An Acad Bras Cienc (2024) 96(1): e20221011 DOI 10.1590/0001-3765202420221011
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Karyotypic changes and diversification time in Epinephelidae groupers (Perciformes). Implications on reproductive isolation

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Abstract: Groupers (Epinephelidae and Serranidae) have attracted special attention to fish farming, and their species offer good opportunities for successful hybridizations. Cytogenetic data allow a better understanding of the role of karyotypic diversification in the acquisition of post-zygotic reproductive isolation (RI). Thus, chromosomal analyses were performed on *E. striatus* (Caribbean Sea), *E. coioides* and *E. tauvina* (Indo-Pacific Region), using standard procedures and mapping of six repetitive DNA classes by the *in situ* hybridization. The three species have 2n=48 chromosomes. The karyotypes of *E. coioides* and *E. striatus* are composed only of acrocentric chromosomes (FN=48), while *E. tauvina* has 8 submetacentric chromosomes (FN=56). Heterochromatin has a preferential centromeric distribution, and the microsatellite repeats are dispersed throughout the chromosomes of all species. The 18S and 5S rDNA sites are unique but show a colocalization arrangement in *E. tauvina* and *E. striatus*. The chromosomal organization suggests that the three species still maintain a significant amount of syntenic regions. The range of the karyotype divergence and the RI levels showed low, but goes turn proportionally greater in relation to the divergence time between the parental species. The slow acquisition of postzygotic RI is consistent with the high karyotype homogeneity presented by Epinephelidae family.

Key words: Karyotype divergence, repetitive DNA, hybrids, post-zygotic barriers.

INTRODUCTION

Interspecific hybridization promotes the arrangement of distinct genomes, which can result in hybrids with multiple adaptive traits (Abbott et al. 2013, Shivaramu et al. 2019). Natural hybridizations frequently occur among fish (Allendorf & Waples 1996, Rahman et al. 2013), mainly due to some conditions such as external fertilization, weak behavioral isolation, uneven abundance between parental species, loss of habitats, overlapping breeding areas, low frequency of sex chromosomes (Campton 1987, Molina et al. 2014b, Nagel et al. 2018), and the slow acquisition of post-zygotic reproductive isolation (RI) (Russell 2003, Stelkens et al. 2010).

Combinations of different biological traits have, in many cases, increased the commercial value of hybrid fish, including the growth rate, environmental tolerance, resistance to cultivation, and production of monosexual stocks. (Rahman et al. 2013, Rimmer & Glamuzina 2017, Shivaramu et al. 2019). Advantageous traits in artificial hybrids have been reported for several cultivated fish groups, such as catfish (Dunham & Smitherman 1983), trout (Dorson et al. 1991), perch (Hooe et al. 1994), carp (Kalsoom et al. 2009), sturgeons (Boscari et al. 2014), cichlids

(Wohlfarth 1994) and groupers (Huang et al. 2016). Groupers of the Epinephelidae family are of particular economic interest (Mitcheson et al. 2013), with around 50 species being exploited in fisheries or aquaculture (Rimmer & Glamuzina 2017, FAO 2019). Some of their hybrids may even have a growth rate 50% higher than their parents (Sugama et al. 2014).

Some fish hybrids present normal and fertile gonads, showing germ cells at different stages of maturation (Moron et al. 2018). On the other way, although normal in size and structure, other hybrids produce abnormal gametes in morphology and/or chromosome sets, or even fertilizable but non-viable gametes (Hooe et al. 1994). The highest degree of RI - the hybrid inviability - may result from the imperfect chromosome pairing during meiosis, a condition that can be overcome by the numerical and syntenic compatibility of chromosomes from homoploid parents (Yoshikawa et al. 2018). Homodiploidy of parental groupers increases the chances of homologous pairing during meiosis in the hybrid genome and may minimize post-zygotic blocks derived from anomalous chromosome segregation. Under natural conditions, homodiploidy is reflected in evolution, making diploid reticulated speciation a fast track for the emergence of new species (Coyne & Orr 2004). The reproductive strategies of groupers encompass one or more patterns of sequential hermaphroditism, mainly protogyny (female to male), but also protandry (male to female), and bidirectional sex changes (Mitcheson & Liu 2008, Avise & Mank 2009). Although sequential hermaphroditism favors cultivation practices, the slow ontogenetic development in some species is a limiting factor and has stimulated the production of hybrids with faster growth rates (Tucker 1994, Mitcheson et al. 2013, Rimmer & Glamuzina 2017). In addition to natural hybrids, a large number of artificial ones (about 20) have been reported in groupers, many of which are regularly used in fish farming (Table I).

