white dot), BINs associated with species inventoried in Vietnam (black square), and some species COI sequence references from Ma et al. (2016, gray triangle), Frable et al. (2019, red triangle), Liu et al. (2013, red triangle), and Johnson and Worthington Wilmer (2019, red triangle). The pattern of divergence among species is represented by a neighbor-joining (NJ) tree based on K2P distances. The relevance of tree nodes are highlighted by bootstrap values (only value > 70% are indicated). *Flags polyphyletic species (more than one BIN but phylogenetically close); °flags paraphyletic species


1
E. epistictus
BOLD:AAF1066 E. epistictus (4)
MK683012 E. fuscomarginatus
MK683014 E. magniscuttis
BOLD:ADB3169 E. epistictus (13)
E. radiatus
E. poecilonotus
BOLD:AAD3854 E. poecilonotus (28)
BOLD:ADC2027 E. morrhua (1)
E. morrhua
BOLD:ACE6821 E. morrhua (14)
E. morrhua
BOLD:AAC6284 E. morrhua (2)
BOLD:AAD3855 E. heniochus (4); E. poecilonotus (1)
Epinephelus epistictus°
Epinephelus fuscomarginatus
Epinephelus magniscuttis
Epinephelus epistictus°
Epinephelus radiatus
Epinephelus poecilonotus
Epinephelus morrhua*
Epinephelus heniochus
2
T. dermopterus
BOLD:AAD9769 T. dermopterus (6)
T. dermopterus
Triso dermopterus
3
A. leucogrammicus
BOLD:AAC8328 A. leucogrammicus (6)
BOLD:AAC8327 A. leucogrammicus (5)
E. corallicola
BOLD:AAD8682 E. corallicola (9)
E. corallicola
E. summana
E. coeruleopunctatus
BOLD:ADL0994 E. coeruleopunctatus (7); E. morrhua (4)
E. erythrurus
BOLD:ACC0501 E. erythrurus (11); E. coeruleopunctatus (4)
E. ongus
BOLD:AAC2802 E. ongus (18); E. coioides (1)
Cromileptes altivelis
BOLD:AAC1876 C. altivelis (16)
BOLD:ABX5597 E. polyphekadion (3)
E. polyphekadion
E. polyphekadion
BOLD:AAD8873 E. polyphekadion (3); E. fuscoguttatus (3)
E. fuscoguttatus
E. fuscoguttatus
BOLD:AAD8872 E. fuscoguttatus (18); E. lanceolatus (1)
E. lanceolatus
BOLD:AAD8914 E. lanceolatus (6)
E. itajara
Epinephelus malabaricus
BOLD:AAB8389 E. malabaricus (27); E. coioides (1)
E. malabaricus
E. coioides
E. coioides
BOLD:AAB8391 E. coioides (79); E. tauvina (27); E. awoara (2); E. malabaricus (1)
E. tukula
E. latifasciatus
BOLD:ADM3791 E. latifasciatus (5)
BOLD:AAD8911 E. latifasciatus (9)
E. daemelii
E. bruneus
BOLD:ACS0266 E. bruneus (3)
JQ518289 E. bruneus
JQ518290 E. moara
E. bruneus
BOLD:AAJ0588 E. bruneus (6)
Anyperodon leucogrammicus*
Epinephelus corallicola
Epinephelus summana
Epinephelus coeruleopunctatus
Epinephelus erythrurus
Epinephelus ongus
Cromileptes altivelis
Epinephelus polyphekadion*
Epinephelus fuscoguttatus
Epinephelus lanceolatus
Epinephelus itajara
Epinephelus malabaricus
Epinephelus coioides
Epinephelus tukula
Epinephelus latifasciatus*
Epinephelus daemelii
Epinephelus bruneus
Epinephelus moara
4
E. macrospilos
BOLD:AAE1882 E. macrospilos (9)
E. trimaculatus
BOLD:AAJ0586 E. trimaculatus (4); E. fario (2)
BOLD:AAC2809 E. quoyanus (18)
E. quoyanus
E. quoyanus
E. bontoides
E. faveatus
BOLD:ACZ9919 E. rivulatus (17)
E. rivulatus
E. rivulatus
BOLD:AAF0433 E. rivulatus (3)
Epinephelus macrospilos
Epinephelus trimaculatus
Epinephelus quoyanus
Epinephelus bontoides
Epinephelus faveatus
Epinephelus rivulatus*
5
BOLD:ADE3544 E. chlorostigma (1)
E. bleekeri
E. bleekeri
BOLD:AAC5362 E. bleekeri (48); E. quoyanus (1); E. akaara (1)
E. areolatus
BOLD:AAA9822 E. areolatus (23)
E. chlorostigma
BOLD:ACC0344 E. areolatus (3)
E. areolatus
E. areolatus
BOLD:AAA9821 E. areolatus (37)
BOLD:ABA7404 E. chlorostigma (5)
E. multinotatus
E. flavocaeruleus
E. cyanopodus
BOLD:AAD1767 E. flavocaeruleus (11); E. cyanopodus (3)
E. aff polylepis
BOLD:AAC5363 E. chlorostigma (14); E. polylepis (5)
E. chlorostigma
BOLD:AAC3591 E. spilotoceps (7)
Epinephelus sp.
Epinephelus bleekeri
Epinephelus aerolatus*
Epinephelus chlorostigma°
Epinephelus multinotatus
E. cyanopodus / E. flavocaeruleus
Epinephelus polylepis
Epinephelus chlorostigma°

