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Cytogenetic characterization of *Rhomboplites aurorubens* and *Ocyurus chrysurus*, two monotypic genera of Lutjaninae from Cubagua Island, Venezuela, with a review of the cytogenetics of Lutjanidae (Teleostei: Perciformes)

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Lutjanidae, commonly known as snappers, includes 105 species, grouped in four subfamilies. In spite of the high number of species and of its worldwide distribution, the family has been little investigated and the phylogenetic relationships among some of its genera and species are still cause for debate. Only a small number of the species has been cytogenetically analysed. This study reports the first description of the karyotype of *Rhomboplites aurorubens* as well as data concerning the distribution of the constitutive heterochromatin and the location of the 18S rRNA and the 5S rRNA genes. Specimens of *Ocyurus chrysurus* from Venezuela were also investigated for the same cytogenetic features. Both species have a 48 uniarmed karyotype, but *R. aurorubens* has a single subtelocentric chromosome pair, the smallest of the chromosome complement, among the other acrocentric chromosomes. The C-positive heterochromatin is limited to the pericentromeric regions of all chromosomes. Both species show a single chromosome pair bearing the Nucleolus Organizer Regions, but NORs are differently located, in a terminal position on the short arms of the smallest chromosomes in *R. aurorubens* and in a paracentromeric position in a chromosome pair of large size in *O. chrysurus*. In *O. chrysurus*, the 5S rDNA gene cluster is located on a medium-sized chromosome pair, whereas in *R. aurorubens* it is syntenic with the 18S rDNA gene cluster on chromosome pair number 24. The obtained cytogenetic data, along with previous cytogenetic, morphological and molecular data for the family, reinforce the proposal to synonymize genus *Ocyurus* with *Lutjanus*. A review of Lutjanidae cytogenetics is also included.

Lutjanidae, comumente conhecidos como *snappers*, inclui 105 espécies, reunidas em quatro subfamílias. A despeito do grande número de espécies e de sua distribuição mundial, a família tem sido pouco estudada e as relações filogenéticas entre alguns de seus gêneros e espécies ainda é motivo de debates. Apenas um pequeno número de espécies foi citogeneticamente analisada. Esse estudo apresenta a primeira descrição do cariótipo de *Rhomboplites aurorubens* assim como dados relativos à distribuição de heterocromatina constitutiva e localização dos genes 18S rRNA e 5S rRNA. Espécimes de *Ocyurus chrysurus* da Venezuela foram também analisados quanto às mesmas características citogenéticas. Ambas as espécies têm cariótipos compostos de 48 cromossomos com um único braço, entretanto *R. aurorubens* tem um único par de cromossomos subtelocêntrico, o menor do complemento cromossômico, entre os outros cromossomos acrocêntricos. A heterocromatina C-positiva é limitada à região pericentromérica de todos os cromossomos. Ambas as species apresentam um único par com Regiões Organizadoras de Nucléolo, mas as RONs são localizadas em posições diferentes, em posição terminal no braço curto dos menores cromossomos de *R. aurorubens* e em posição paracentromérica no braço longo de um par de cromossomos grandes de *O. chrysurus*. Em *O. chrysurus*, os genes 5S rDNA estão localizados em um par de cromossomos de tamanho médio, enquanto em *R. aurorubens* eles são sintenicamente localizados com os genes 18S rDNA no par de cromossomos número 24. Os dados citogenéticos obtidos, junto com os dados morfológicos e moleculares disponíveis para a família reforçam a proposta de sinonimizar o gênero *Ocyurus* com *Lutjanus*. Uma revisão da citogenética dos Lutjanidae é também apresentada.

Key words: C-banding, FISH, Karyotype, NORs, Ribosomal genes.