The karyotype features and genome diversification of epinephelids have become better known in recent years (Wang et al. 2020, Amorim et al. 2021). Epinephelidae species are characterized by an intermediate rate of karyotype changes regarding other Perciformes groups (Molina 2007, Molina et al. 2014b, Motta-Neto et al. 2019). The diploid value (2n=48) is a symplesiomorphic trait, shared by all grouper species analyzed so far. Among them, about 60% have a basal karyotype composed of acrocentric chromosomes (FN=48). The remaining species have karyotypes diversified by structural rearrangements, with FN greater than 48 (Motta-Neto et al. 2019, Amorim et al. 2021). To date, cytogenetic studies in groupers have focused on cytogenetic characterization aspects. Preliminars genetic divergences and cytogenetic characteristics of the epinephelids have been associated with the hybridization processes in this family (Rahman et al. 2013, Tseng & Shih 2018), however, the quantification of the karyotype divergences and its relation with the post-zygotic effects on the hybrids are unknown. Here, are presented the microestructural chromosome divergences among on three cultived species of groupers, *E. coioides*, *E. striatus* and *E. tauvina*, by chromosomal mapping of six repetitive DNA classes [18S and 5S rDNA, microsatellites $(CA)_{15}$, $(GA)_{15}$, $(CAA)_{10}$ and $(CGG)_{10}$], and the association between the karyotype divergences and the ontogenetic effects on epinephelid hybrids. These repetitive sequences have a fast evolutionary dynamics and offer a varied comparative set of chromosomal markers. The combined approach involving cytogenetical, phylogenetical and temporal divergence contributed to elucidate new aspects of the acquisition of post-zygotic barriers in these reef fishes.

Table I. Interspecific crosses in Epinephelidae and Serranidae species. Karyotypes (adapted from Amorim et al. 2021), ΔFN – difference in the number of chromosome arms (FN) between parental karyotypes, (D) genetic distances from the 16S mtDNA, (M.a) divergence times among species, and ontogenetic effects (OE) on hybrids. +: parameters with values up to 30%; ++: 50%; and +++: >70% in relation to the parental species. F = fertilization, E = eclosion, G = growth, S = survival.

Parental Species – karyotypes					ΔFN	D (%)	M.a	Ontogenetics Effects - OE				Ref.
								early		later		
								F	E	G	S	
***Epinephelus* - congeneric hybrids**												
E. costae	48a	x	E. marginatus	48a	0	1.6	~ 4	++	+++	++	+	9
E. coioides	48a/2sm+46a	x	E. akaara	48a	0-2	3.8	~9	++	++	+++	+++	8
E. bruneus	4sm+44a	x	E. akaara	48a	4	4.1	~ 10	+	+++	++	+	21
E. lanceolatus	6sm+42a/8sm+40a	x	E. moara	4sm+44a	2-4	4.2	~ 10	+	++	++	++	17
E. lanceolatus	6sm+42a/8sm+40a	x	E. tukula	2sm+46a	4-6	4.2	~ 10	+	-	-	-	10
E. coioides	48a/2sm+46a	x	E. fuscoguttatus	2sm+46a	0-2	4.6	~ 11	+++	+	+	+	5,24
E. fuscoguttatus	2sm+46a	x	E. coeruleopunctatus	2sm+46a	0	4.6	~ 11	+	-	-	-	10
E. fuscoguttatus	2sm+46a	x	E. tukula	6sm+42a	4	4.6	~ 11	+	+	++	++	19
E. fuscoguttatus	2sm+46a	x	E. corallicola	-	-	4.6	~ 11	+	+	-	-	3
E. fuscoguttatus	2sm+46a	x	E. lanceolatus	6sm+42a/8sm+40a	4-6	4.8	~ 11	+++	+++	++	++	11,12,20
E. fuscoguttatus	2sm+46a	x	E. polyphekadion	6sm+42a	4	4.8	~ 11	++	++	++	+++	13,14
				ΔFN average	**2.2/3.2**	**OE average**		<++	++	++	++	
E. lanceolatus	6sm+42a/8sm+40a	x	E. polyphekadion	6sm+42a	0-2	5.2	~ 12	+	-	-	-	3
E. fuscoguttatus	2sm+46a	x	E. akaara	48a	2	5.4	~12.5	+	+	++	+	22
E. coioides	48a/2sm+46a	x	E. lanceolatus	6sm+42a/8sm+40a	4-8	5.4	~12.5	++	++	++	++	6,7,24
E. amblycephalus	-	x	E. akaara	48a	-	5.8	~ 14	+++	+	++	+	4
E. lanceolatus	6sm+42a/8sm+40a	x	E. akaara	48a	6-8	6.6	~ 16	++	++	++	++	16,25
E. marginatus	48a	x	E. aeneus	-	-	7.0	~ 17	++	++	++	++	18
				ΔFN average	**3.0/5.0**	**OE average**		<++	<+	++	<++	
Intergeneric hybrids or between non-***Epinephelus*** genera												
P. maculatus	-	x	P. leopardus	48a [n/a]	-			+++	+	++	+	23
C. aurantia	-	x	C. spiloparaea	-	-	1.4	~3.5	+	+	+++	+++	1
C. fulva	48a	x	P. furcifer	-	-	4.6	~ 11	+	+	+++	+++	2
E. lanceolatus	6sm+42a/8sm+40a	x	Cr. altivelis	2sm+46a	4-6	6.0	~ 14	+	+	+++	++	15
C. fulva	48a	x	E. guttatus	48a	0	10.0	~ 24	+	+	-	+	2
E. morio	-	x	Ce. striata	24m+22sm+2a	-	18.0	~ 43	o	o	o	o	2
				ΔFN average	**-**	**OE average**		<++	<+	<++	<++	