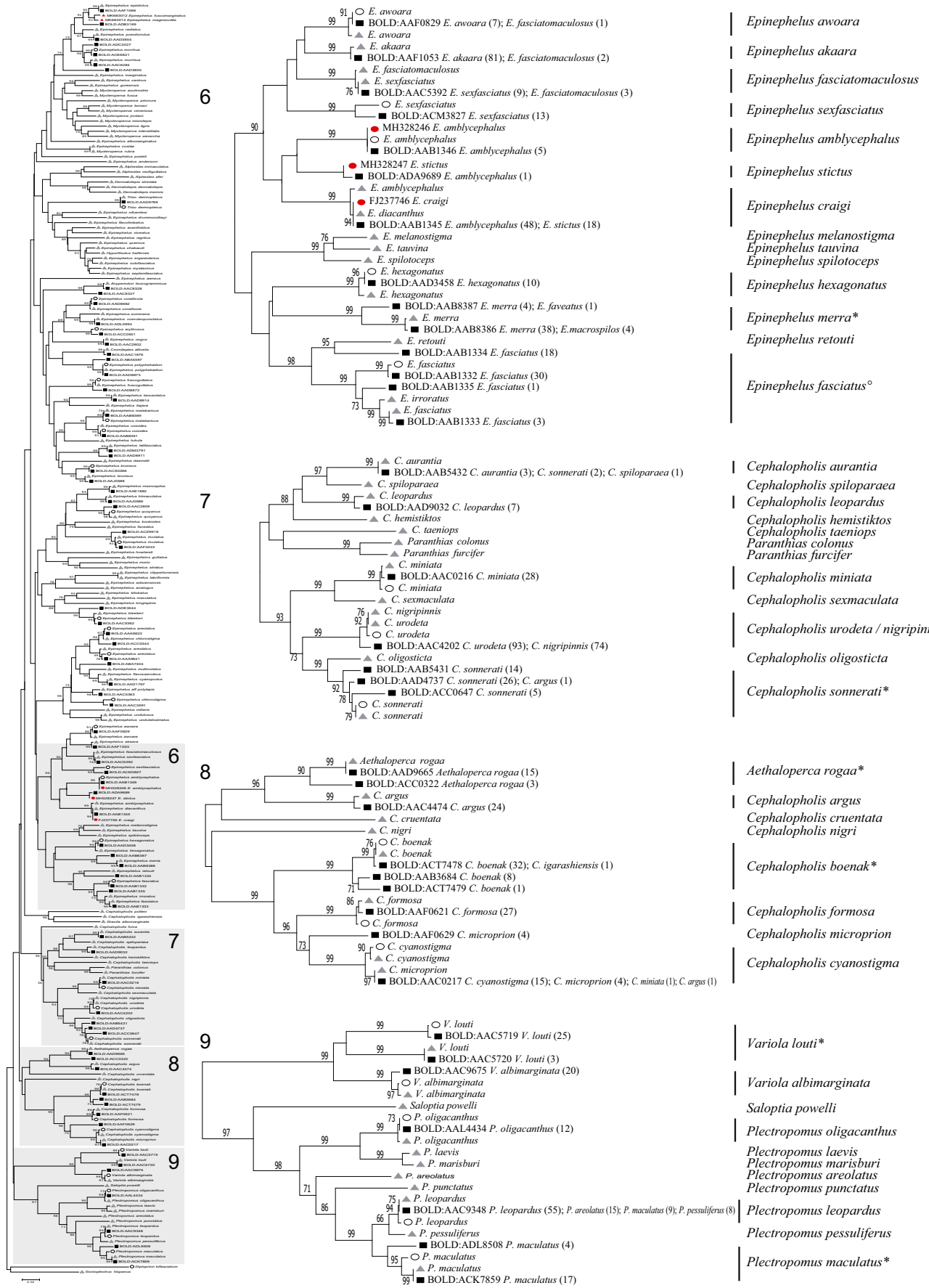


Fig. 3 (continued)

restricted to the Indian Ocean (Fig. 4b); *Cephalopholis boenak* with two BINs present in Western Australia whereas BOLD:AAB3684 was restricted to Australian shores (Fig. 4c); *Epinephelus areolatus* with two BINs present in Vietnam while BIN BOLD:AAA9822 was mainly observed in the Indian Ocean (Fig. 4f), and *E. merra* (Fig. 4k) with two BINs observed in Indonesia while their distribution range were allopatric outside this location and presented a clear separation between the Indian and the Pacific Oceans.

