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Chromosomal distribution of two multigene families and the unusual occurrence of an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in the dolphinfish (Coryphaenidae): An evolutionary perspective

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ABSTRACT. Dolphinfishes (Coryphaenidae) are pelagic predators distributed throughout all tropical and subtropical oceans and are very important for commercial, traditional, and sport fishing. This small family contains the *Coryphaena hippurus* and *Coryphaena equiselis* species whose chromosomal aspects remain unknown, despite recent advances in cytogenetic data assimilation for Perciformes. In this study, both species were cytogenetically analyzed using different staining techniques (C-, Ag-, and CMA_3 banding) and fluorescence *in situ* hybridization, to detect 18S rDNA and 5S rDNA. *C. hippurus* females exhibit 2n = 48 chromosomes, with 2m+4sm+42a (NF = 54). In *C. equiselis*, where both sexes could be analyzed, females displayed

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2n = 48 chromosomes (2m+6sm+40a) and males exhibited 2n = 47 chromosomes (3m+6sm+38a) (NF = 56), indicating the presence of $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosomes. Sex-chromosome systems are rare in Perciformes, with this study demonstrating the first occurrence in a marine pelagic species. It remains unknown as to whether this system extends to other populations; however, these data are important with respect to evolutionary, phylogenetic, and speciation issues, as well as for elucidating the genesis of this unique sex system.

Key words: Marine fish; Karyotypic differentiation; $X_1X_1X_2X_2/X_1X_2Y$ system; Robertsonian fusion

INTRODUCTION

Cytogenetic data remain limited for large marine pelagic fish, primarily because of the logistics involved in sample collection; yet, these taxonomic groups represent a relevant component of ocean biodiversity. These taxonomic groups are a particularly valuable model of karyotypic evolution, in contrast to less vagile demersal reef species. Efficient biogeographic barriers for demersal species are often insufficient to restrain the gene flow of large pelagic migrators (Palumbi, 1994), possibly hindering the karyotypic diversification of these species.

Among marine Perciformes, the suborder Carangoidei, which is part of the small family Coryphaenidae (Springer and Smith-Vaniz, 2008), has revealed significant diploid conservatism, where most of the species display 2n = 48 chromosomes (Arai, 2011; Accioly et al., 2012). Coryphaenidae is composed of just 2 epipelagic species: *Coryphaena hippurus* Linnaeus, 1758 (Common dolphinfish) and *Coryphaena equiselis* Linnaeus, 1758 (Pompano dolphinfish). Both species are distributed in all tropical and subtropical oceans (Figure 1). *Coryphaena* species provide an important resource for industrial, traditional, and sport fishing and are exploited by the fishing fleets of many countries (Lasso and Zapata, 1999). Enzymatic analyses of these two species reveal exclusive alleles with marked differentiation (Pujolar and Pla, 2002). Recent investigations using mitochondrial DNA identified three phylogroups of *C. hippurus* in the Eastern Atlantic, Indo-Pacific, and Mediterranean Sea (Diaz-Jaimes et al., 2010).

Morphological characteristics, such as the presence of two ossified prenasal canals, which are rare for Percoidei, clearly place Coryphaenidae in the suborder Carangoidei. This suborder is a monophyletic group that is composed of the families Echeneidae, Rachycentridae, Carangidae, Nematistiidae, and Coryphaenidae (Reed et al., 2002; Springer and Smith-Vaniz, 2008). This division has been recently supported by molecular phylogenetic analyses (Gray et al., 2009).

While sex chromosomes are rare in fishes, they are fairly diverse, presenting a remarkable number of single and multiple systems (XX/XY, ZZ/ZW, ZZ/ZW_1W_2, XX/XY_1Y_2, $X_1X_1X_2X_2/X_1X_2Y$). These systems might be in the stages of early differentiation (Nanda et al., 2000), or highly differentiated according to morphology and/or structure. Multiple sex chromosomes usually arise from centric/tandem fusions or translocations between ancestral sex chromosome and autosomes. A Y-autosome rearrangement leads to $X_1X_1X_2X_2$ sex chromosomes in females and X_1X_2Y in males. Despite the large number of living species, the occurrence of cytologically differentiated sex chromosomes appears to be rare in marine fishes.