ΔFN – difference in the number of chromosome arms (FN) between parental karyotypes; D – genetic distance. References - 1) Randall & Justine 2008; 2) Tucker 1994; 3) Addin & Senoo 2011; 4) Tseng & Poon 1983; 5) Koh et al. 2008; 6) Chu et al. 2010, 7) Huang et al. 2016; 8) Liufu et al. 2007; 9) Glamuzina et al. 2001; 10) Rimmer & Glamuzina 2017; 11) Senoo 2006, 12) Ching et al. 2018, 13) James et al. 1999; 14) Ismi et al. 2013; 15) Chen et al. 2017; 16) Kim et al. 2018; 17) Chen et al. 2018; 18) Glamuzina et al. 1999; 19) Cheng et al. 2019; 20) Tan et al. 2018; 21) Kang et al. 2020; 22) Noh et al. 2015; 23) Frisch & Hobbs 2007; 24) Koh et al. 2010; 25) Noh et al. 2019.

MATERIALS AND METHODS

Samples and standard chromosomal analyses

E. coioides, one of the most economically important fish farmed in China and Southeast Asia, and *E. tauvina* and *E. striatus* groupers were analyzed in this study. *E. coioides* (n=5) and *E. tauvina* (n=5) were obtained, from the Andaman Sea (11°04'00"N and 95°44'34"E), and *E. striatus* (n= 10) were juvenile specimens of the coast of Florida – USA (25°09'40"N, 80°45'83"W) (Figure 1), and obtained from an experimental research laboratory.

Individuals were previously submitted to mitotic stimulation by muscular and intraperitoneal injection of attenuated antigen complexes (Molina et al. 2010), for a period of 24 hours. Next, the animals were euthanized with an overdose of clove oil. Chromosome preparations were obtained by short-term culture (Gold et al. 1990) of the cell suspensions from anterior region of the kidney. The cell suspension were hypotonized with KCl 0.075M solution, preserved with methanol: acid acetic (3:1) fixative solution and dripped onto a slide covered with a film of distilled water heated to 60°C. Chromosomes were stained with a 5% Giemsa solution diluted in phosphate buffer pH 6.8 for 8 min to determine the diploid chromosome number (2n) and the composition of the karyotype. The heterochromatic regions were analyzed using the C-banding method (Sumner, 1972), and the nucleolar organizer regions (NORs), by silver nitrate impregnation (Howell & Black 1980).

Probes for chromosomal hybridization

The 5S (~200 bp) and 18S rDNA (~1400 bp) probes were obtained by PCR from the nuclear DNA of *Rachycentron canadum* (Rachycentridae), using the primers A 5'-TAC GCC CGA TCT CGT CCG ATC-3' and B 5'-CAG GCT GGT ATG GCC GTA AGC-3' (Pendás et al. 1994) and NS1 5'-GTA GTC ATA TGC TTG TCT C-3' and NS8 5'-TCC GGT GCA TCA CCT ACG GA -3' (White et al. 1990), respectively. The 5S rDNA and 18S rDNA probes were labeled by nick translation, respectively, with biotin-14-dATP and digoxigenin-dUTP-11, according