– Lastly, for some multi BIN species a phylogeographic structure was also observed within a BIN. This was the case for: *Plectropomus maculatus* (Fig. 4o) with the BIN BOLD:ACK7859 that showed two sets of haplotypes either distributed along the Australian shore or in the South East Asia, and *Variola louti* (Fig. 4p) with the BIN BOLD:AAC5719 that presented three subclades restricted to the Red Sea, the Andaman Islands and the Indian Ocean.

Discussion

Grouper diversity in Vietnam

To date, the species checklist for Vietnam consisted of 40 groupers and, while this study identified 30 species, up to nine are new records. As a result, up to 50% of known Vietnamese grouper diversity was not recovered in this study, despite sampling on a large spatial and temporal scale. Several species listed are, however, uncommon and can present a patchy geographical distribution or occurrence in Vietnam that is limited to some provinces. This is the case for *Anyperodon leucogrammicus*, *Cephalopholis leopardus*, *C. microprion*, *Epinephelus akaara*, *E. craigi*, *E. cyanopodus*, etc. Furthermore, the absence of some species may be a consequence of the intense fishing pressure on many groupers. In 1996, 43.5% of groupers were considered as threatened and two, *Cromileptes altivelis* and *Epinephelus akaara* as endangered, in the IUCN red list (Moriss et al. 2000). These two endangered species were not observed in the present study despite historical records in Vietnam. Consequently, our results suggest that more grouper species may be locally endangered but the absence, in Vietnam, of temporal series of fish biomass landing hampers our ability to monitor population dynamics and evaluate species status (Stocks et al. 2019). Life history traits of groupers, such as large body size, slow growth, long life-span, late reproduction, plus behaviors such as territoriality and spawning aggregations, make their survival uncertain and the chances of local population collapse high (Moriss et al. 2000; Sadovy de Mitcheson et al. 2012). Beyond biological reasons that render groupers vulnerable to over-exploitation, pollution, and habitat destruction are also a major threat. Urbanization of coastal areas in Vietnam has degraded and even destroyed many aquatic habitats. Mangrove forests, known to be important nursery areas for marine fishes including groupers, declined dramatically from 4000 km^2 in 1950 to 1570 km^2 in 1994 (Son and Thuoc 2003), due to conversion for agriculture or pond aquaculture, and unsustainable use for timber (Hong 1993). Similarly, coral reefs that concentrate a high proportion of grouper species have decreased to 15–20% of their original surface due to pollution, siltation, and blast fishing (Lutaenko 2011). For all these reasons, it is likely that many groupers species in Vietnam have become rare, so explaining their absence in the present sampling.

Curiously, up to nine species are new records for Vietnam. This contrasts with the species rarefaction described above. These new records may also, however, be explained by exploitation. Vietnamese fisheries are primarily small-scale and inshore, but, due to the decline of many high-value inshore resources, many fishermen now focus on deeper waters (Thuoc 2001). As a consequence, some species that are not present inshore and thus were not targeted historically by the small scale fisheries, have become more visible on fish markets (Sadovy de Mitcheson et al. 2012). Among the nine new records up to six (*E. bruneus*, *P. maculatus*, *P. oligacanthus*, *T. dermoptrus*, *V. albimarginatus* and *V. louti*) present a vertical distribution extending into deep water below above 100 m. None are, however, strictly restricted to deep waters, which limits this interpretation such that it cannot by itself explain why these species were not previously recorded. Nevertheless, in recent Vietnamese investigations, six of these species were observed (Le et al. 2011; Tran et al. 2015; Vo et al. 2016; Vo 2018) while *E. fuscoguttatus* is known to be reared in aquaculture at Quang Ninh based on larvae fished in the wild (Le 2004). Last, there are still two species: *E. polyphekadion* and *V. albimarginata* that remain new records for Vietnam whatever species checklist is considered and regardless of any aquaculture practices in Vietnam. The distribution range of some groupers are known to be very patchy, such as *E. polyphekadion*, such that they may be limited to small areas in Vietnam and have therefore not been recorded before. Concerning *V. albimarginata*, it is more difficult to explain why it was not recorded before because the species seems abundant in Vietnam (pers obs) and is difficult to misidentify with other groupers.