Few fish species have been described as having sex chromosomes (Arai, 2011).

The present study provides cytogenetic data for two species belonging to the Coryphaenidae family, *C. hippurus* and *C. equiselis*. The heterochromatic patterns, GC-rich regions, and chromosomal mapping of 5S and 18S ribosomal genes of these two species are identified using dual-color FISH assays. A multiple sex chromosome system is described for *C. equiselis*, representing the first description of sex chromosomes in large pelagic Perciformes. Because of the significant migratory potential of the *Coryphaena* genus (Oxenford and Hunt, 1986), the existence of this system in *C. equiselis* represents an important characteristic, providing inferences about its origin and ancestry, with possible phylogenetic implications.

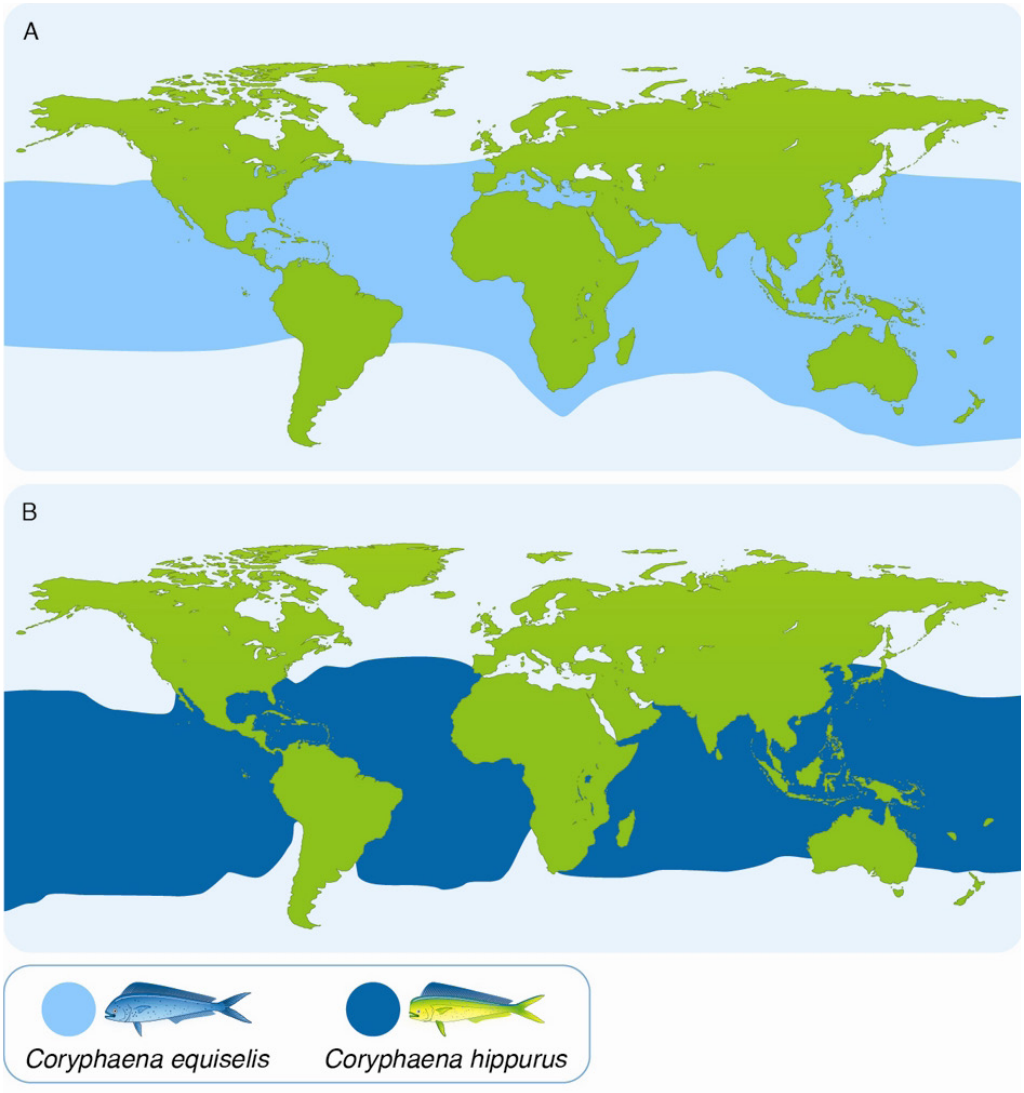

Figure 1. Geographical distribution of the species *Coryphaena equiselis* (**A**) and *Coryphaena hippurus* (**B**).