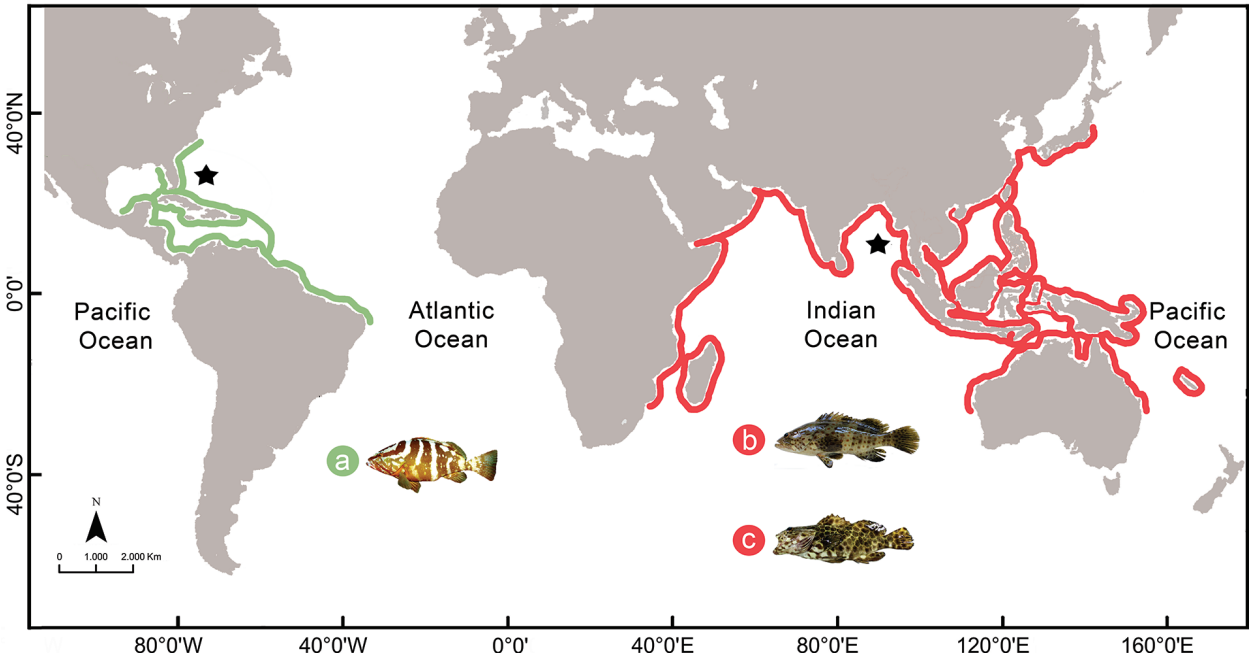

Figure 1. Geographic distribution of (a) *Epinephelus striatus* (in green), (b) *Epinephelus coioides* and (c) *Epinephelus tauvina*, with a sympatric area indicated in red. The collection points are highlighted by black stars.

to the manufacturer's specifications (Roche®, Mannheim, Germany). The oligonucleotides $(CA)_{15}$, $(GA)_{15}$, $(CAA)_{10}$ and $(CGG)_{10}$ were labeled with AlexaFluor 555 at the 5' terminal position during synthesis (Invitrogen, Thermo Fisher Scientific, California, USA).

Chromosomal hybridization

FISH experiments were performed following Pinkel et al. (1986). Hybridization signals were detected using anti-digoxigenin rhodamine-conjugated, for the 18S rDNA probe, and streptavidin-FITC (Roche®, Mannheim, Germany), for the 5S rDNA probe. The chromosomes were counterstained with Vectashield/DAPI (1.5 µg/ml) (Vector Laboratories, Burlingame, CA, USA). The hybridization of the simple sequence repeats (SSRs) was performed according to Kubat et al. (2008).

Image processing

Approximately thirty mitotic metaphases of each individual were photographed using an OlympusTM BX51 epifluorescence microscope coupled to an Olympus DP73 digital capture system, using cellSens® software (Olympus Corporation, Ishikawa, Japan). Chromosomes were classified regarding the arms ratio (AR) in metacentric (m), with AR ranging from 1.00-1.70; submetacentric (sm), AR=1.71-3.00; subtelocentric (st), AR= 3.01-7.00; and acrocentric (a), AR>7.01 (Levan et al., 1964). The fundamental number (FN) (i.e. number of chromosome arms), was defined considering the m, sm and st chromosomes to have two arms, while the acrocentric chromosomes only one arm.

Mitochondrial 16S sequences

Partial sequences of the 16S mitochondrial gene from 24 Epinephelidae parental species of interspecific crosses were obtained from the GenBank (Supplementary Material - Table SI). The sequences were aligned using MUSCLE (Edgar 2004), and the average rates of genetic divergence (Kimura-2p model) were obtained using the MEGA 6 software (Tamura et al. 2013). The temporal divergence per million years was estimated from Domingues et al. (2005), considering 1.0% of genetic divergence by 2.4 My.

RESULTS

The three species analyzed have 2n=48 chromosomes, but with some variations in the karyotype formula. The karyotypes of *E. striatus* and *E. coioides* are exclusively composed of acrocentric chromosomes (FN=48), with a small differentiation in size among the sequential pairs, while the karyotype of *E. tauvina* is composed of 8sm+40a (FN=56) (Figure 2). The heterochromatin has a reduced amount, mainly located at the centromeric and pericentromeric regions of chromosomes. In *E. striatus* and *E. coioides* the Ag-NORs sites are situated on the short arms of pair 24. In *E. tauvina* they are also located on the short arms, but in a larger pair, the 20th one (Figure 2).

A single locus of the 18S and 5S rDNA sequences was identified in all species. However, in *E. coioides* the 18S rDNA is located on the short arms of pair 24, while the 5S rDNA is located on the short arms of pair 23 (Figure 2). Differently, in *E. striatus* and *E. tauvina* the 18S and 5S rDNA sites are colocalized on the short arms of pairs 24 and 20, respectively (Figure 2). The microsatellites $(CA)_{15}$, $(GA)_{15}$, $(CAA)_{10}$ and $(CGG)_{10}$ have a dispersed chromosomal distribution in the three species (Supplementary Material - Figure S1).