BIN diversity in Indo-Pacific groupers present in Vietnam

Specimens mislabelled in BOLD may have two origins: taxonomic confusion or hybridization. Grouper taxonomy is traditionally based on color patterns, morphological

distinctiveness, and osteological characters (Heemstra and Randall 1993) but, for some species, these criteria overlap from a species to another leading to confusion (Alcantara and Yambot 2016; Qu et al. 2018). Furthermore, recent taxonomical investigations describing new species confound with other species in previous DNA barcoding studies. This is the case for *E. craigi* recently described by Frabe et al. (2018) and previously confounded or still misidentified with *E. amblycephalus* and *E. stictus.* Similarly, despite the redescription of *E. moara* by Liu et al. (2013), this species is still ignored in BOLD and its BIN:BOLD:AAJ0588 is associated with *Epinephelus bruneus*.

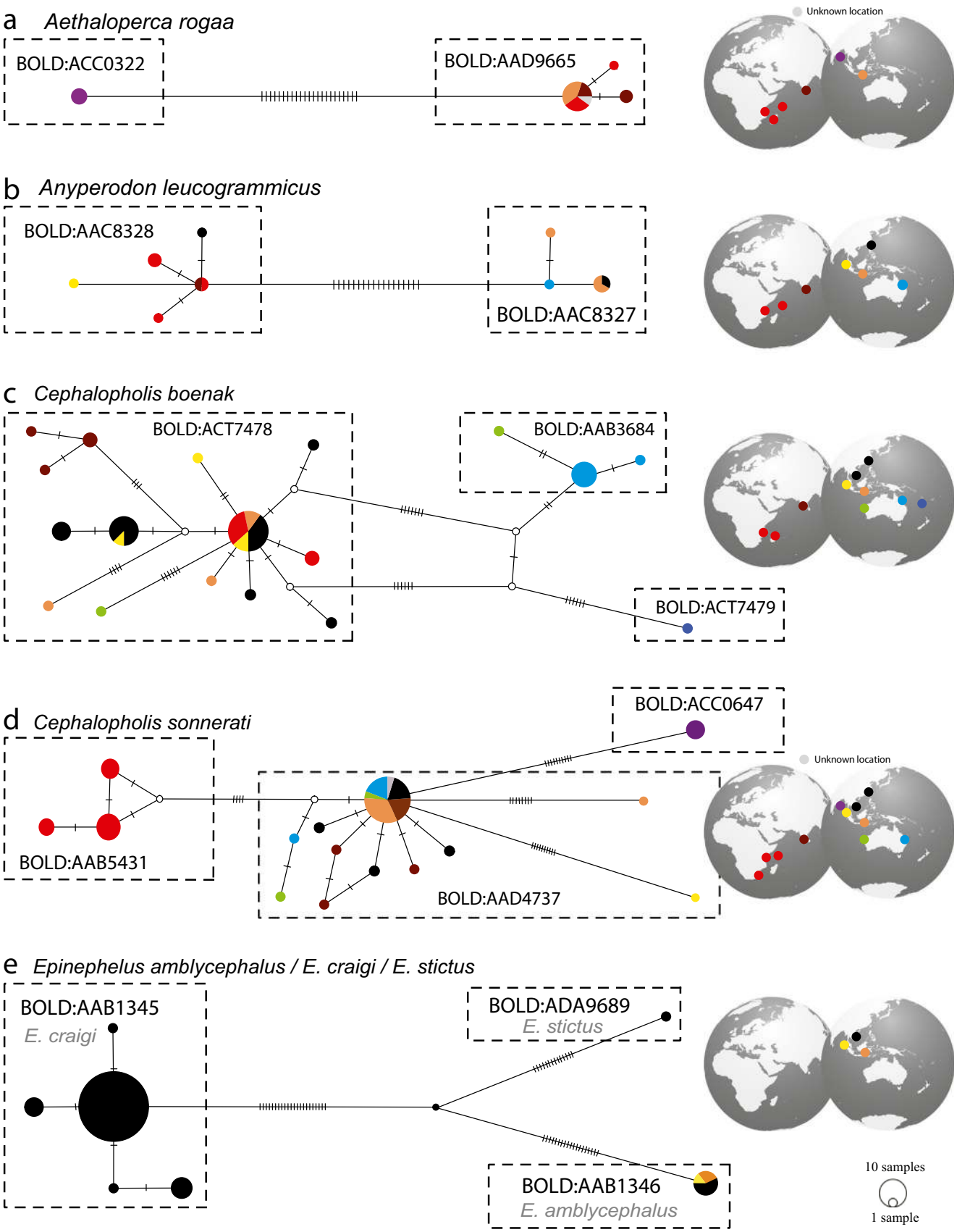
In addition, hybridization within groupers can produce specimens with atypical body coloration, influencing our ability to recognize overall species. Several studies already identified grouper hybrids in the wild (Bostrom et al. 2002; Frisch and van Herwerden 2006; Randall and Justine 2008; Payet et al. 2016; Qu et al. 2018). Genetic characterization of a hybrid requires the comparison of its phylogenetic position estimated by a mitochondrial marker such as the COI (maternally inherited) against a nuclear marker (bi-parentally inherited), as proposed by Payet et al. (2016) or Qu et al. (2018). However, hybridization does not necessarily lead to conflicting phylogenies since an absence of interspecies differentiation by genetic markers can also be interpreted as a consequence of hybridization and introgression. This was already pointed out for two couples of species that share an unique BIN:BOLD:AAC4202 for *Cephalopholis urodeta* and *C. nigripinnis* and BOLD:AAD1767 for *E. cyanopodus* and *E. flavocaeruleus*. Despite clear differences of color pattern, these species share a unique BIN and are indistinguishable with nuclear markers (Payet et al. 2016; Qu et al. 2018). According to Payet et al. (2016), such absence of interspecies differentiation maybe due either to hybridization and introgression, taxonomical characters that are phylogenetically irrelevant, or incomplete lineage sorting. While Qu et al. (2018) suggested that *E. cyanopodus* and *E. flavocaeruleus* may be considered as a unique species, Payet et al. (2016) considered *Cephalopholis urodeta* and *C. nigripinnis* as real species that able to interbreed because these authors depicted various intermediate phenotypes between the two species where they occur in sympatry.