MATERIAL AND METHODS

Cytogenetic analyses were conducted using female specimens of *C. hippurus* and male and female specimens of *C. equiselis*. The fish were collected by fishing boats in the São Pedro and São Paulo Archipelago (00°55'N and 29°21'W) in the Mid-Atlantic region. All individuals were sexed by macroscopic gonadal examination and taxonomic identification according to Gibbs and Collette (1959). Kidney tissue fragments were used to obtain mitotic chromosomes, via *in vitro* interruption of the cell cycle (Gold et al., 1990). Nucleolus organizer regions (NORs) were detected by silver nitrate impregnation (Ag-NORs), according to Howell and Black (1980), whereas heterochromatic regions were evidenced by C-banding (Sumner, 1972). Fluorochrome staining (chromomycin A_3 and DAPI) was used to show GC or AT-rich chromosomal regions (Carvalho et al., 2005). Dual-color FISH assays were performed according to Pinkel et al. (1986), with slight adaptations for cytogenetic mapping of ribosomal genes using 5S and 18S rDNA probes.

To obtain the 18S probe, we used a 1400-pb tandemly repeated DNA sequence isolated from the DNA of the fish *Prochilodus argenteus* (Hatanaka and Galetti, 2004), labeled by nick translation with digoxigenin-11-dUTP (Roche, Germany), following manufacturer protocol. The 5S rDNA probe, with 500-200pb, was obtained from the nuclear DNA of the fish species *Leporinus elongatus* (Martins and Galetti, 1999) labeled by nick translation with biotin-14-dATP (Roche), also following manufacturer protocol.

Metaphases were photographed under an Olympus™ BX50 (Olympus, Tokyo, Japan) epifluorescence photomicroscope with an Olympus DP70 digital capture system. Chromosomes were classified as metacentric, submetacentric, and acrocentric (Levan et al., 1964).

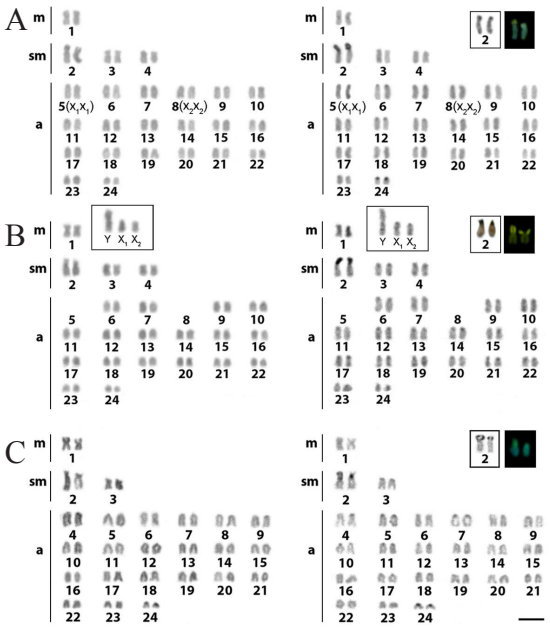

Figure 2. Karyotypic pattern of female (**A**) and male (**B**) *Coryphaena equiselis* and female *Coryphaena hippurus* (**C.**) Conventional Giemsa staining (left) and C-banding (right); in **B.** the X_1X_2Y chromosomes are highlighted. Ag-NOR sites, with overlapping CMA_3^+/$DAPI^-$ patterns (pair 2), are illustrated in the charts on the right. Scale bar = 5 µm.