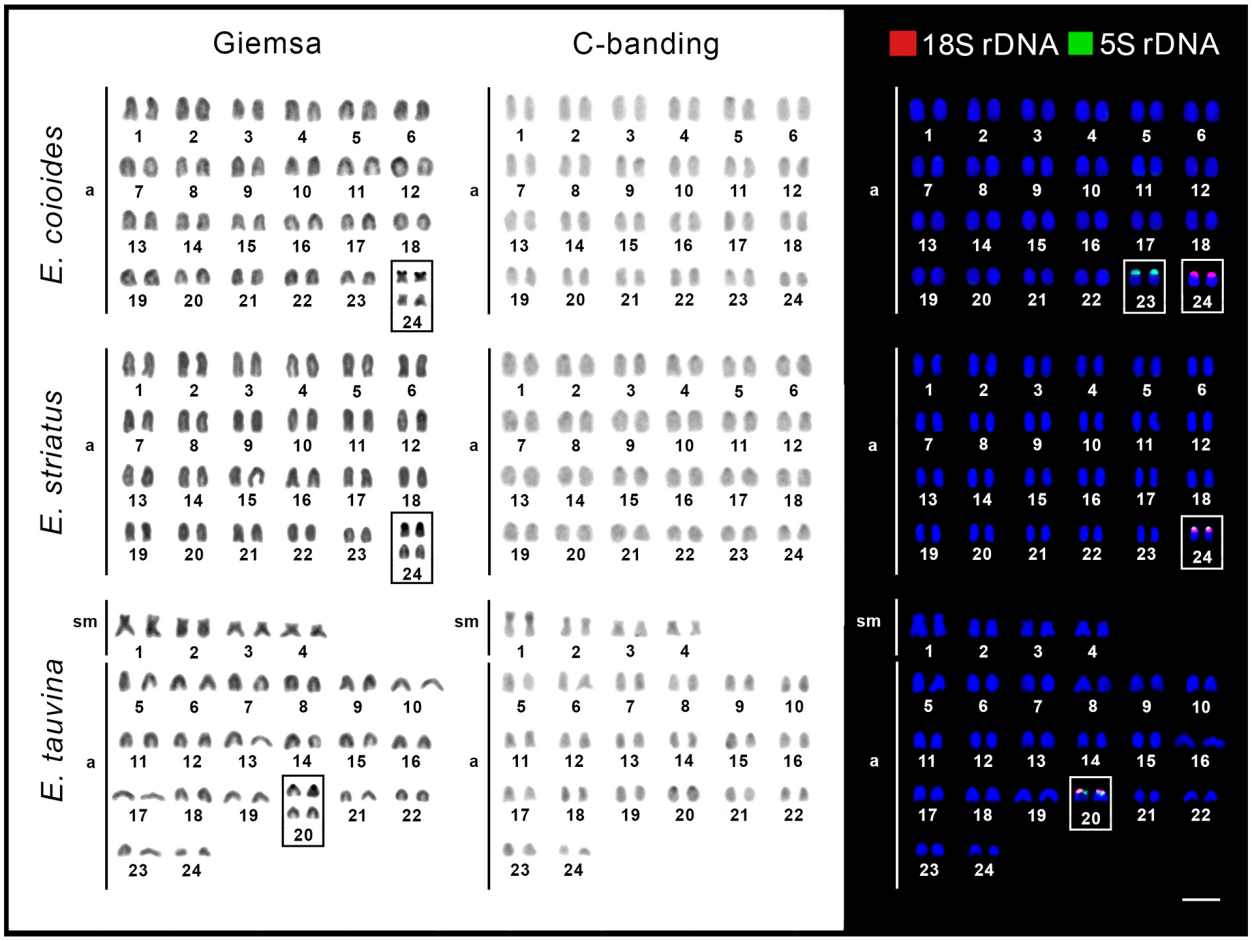

Figure 2. Karyotypes of *E. striatus*, *E. coioides* and *E. tauvina* under Giemsa staining, C-banding and fluorescence *in situ* hybridization (FISH) with rDNA probes. Chromosome pairs bearing Ag-NORs/18S rDNA (red) and 5S rDNA (green) sites are highlighted in boxes. A syntenic 18S/5S rDNA array occurs on the 24 and 20 pairs of *E. striatus* and *E. tauvina*, respectively. Scale bar = 5µm.

DISCUSSION

The slow acquisition of RI among groupers agrees with the relatively low quantitative divergence of their karyotypes. In fact, several cytogenetic and biological conditions seem to favor a significant number of viable and fertile hybrids, thus highlighting a limited effect of post-zygotic barriers in Epinephelidae. Among these several cytogenetic features deserve to be highlighted, such as the sharing of homoploid karyotypes and the significant conservation of extensive syntenic and colinear stretches in the genome of the species (Wang et al. 2020, Yang et al. 2021, Amorim et al. 2021). Chromosome homologies allow correct pairing, recombination, and uniform segregation. In addition, the asynchronous hermaphroditism minimizes the genomic divergences between sexes, including the differentiation of sex chromosomes, since the same genome transits between the two sexes during the ontogenetic history (Wright et al. 2016). In fact, the presence of differentiated sex chromosomes in one or both parents can alter the gene balance, promoting the sterility or infeasibility of heteromorphic sex in hybrids (Haldane 1922).

The three species, *E. striatus*, *E. coioides*, and *E. tauvina* and all other karyotyped

groupers (~ 50 species) share the same diploid value (2n=48). Among these species, 61% share structurally similar karyotypes formed entirely by acrocentric chromosomes (FN=48), as *E. coioides* and *E. striatus*, while the others, including *E. tauvina*, exhibit some additional structural changes in the chromosomes (FN=48-96) (Motta-Neto et al. 2019, Amorim et al. 2021). In fact, similar to *E. tauvina*, more than 40% of Epinephelidae species show some karyotype diversification associated with pericentric inversions. The changes by inversions may be related with adaptive processes (Kirubakaran et al. 2016), and act as post-zygotic barriers (Ortiz-Barrientos et al. 2016).