The question that arises for species that present two to four specific BINs is of their evolutionary significance. Among 15 species presenting this situation, only three, *E. chlorostigma*, *E. fasciatus*, and *E. epistictus* were associated with BINs that does not form monophyletic clade. Identification of *E. chlorostigma* seems confused in light of the phylogenetic tree based on sequences of various origin (Fig. 3). While the COI barcode of the specimen of Ma et al. (2013) corresponded to *E. areolatus* (for this study and for BOLD), among the BINs associated with *E. chlorostigma* in BOLD, none corresponded to the Vietnamese specimens that have been

Fig. 4 Haplotypic networks recovered in multi BIN grouper species for ▶ which BINs form a clade in Fig. 3. Dot color in the network represents the geographic origin of the haplotypes, while its size represents the abundance in BOLD. **a** *Aethaloperca rogaa*; **b** *Anyperodon leucogrammicus*; **c** *Cephalopholis boenak*; **d** *Cephalopholis sonnerati*; **e** *Epinephelus amblycephalus*, *Epinephelus stictus*, *Epinephelus craigii*; **f** *Epinephelus areolatus*; **g** *Epinephelus bruneus*, *Epinephelus moara*; **h** *Epinephelus epistictus*; **i** *Epinephelus fasciatus*; **j** *Epinephelus latisfasciatus*; **k** *Epinephelus merra*; **l** *Epinephelus morrhua*; **m** *Epinephelus polyphekadion*; **n** *Epinephelus rivulatus*; **o** *Plectropomus maculatus*; **p** *Variola louti*

unambiguously identified as *E. chlorostigma*. By contrast, the BIN of the Vietnamese *E. chlorostigma* (BOLD:AAC3591) was associated in BOLD to seven specimens identified by the same institute as *E. spilotoceps* and collected in the South China Sea close to the Vietnamese sampling location. These specimens have been misidentified considering that (1) available pictures do not present the four blackish brown saddle-blotches at the base of the dorsal fin that are characteristic of *E. spilotoceps* (FAO), (2) the specimen identified by Ma et al. (2016) as *E. spilotoceps* belongs to a different phylogenetic lineage (Fig. 3), and (3) there is a BIN (BOLD:AAC3590) consisting of 17 members (including the specimen of Ma et al. 2016) that was unambiguously identified by BOLD as *E. spilotoceps*. Finally, there are two unrelated BINs (BOLD:AAC3591 and BOLD:ABA7404) associated to the name *E. chlorostigma* which suggest that there is a cryptic species in this taxon. Considering the type locality of *E. chlorostigma* (Seychelles), and BIN's geographic distributions which are based on only a few specimens, more work is needed to determine the BIN that corresponds to *E. chlorostigma*.