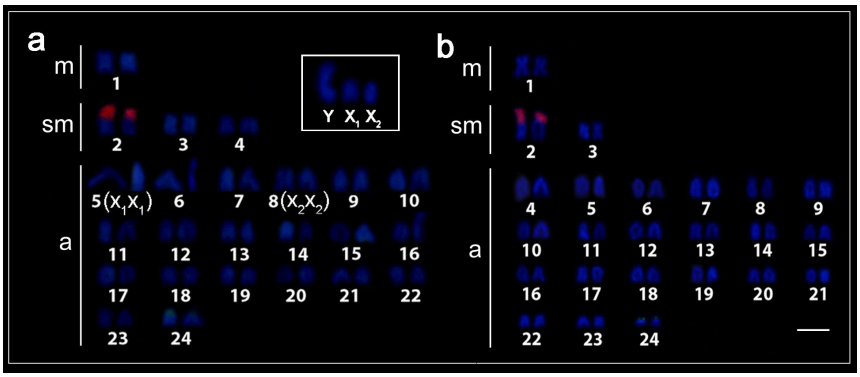

Figure 3. Chromosomal mapping of 18S (red) and 5S (green) rDNA sites, identified using dual-color FISH, for *Coryphaena equiselis* (**a**) and *Coryphaena hippurus* (**b**). Female karyotype of *C. equiselis* ($X_1X_1X_2X_2$) (**a**), with the heterogametic system present in the male karyotype (X_1X_2Y) being highlighted. The 18S and 5S rDNA sites are located on the same chromosomes (2 and 24, respectively) in both species. Scale bar = 5 µm.

RESULTS

The *C. equiselis* species exhibited intersex differences in diploid values, with females displaying 2n = 48 chromosomes and a karyotypic formula composed of 2m+6sm+40a (NF = 56) and males displaying 2n = 47 chromosomes and a karyotypic formula composed of 3m+6sm+38a (NF = 56) (Figures 2a, b). Three non-homologous chromosomes were present in the male karyotype; of which two were acrocentric, and tentatively corresponded to numbers 5 and 8 in the female karyotype, and one was a large metacentric chromosome, which was absent in females (Figure 2b, highlighted). Female specimens of *C. hippurus* displayed 2n = 48 chromosomes, with a karyotype composed of 2m+4sm+42a (NF = 54) (Figure 2c). We were unable to obtain male specimens of this species.

In both species, Ag-NOR sites were located on the short arms of chromosome 2 (subtelocentric) (Figure 2, highlighted), which also displayed positive CMA_3 and negative DAPI staining. Dual-color FISH assays with 18S and 5S rDNA probes demonstrated that these ribosomal subunits are located on different chromosomes (Figure 3). In both species, the 18S site corresponded to the location of Ag-NORs. In contrast, the 5S site was located on the short arms of the smaller pair (pair 24) in both species.

Heterochromatic blocks were slightly reduced in the centromeric chromosomal region, but more so in the regions corresponding to the 18S NOR/rDNA and 5S rDNA sites (Figure 2). The heterochromatic pattern of the metacentric chromosome, which is exclusive to *C. equiselis* males, revealed an interstitial heterochromatic segment on the long arm, which appeared to be similar to those present in the equivalent position on the chromosome 5 homologues of females.

DISCUSSION

The karyotypic characteristics of the males and females of *C. equiselis* indicate that this species has an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system. Within this system, the large metacentric chromosome that is exclusive to males corresponds to the neo-Y chromo-

some, and the X_1 and X_2 chromosomes probably correspond to numbers 5 and 8 of the female karyotype. The two species of the monotypic genus *Coryphaena* exhibit sexual dimorphism during the adult phase, in which males develop a characteristic bone crest on the front of the head (Collette, 1981). The relationship between sexual dimorphism and differentiated sex chromosomes has been poorly established for fish species. Differentiated sex chromosomes might play an important role in the evolution of sexual dimorphism (Rice, 1984), as they are the only part of the genome that differs between sexes. Thus, at least in *C. equiselis*, this correlation might be valid, considering the chromosomal and morphological dimorphism between the sexes in this species. Moreover, in addition to the ontogenetic and physiological aspects, chromosomal heterogamety is also able to promote or provide speciation through reproductive isolation among species, especially in the marine environment, because of the reduced number of geographical barriers or varying degrees of population isolation (Kitano et al., 2009).