Recent data showed an increase in the karyotype diversification associated to the historical biogeographic expansion of groupers species. Indeed, in the Atlantic Ocean, 87% of the analyzed species have conserved 2n=48 basal karyotype, this pattern is reduced to 56% of the Pacific, 55% of the Indo-Pacific, and only to 33% of the Indian Ocean species (Amorim et al. 2021). Apparently, the progressive historical karyotype divergence observed in groupers (Amorim et al. 2021) was promoted by reach of new areas generating conditions for distinct evolutionary opportunities (Rohde & Muller 2005, Carpenter et al. 2011).

Despite this, the organization and distribution of repetitive sequences in the chromosomes still offer indications of chromosomal conservatism (Amorim et al. 2021).

The remarkable chromosomal conservatism in Epinephelidae species is particularly noteworthy when comparing *P. leopardus* (2n=48a) and *E. akaara* (2n=48a) karyotypes (Wang et al. 2020). These species have an estimated divergence time of more than 35 Mya (Ma et al. 2016), but still show a clear one-to-one relationship among their chromosomes, highlighting the synteny among of their 24 linkage groups (Wang et al. 2020). Indications of similar high genomic conservatism also occur between *E. fuscoguttatus* (2n=2sm+46a) and *Plectropomus leopardus* (2n=48a), whose divergence time is about 49.3 (32.5–65.9) million years ago (Yang et al. 2020).

The Ag-NOR sites are located in a single pair of chromosomes in the three analyzed species, in a medium-sized pair in *E. tauvina* and the smallest pair of the karyotype in *E. striatus* and *E. coioides*. The occurrence of ribosomal sites in the same position and on the smallest pair of chromosomes is also a significantly recurrent conservative condition among grouper species (Tseng & Shih 2018, Amorim et al. 2021). In general, the 18S and 5S rDNA sites are also not syntenic in groupers (Minglan et al. 2014, Paim et al. 2017). Therefore, the co-localization of 18S/5S rDNA in *E. striatus* and *E. tauvina* points to the potential evolutionary dynamism of these regions, which may eventually promote microstructural reorganizations in the chromosomes. However, different cytogenetic markers, including rDNA regions and other repetitive sequences (Amorim et al. 2021, present study), support the substantial syntenic conservatism in grouper chromosomes. Comparative analyses of the repetitive sequences allow tracking its evolutionary dynamics in karyotypes, in view of their rapid evolutionary rates (Vicari et al. 2010, Cioffi & Bertollo 2012), including fish groups with slow chromosomal divergence (Costa et al. 2013, 2015). In the three *Epinephelus* species, the $(CA)_{15}$, $(GA)_{15}$, $(CAA)_{10}$ and $(CGG)_{10}$ repeats do not show clear differences in their genomic distribution, being equally dispersed in eu- and heterochromatic regions, without detectable accumulation points. This diffuse organization does not signal remaining rearrangements in the karyotypes. In fact, it may be a limiting factor for karyotypic alterations (Molina 2007), mainly due to its small or non-close association

with other repetitive elements (Piscor & Parise-Maltempi 2016).

The maintenance of chromosomal and genomic conservation over tens of millions of years plays a significant role in the slow acquisition of post-zygotic barriers among Epinephelidae fish. In fact, negative epistatic interactions and consequent RI increase are more likely to occur when there are divergences in the number and structure of chromosomes of the two hybridizing taxa (King 1993, Cursino et al. 2014, Moran et al. 2019). The generalized homoploid condition of Epinephelidae fish overcomes blocks imposed by RI (Buggs et al. 2011), ensuring a greater hybrid viability (Rahman et al. 2013). Indeed, evidence of RI breaks is reported in Haemulidae (Marceniuk et al. 2019), Lutjanidae (Batista et al. 2012), Pomacantidae (Pyle & Randall 1994) and Chaetodontidae (Montanari et al. 2012), all fish families showing a slower rate of karyotypic changes (Molina 2007, Molina et al. 2014a).

Karyotype divergence and ontogenetic effects in interspecific Epinephelidae hybrids

Inversions are the main detectable rearrangements in Epinephilidae karyotypes (Amorim et al. 2021). It is known that inversions can interfere with normal chromosomal pairing and recombination during meiosis (Rieseberg 2001, Ortiz-Barrientos et al. 2016), and that even a single event can generate barriers driving to speciation (Ayala et al. 2013). However, inversions can be also related with adaptation processes (Wellenreuther & Bernatchez 2018, Faria et al. 2019). In *Gadus morhua* (Gadidae), for example, inversions cover more than 6% of the genome, and are associated with eco-adaptations of widely migratory ecotypes (Kirubakaran et al. 2016, Wellenreuther & Bernatchez 2018).