Concerning BINs recovered in specimens identified as *E. fasciatus*, they form a monophyletic clade that is paraphyletic with *E. retouti* and *E. irroratus* (Fig. 3). *E. irroratus* is endemic to the Marquesas Island and shares the BIN BOLD: AAB1333 with *E. fasciatus*, which is mainly observed in the SW and Central Pacific (Fig. 4i). By contrast, the Indo-Pacific *E. retouti* was associated with a BIN (BOLD:AAX8350) that did not correspond to any BINs identified in *E. fasciatus* but was closely phylogenetically related to the *E. fasciatus* BIN (BOLD: AAB1334). This last BIN (BOLD:AAB1334) diverged deeply from all other *E. fasciatus* BINs (Fig. 3) and presented a strong allopatric distribution limited to the Red Sea and the West Indian Ocean (Fig. 4i). All these results stress the presence of a species complex in *E. fasciatus* and, because the type locality of this species is the Red Sea, this suggests that the valid *E. fasciatus* should be limited to the Red Sea and the West Indian Ocean (= the BIN BOLD:AAB1334). All other BINs associated with the name *E. fasciatus* may correspond to another species for which phylogenetic relationships with *E. irroratus* is not clear. More taxonomic and phylogenetic work is necessary to solve


a Aethaloperca rogaa
BOLD:ACC0322
BOLD:AAD9665
Unknown location
b Anyperodon leucogrammicus
BOLD:AAC8328
BOLD:AAC8327
c Cephalopholis boenak
BOLD:ACT7478
BOLD:AAB3684
BOLD:ACT7479
d Cephalopholis sonnerati
BOLD:ACC0647
BOLD:AAB5431
BOLD:AAD4737
Unknown location
e Epinephelus amblycephalus / E. craigi / E. stictus
BOLD:AAB1345
E. craigi
BOLD:ADA9689
E. stictus
BOLD:AAB1346
E. amblycephalus
10 samples
1 sample

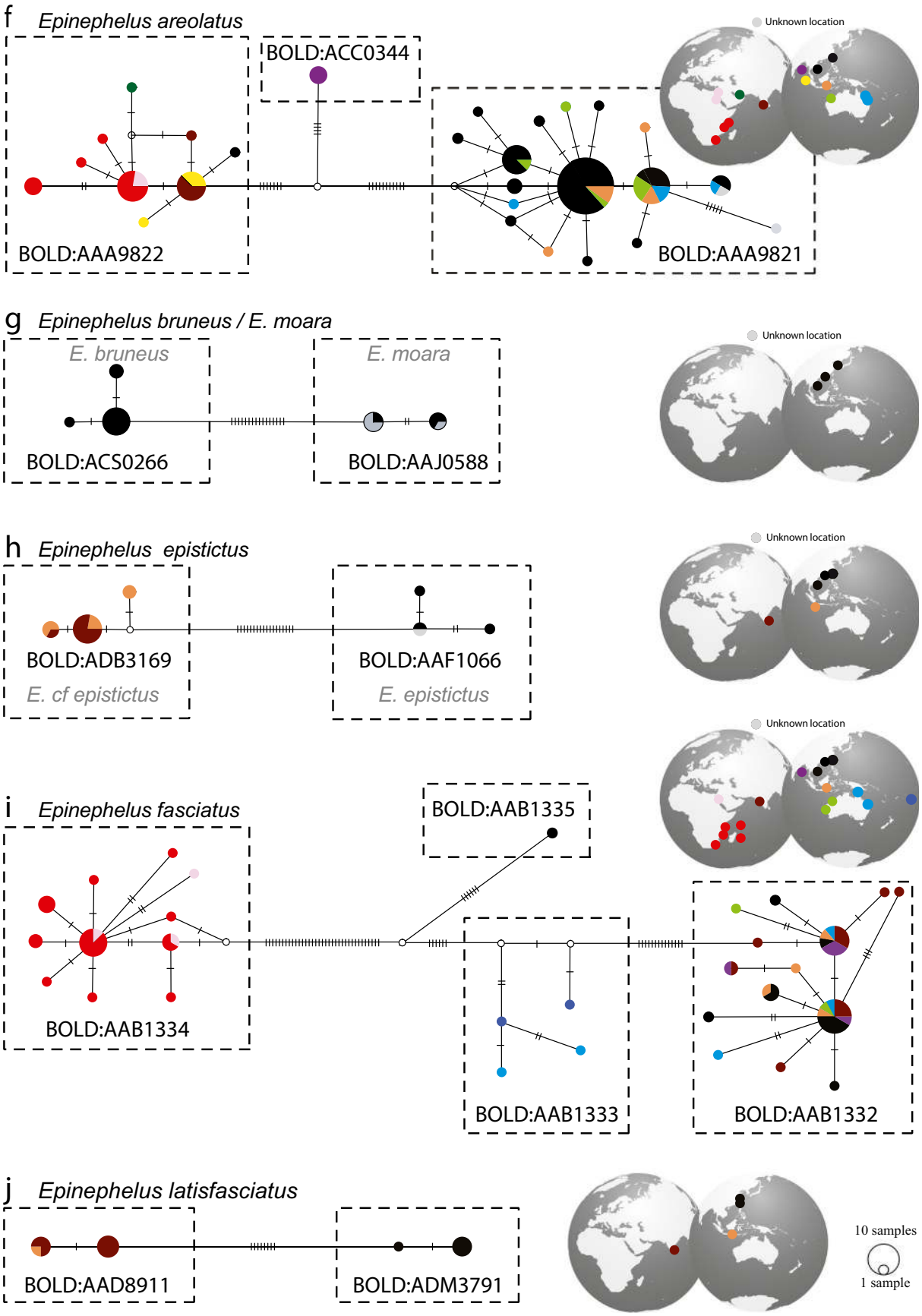


Fig. 4 (continued)