The general karyotypic pattern of the two *Coryphaena* species, which display well documented migratory habits (Oxenford and Hunt, 1986) and wide geographic distribution, could be similar to the conserved karyotypic patterns found in demersal reef species that have potential larval dispersion, which, for some families, tends to reduce karyotypic diversity (Molina and Galetti, 2004a). Indeed, both species analyzed here exhibited a diploid value of 2n = 48 chromosomes, which is considered basal for Perciformes (Galetti et al., 2000). Furthermore, other conserved chromosomal patterns emerge from the karyotypes of Coryphaenidae, such as the primary ribosomal sites and reduced heterochromatic content in centromeric or terminal regions.

The existence of male chromosomal heterogamety in *C. equiselis*, due to a system of $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosomes, is a derived condition and represent the first occurrence of sex chromosomes in Echeneoidea (Rachycentridae + Coryphaenidae + Echeneidae) and for the monophyletic suborder Carangoidei. Analyses of different groups of fish have indicated that systems of differentiated sex chromosomes are able to emerge repeatedly in the same taxon through independent events (de Bello Cioffi et al., 2011). Regarding Coryphaenidae, the absence of data for *C. hippurus* males precludes delimiting the extent and dating of the origin of this multiple system in periods before or after the speciation of these species, but it might represent a strong reproductive barrier between these 2 species, especially in a situation of geographic overlap.

Differentiated sex chromosomes are relatively rare in marine species (Galetti et al., 2000). Among these cases, multiple $X_1X_1X_2X_2/X_1X_2Y$ systems have been the most widely reported (e.g., Ueno and Takai, 2008). In Perciformes, the genesis of multiple systems is largely attributed to Robertsonian fusion mechanisms, involving acrocentric chromosomes (Galetti et al., 2006). Furthermore, in addition to the formation of multiple sex systems, Robertsonian translocations are associated to the variability and karyotypic diversification in the autosomes of various groups of marine fish. The fixation of reciprocal translocation has been identified in several groups, including species from the Gobiidae families (e.g., Thode et al., 1988), and in transitory polymorphic form in other groups, such as the pomacentrids of the subfamily Chrominae (Molina and Galetti, 2002).

In *C. equiselis*, the neo-Y chromosome is approximately twice as large as the largest acrocentric chromosomes of the karyotype. This chromosome seems to have originated from the centric fusion of 2 acrocentric chromosomes, corresponding to one of the homologues of chromosomes 5 and 8 of the female karyotype. Thus, in parallel with the formation of the neo-Y chromosome, the remaining chromosomes from each of these pairs represent chromosomes X_1 and X_2, respectively. The presence of a discrete heterochromatic block in an interstitial

position in chromosome 5 and on the long arm of Y, indicates the possible involvement of this chromosome in the formation of neo-Y by centric fusion.

In the X_1X_2Y system described for *C. equiselis*, reduced heterochromatic content exhibited little evolutionary dynamism, given that, except for the interstitial block on the long arm of the neo-Y, it reflected the widely shared basal condition of Perciformes. This small amount of heterochromatin, which shows a neutral response to the fluorochromes used, is found in chromosomes involved in translocation (X_1/X_2). Initially, multiple systems must be established by the polymorphic state, with a tendency to fixation, in contrast to the varying degrees of heterochromatization present in a number of simple systems, indicating that a growing chromosome Y might have initial states of differentiation (Ueno and Takai, 2008). These studies also indicate that after chromosomal rearrangement, no subsequent differentiation occurs between sex chromosomes, corroborating the concept that chromosomal rearrangements are essential for multiple sex chromosome differentiation where additional heterochromatization is not required (de Bello Cioffi et al., 2011).