Unfavorable effects of inversions do not seem to be significant among grouper species regarding the hybrid viability and fertility (Table I). The previous description of the karyotypes of *E. coioides*, which classified the Ag-NOR pair as submetacentric chromosomes (2sm+46a; Wang et al. 2010), and *E. lanceolatus* (8sm+40a; Jiun & Mei 2009) were considered in the estimates of karyotypic divergences of the species involved in interspecific crossings. Hybrids of *E. coioides* ♀ (48a/2sm+46a; FN=48/50) X *E. lanceolatus* ♂ (2n=6sm+42a/8sm+40a; FN=54/56), with at least four detectable pericentric inversions (ΔFN=4-8), reach maturity and normal gonadal development (Li et al. 2018). Hybrids from phylogenetically close lineages may even show a greater growth and adaptability than their parental species (Senoo 2006, Liufu et al. 2007, Huang et al. 2016). Such a heterotic condition can even occur with some chromosomal diversification (Table I), probably due to sufficient levels of parental gene balance (Birchler & Veitia 2007), and the hybrid genome generating large adaptive effects (Dagilis et al. 2019). Some grouper hybrids, such as *E. lanceolatus* (2n=6sm+42a/8sm+40a) x *E. fuscoguttatus* (2n=2sm+46a), present a number of more favorable characters than their parental species, including incubation time, fertilization rates and hatching, growth, survival, adaptability and disease resistance (Ching et al. 2018). Therefore, favorable zootechnical characteristics (Senoo 2006, Liufu et al. 2007, Huang et al. 2016, Table I) demonstrate that hybridization is an important and effective strategy in grouper cultivation.

The time of divergence generally increases the rate of post-zygotic barriers among fish. Sterility in one or both sexes corresponds to the first level of RI, which progresses to the hybrid infeasibility when the average parental divergence reaches about ten million years (Russell 2003). Interspecific group hybrids have been obtained from parents bearing similar or structurally diversified karyotypes (Table

I). The analysis of parental karyotypes and their divergence times allowed us to infer the ontogenetic development of the hybrids and RI. The divergence time between the parental species, estimated from the percentage differences in the 16S mtDNA sequences, ranged from 1.6% (*E. costae* x *E. marginatus*) to 7.0% (*E. marginatus* x *E. aeneus*) (4-17 Mya), or 1.4% in *Cephalopholis* (*C. aurantia* x *C. spiloparaea* - ~3.5 Mya). Data on hybrid biological traits (eg, fertilization and hatching rates) suggest that ontogenetic parameters are not directly affected by the parental time divergence for most of the interspecific crosses (Table I). Parental species with considerable hybrid production, such as *E. fuscoguttatus* x *E. lanceolatus* and *E. fuscoguttatus* x *E. polyphekadion*, show a genetic distance of 4.8%, indicating an evolutionary divergence of ~11 Mya (Table I). In all of these crosses, the hybrid products were viable. Although fertility aspects are not available for all crosses, some hybrids were also fertile. Hybrids of *E. coioides* x *E. lanceolatus* (5.4% genetic divergence) and *E. lanceolatus* x *E. fuscoguttatus* (4.8% genetic divergence) showed even greater growth, survival and adaptability to captivity than their parents (Table I). Regarding intergeneric crosses, the genetic distance ranged from 4.6% (*C. fulva* x *P. furcifer* - 11 Mya) to 18% (*Epinephelus morio* x *Centropristis striata* - 43 Mya). The later is the high value among the species pairs and resulted in hybrid inviability (Table I), with larval lethality three days after fertilization. Crosses among *Epinephelus*, *Cromileptes* and *Cephalopholis* species, with an evolutionary divergence between 14 to 24 Mya, indicated the occurrence of post-zygotic barriers regarding the performance of ontogenetic parameters. However, some crosses between *Cephalopholis* and *Paranthias* species, diverging around 11 Mya, can still produce viable larvae (Tucker 1994). Likewise, hybrids between *Cephalopholis* and *Epinephelus* species (~11 Mya) may have shorter incubation time and higher growth than the parental species (Ching et al. 2018). On the other hand, crosses between *E. morio* × *C. striata*, with a very high evolutionary divergence time (~40 Mya), have resulted in few days of larval survival after hatching (Tucker 1994, Table I). But in this case, in addition to the divergence time, cannot be ruled out some influence of the significantly diversified karyotype of *C. striata*: 2n=24m+22sm+2a; FN=94 (Moran et al. 2019).

The diversification of groupers was significantly influenced by major biogeographic barriers. The main barriers during the Pliocene and Pleistocene periods, which resulted from the sea level reduction at 5.3-0.01 Mya (Ma et al. 2016), were particularly preponderant. However, this period of divergence is much shorter than the estimated mean time for the acquisition of an effective RI (Russell 2003) (Figure 3), thus suggesting that allopatry or pre-zygotic reproductive barriers probably played a more important role in that process. The genetic cohesion of species is intrinsically related to their evolutionary histories and degrees of lineage relationships (Marques & Ferreira 2008, Papadaki et al. 2018). The ontogenetic developmental indices of grouper hybrids apparently support that the divergence time of the clades was not sufficient in establishing an effective RI yet.

CONCLUSIONS

Groupers stand out as successful species for marine fish farming and hybrid production, but their level of introgression is still being better evaluated. In this study, in addition to new data on the karyotypic organization of some grouper species, ontogenetic effects of hybridization and the time of evolutionary divergence of the hybridizing species were also analyzed.