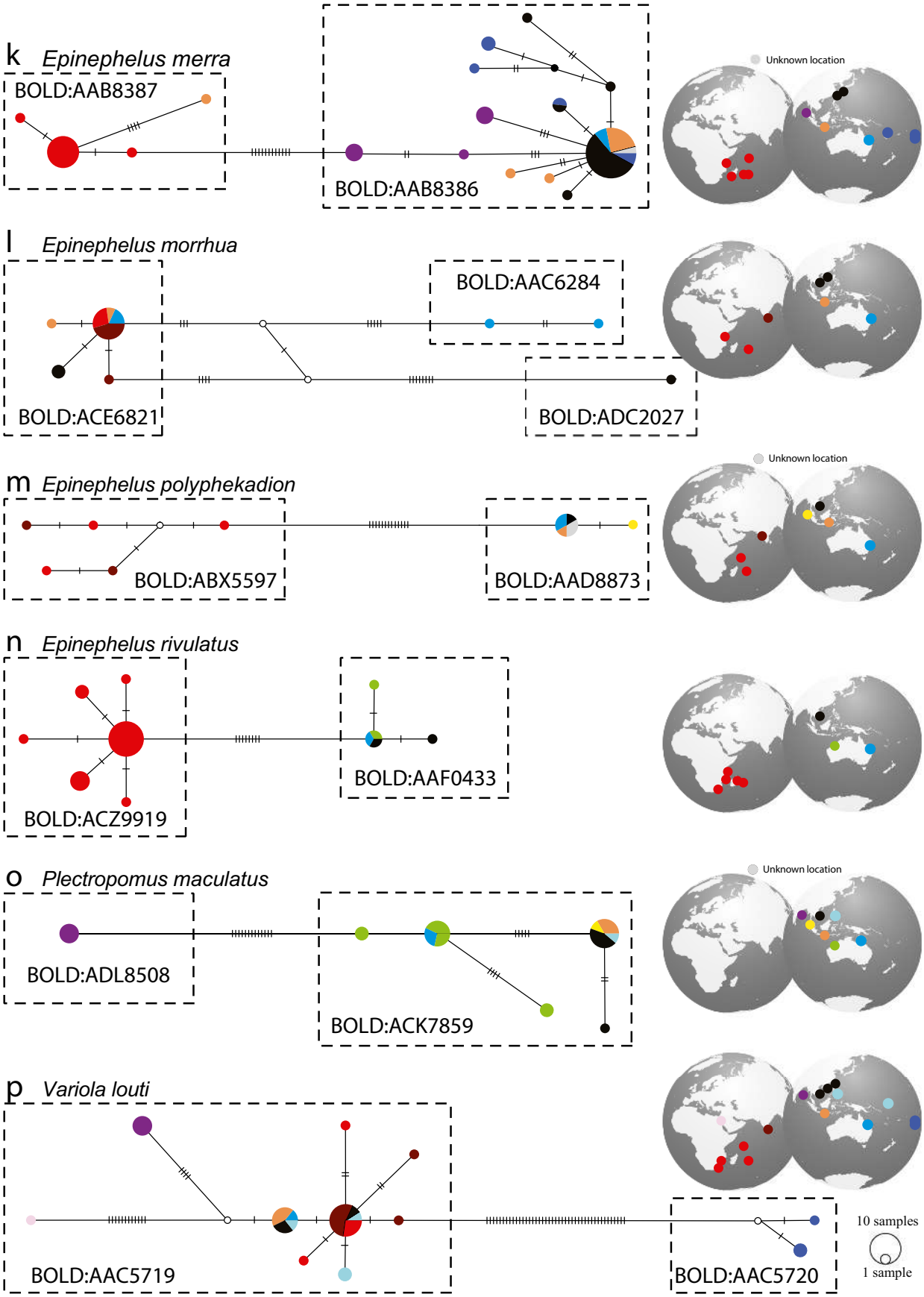


Fig. 4 (continued)

this question, given that genetic data concerning *E. retouti* is limited to only 7 COI barcodes.