This study showed that *C. equiselis* and *C. hippurus* female karyotypes (homogametic) have the same diploid number, with some karyotypic differences. Diploid number of *C. hippurus* female (2n = 54) was smaller compared to those of *C. equiselis* (2n = 56), because of the presence of an acrocentric pair in the former species, rather than the submetacentric pair observed in the latter species. This discernible structural difference might be attributed to pericentric inversion, one of the most common mechanisms in the karyotypic diversification of Perciformes (Molina, 2007). Despite preliminary indications, and given the phylogeny yet to be resolved by existing molecular studies (Gray et al., 2009), the presence of a greater number of acrocentric chromosomes in *C. hippurus* might indicate that this species has a more basal condition compared to *C. equiselis*.

The mapping of the 5S and 18S rDNA sequences in apparently homologous chromosomes in the two species of Coryphaenidae revealed little evolutionary dynamism associated to the multigenic families of this group. Rachycentridae is the sister group of Coryphaenidae that is formed by the monotypic species *Rachycentron canadum*. Comparison with this group, and a number of Carangidae (e.g., Rodrigues et al., 2007), indicates that the location of the 18S sequences on the short arms of a single chromosomal pair of equal size, is a basal condition, suggesting extensive homeology. Equilocal labeling of the 18S rDNA sequences and Ag-NOR sites rules out the existence of additional polymorphic or latent sites. Mapping data of the ribosomal subunit 5S in Carangoidei remains scarce. In *C. hippurus* and *C. equiselis*, these sequences are also simple and invariant, and are exclusively conserved on the smallest chromosome pair (24), which is in direct contrast to the presence of two 5S sites in the only representative of the Rachycentridae family that has been analyzed (Jacobina et al., 2011). In many vertebrates, such as Coryphaenidae, 5S rDNA genes are located on a single chromosome pair; however, in fish, these genes may be found on just one pair or several chromosomes (Mazzei et al., 2004). The 5S sequences that are located on just 1 chromosome pair have been reported for several fish species (Sola et al., 2000). This characteristic appears to represent a basal condition for the group (Martins and Galetti, 1999, 2001), given that it is the most frequent in Teleostei with typically basal karyotypes (2n = 48 acrocentric). In contrast to the terminal position observed in Coryphaenidae, these sites are most commonly observed at an interstitial position in fish, indicating some degree of protection against disruptive events promoted by structural rearrangements (Martins and Galetti, 2001).

Genetic and cytogenetic patterns in fish have been associated to their dispersal potential (e.g., Molina and Galetti, 2004a). Indeed, it is well established that the fixation of inversions and translocations are marked by vicarious processes leading to karyotypic diversification (Sites and Moritz, 1987). In the marine environment, where there are fewer biogeographic partitions compared to continental areas, species generally seem to exhibit less karyotypic diversification (Brum, 1996).

Some families of reef fish display marked karyotypic derivation, such as Pomacentridae and Gobiidae, among others (Ene, 2003; Molina and Galetti, 2004b). In contrast, little is known about the karyotypic patterns of large neritic pelagic fish, which include migratory adults that are able to undertake long journeys to reproductive sites. The scarcity of cytogenetic information, as well as the basic analyses normally used, restricts the establishment of inferences about the level of karyotypic diversification displayed by these fish groups. In Coryphaenidae, despite high vagility and large population size, the karyotypic macrostructure present in the family (2n = 48; NF = 54-56) demonstrates karyotypic patterns similar to those observed in other Carangoidei, such as Carangidae (46 < 2n < 56; NF = 48–78) and Rachycentridae (2n = 48; NF = 54) (Arai, 2011). However, despite this relative conservation, it is important to underscore the differentiation of the X_1X_2Y system in *C. equiselis*, which represents a derived characteristic when compared to other species of large pelagic Perciformes. It is worth highlighting that in *Rachycentron canadum*, a monotypic species belonging to the Rachycentridae family, which forms a monophyletic group with Coryphaenidae (Gray et al., 2009), there is no evidence of differentiated sex chromosomes (Jacobina et al., 2011). Hence, it might be possible to extend the present study on *C. hippurus* males to determine whether differentiated sex chromosomes are present in this species; therefore, this study might potentially contribute towards characterizing synapomorphy in the Coryphaenidae family, or the exclusive autapomorphy of *C. equiselis*, as well as providing possible implications of this sex chromosome system for the speciation in this group.

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