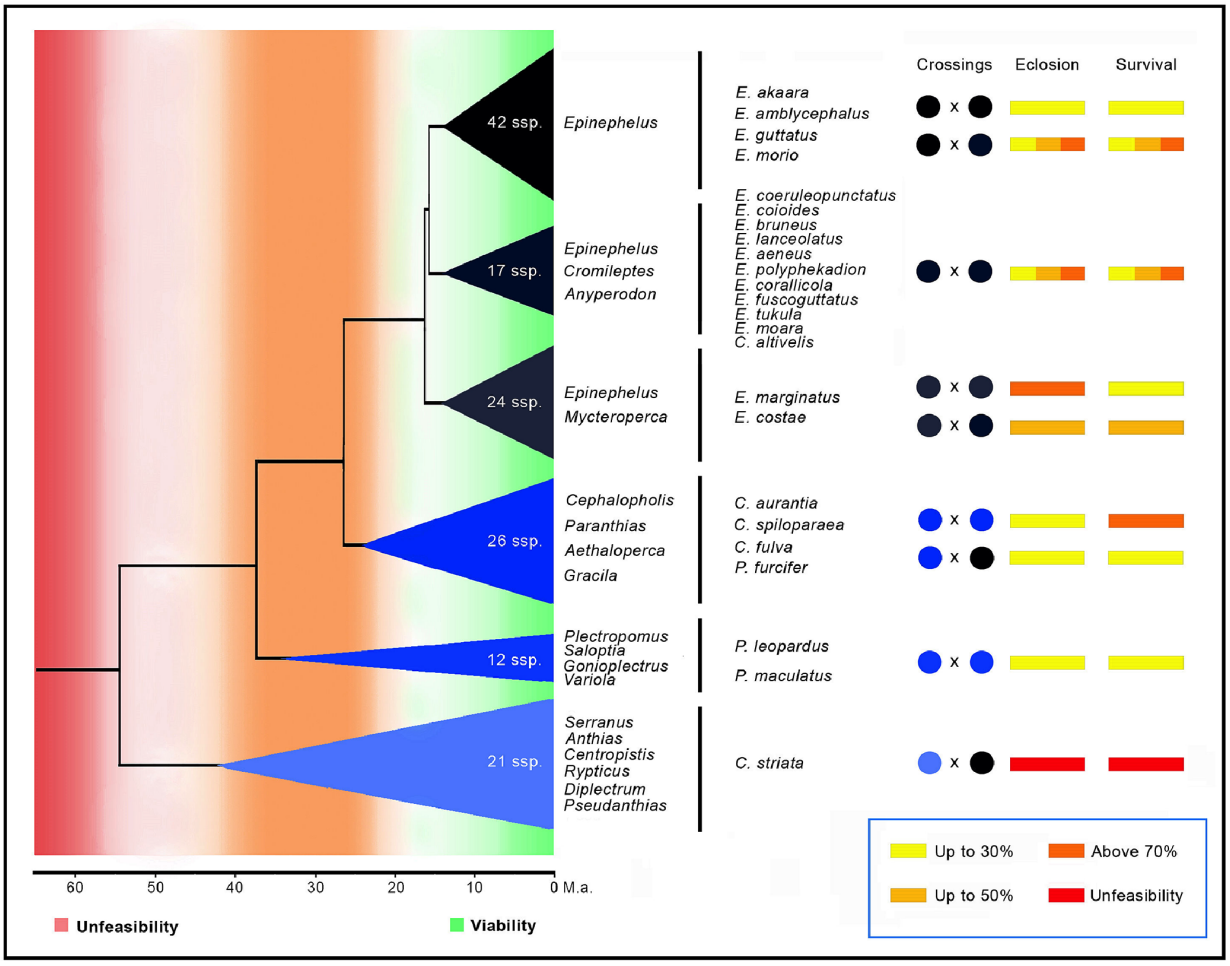

Figure 3. Post-zygotic ontogenetic effects in interspecific Epinephelidae hybrids, under a phylogenetic and temporal perspective (adapted from Ma et al. 2016).

Although the divergence time is a relevant factor for reproductive isolation, the general scenario that stands out in groupers is that post-zygotic reproductive isolation is not expressive yet. On the other hand, the high rate of karyotypic conservatism in this and other marine fish groups is consistent with their hybridization success. Therefore, the cytogenomic characterization of parental species stands out as a useful tool for analyzing hybridization and its traceability, as well as for the biological conservation and evolutionary approaches of groupers.

Acknowledgments

The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the financial support (Process nº 442626/2019-3), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship granted to KDJA. They also thank IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) for the license to collect the specimens (Process No. 19135-8) and UFRN (Universidade Federal do Rio Grande do Norte) for allowing this study to be carried out.

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SUPPLEMENTARY MATERIAL

Table SI.
Figure S1.

How to cite
AMORIM KDJ ET AL. 2024. Karyotypic changes and diversification time in Epinephelidae groupers (Perciformes). Implications on reproductive isolation. An Acad Bras Cienc 96: e20221011. DOI 10.1590/0001-3765202420221011.

Manuscript received on November 17, 2022;
accepted for publication on June 26, 2023

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Author contributions

KDJA and WFM conceived and the study; KDJA, CCMN, AT, RXS, ATB, and GWWFC, conducted the experiments; KDJA, GWWFC, and WFM, analyzed the data; KDJA, WFM, MBC, LACB, DDB, and GWWFC, wrote the manuscript; all authors read and approved the final version.