Last, the two BINs (BOLD:AAF1066 and BOLD:ADB3169) associated with specimens identified as *E. epistictus* form a well-supported clade that also included the recent described species *E. fuscomarginatus* and its sister species *E. magniscuttis* (Fig. 3). This result confirmed findings of Johnson and Worthington Wilmer (2019) who demonstrated that *E. epistictus* is a species complex composed of at least 4 species. Because the type locality is Nagasaki, Japan, it is probable that the BIN BOLD:AAF1066 observed in specimens collected in the NW Pacific (Taiwan and China) corresponds to *E. epistictus*, but see Johnson and Worthington Wilmer (2019) for a more detailed and complete analysis.

For all other polyphyletic species or multi-BIN species, the present study demonstrated that their BINs form well supported clades in the majority of cases (Fig. 3). This result may be interpreted in three ways: BINs of a species correspond to (1) undescribed or cryptic sister species; (2) intraspecific biogeographic lineages, or (3) artifacts due to bad nucleotide scoring in the sequencing file (ab1 file) generated by the sequencing apparatus. This last situation cannot be excluded when a BIN is observed at only one location and thus consists of few members analyzed by only one institute. Up to seven BINs correspond to this situation and, curiously, 4 of them where described only in the Andaman Islands, such as BOLD:ACC0322 (*Aethaloperca rogaa*), BOLD:ACC0647 (*Cephalopholis sonnerati*), BOLD:ACC0344 (*Epinephelus areolatus*), and BOLD:ADL8508 (*Plectropomus maculatus*). Given that it is rare in groupers that a BIN is restricted to only one location and unless there is a hotspot of diversity in the Andaman Islands, such results remain doubtful and call for further investigation.

Now, when polyphyletic species are composed of BINs with members from different geographic origin that do not match any geographic pattern, it is probable that these BINs flag sister species. This is the case for BINs highly associated with the name *E. amblycephalus* and that correspond to three sister species: *E. amblycephalus*, *E. stictus*, and the recently described species *E. craigi* (Figs. 3 and 4e). This is also true for the two BINs associated with the species name *E. bruneus*, since one of these corresponds to the COI sequence of the species *E. moara* that is still not recognized as valid in Eschmeyer's Catalog of Fishes (Fricke et al. 2019), despite arguments provided by Liu et al. (2013).

Lastly, in all remaining multi-BIN species, a phylogeographic structure has been revealed, with BIN geographic distributions that match biogeographic units. This suggests that the presence of more than one BIN in a species mirrors a complex evolutionary history. Several studies have already stressed phylogeographic structures in groupers such as *Cephalopholis argus*, *Epinephelus areolatus*, *E. polyphekadion*, *E. merra*, *Plectropomus areolatus*, and *P. leopardus* (Gaither et al. 2011; DiBattista et al. 2013; Borsa et al. 2016; Ma et al. 2018). If we exclude *Plectropomus areolatus* that was not reviewed in this study due its absence in Vietnam, three of five of these species are multi BIN species. Most of these studies interpret the genetic structure of these species as a result of paleogeographic changes that directly impact the phylogeographic structure of groupers (Gaither et al. 2011; DiBattista et al. 2013; Borsa et al. 2016). A number of important biogeographic barriers in the Indo-Pacific have shaped the present-day genetic structure, such as the mid Indian Barrier or the Indo/Pacific Ocean divide. But Ma et al. (2018) also noticed that reproductive behavior is an important factor that can modulate the strength of a biogeographic barrier by either limiting or increasing population connectivity. According to Ma et al. (2018) discrete reproduction in small groups of *Plectropomus leopardus* individuals may have favored a stronger genetic structure of Pleistocene origin in the West Indo-Pacific than *Plectropomus areolatus* and *Ephinephelus polyphekadion*, which breed in large numbers on a few spawning grounds. Secondary contact and genetic connectivity may be stronger for species presenting such behavior, explaining why some genetic breaks in the West Indian Pacific are not observed.

To conclude, the association of several BINs to a species cannot be interpreted straightforwardly. If, in a number of cases, misidentification and hybridization artificially increase the apparent genetic diversity of a species, the presence of several BINs can also flag putative cryptic species or a strong phylogeographic structure. More barcoding studies focusing on fish families are needed to clarify the association between a species and BINs and, thereby, improve the validity and reliability of the species identification engine provided by BOLD. In any case, beyond species identification, once clarified for multi-BIN species, it would certainly be possible to use the BINs not only to identify a specimen but also to track its biogeographic origin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Sampling and field studies All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities and are mentioned in the acknowledgements, if applicable.

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files]. DNA barcodes generated during the current study are available in the project file SERV: Epinephelidae of Vietnam on BOLD systems website (http://boldsystems.org).

Author contribution JDD conceived and designed research. JDD, MHP, TTVY, DHH, and QVV conducted experiments. JDD analyzed data and wrote the manuscript. All authors read and approved the manuscript.

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