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Introduction

Snappers (Perciformes, Lutjanidae) are reef-associated marine fish which are distributed worldwide in the tropical and sub-tropical regions. The family includes 105 species grouped in four subfamilies (Paradichthyinae, Etelinae, Lutjaninae and Apsilinae) and 17 genera (Nelson, 2006). Lutjaninae is the largest subfamily and includes approximately 70 species, grouped in six genera: *Lutjanus*, with 64 species, *Macolor* and *Pinjalo*, with two species each, and three monotypic genera, *Hoplopagrus*, *Ocyurus* and *Rhomboplites* (Nelson, 2006). In spite of the high number of species and its worldwide distribution, the family has been little investigated, and contradictory results have been obtained concerning the phylogenetic relationships and the taxonomic status of some of its genera and species. For example, the validity of the genus *Ocyurus* has been extensively discussed (Anderson, 1967; Davis & Birdsong, 1973; Domeier & Clarke, 1992; Chow & Walsh, 1992; Loftus, 1992), leading some authors to propose the synonymization of *Ocyurus* with the genus *Lutjanus* (Loftus, 1992; Clarke *et al*., 1997).

Until quite recently, the number of snapper species whose karyotype had been described was limited to four (Rishi, 1973; Choudhury *et al*., 1979; Raghunath & Prasad, 1980; Ueno & Ojima, 1992) and for three of them only the Giemsa features had been reported. However, in 2008, three different studies added new data, mainly for species of the genus *Lutjanus* (Nirchio *et al*., 2008; Rocha & Molina, 2008; Ueno & Takai, 2008), reporting karyotypes as well as several finer cytogenetic features. All these studies have shown that a general chromosome homogeneity occurs within the family, but that derived karyotypes can also be found, whose phylogenetic interpretation remains unclear. Therefore, further data on other snapper species are needed to obtain a more general picture of the karyoevolutive trends in the family.

This study continues a cytogenetic survey of Venezuelan snappers (Nirchio *et al*., 2008, Table 1) by extending the investigation to the two monotypic genera, *Ocyurus* and *Rhomboplites*, which, along with *Lutjanus*, represent the three genera of Lutjaninae, with a total of 12 species, existing in Venezuela (Cervigón, 1993). The two monotypic species, *Ocyurus chrysurus*, the yellowtail snapper, and *Rhomboplites aurorubens*, the vermilion snapper, have an almost overlapping western Atlantic distribution, extending southwards from Massachusetts, USA, and Bermuda to southeastern Brazil, including the Gulf of Mexico and Caribbean Sea (Allen, 1985; Froese & Pauly, 2008). This study reports the first description of the karyotype of *R. aurorubens* and reports, in both species, data on the distribution of the constitutive heterochromatin and the locations of the 18S rRNA and the 5S rRNA genes obtained by conventional (Giemsa staining, C-banding, silver staining) and molecular techniques (Fluorescent *In Situ* Hybridization - FISH). A review of the available karyological literature of Lutjanidae is also presented.

Material and Methods

Cytogenetic analyses were performed on nine specimens (five males and four females) of *Rhomboplites aurorubens* and on four unsexed specimens of *Ocyrus chrysurus* captured in Cubagua Island, Venezuela. Voucher specimens were deposited at the Ichthyology collection of the Escuela de Ciencias Aplicadas del Mar (ECAM), Universidad de Oriente.

Twenty four hours prior to chromosome preparations, the fish were intramuscularly injected with a yeast glucose solution (Lee & Elder, 1980) for mitosis stimulation. Chromosomes were obtained from kidney cells according to Foresti *et al*. (1993). C-bands were obtained according to the method described by Sumner (1972). For the detection of the active Nucleolus Organizer Regions (NORs), the chromosome sites where major ribosomal genes -18S, 5.8S and 28S- are clustered, slides were stained with silver nitrate using the method of Howell & Black (1980).

The 18S and 5S rDNA sites were also identified by Fluorescent *In Situ* Hybridization (FISH), according to the method of Pinkel *et al*. (1986). A sequence of 1,800 base pairs of the 18S rRNA gene of *Oreochromis niloticus* (Nile tilapia), cloned in pGEM-T plasmid, was used as a probe to localize sites of 45S rDNA. Polymerase Chain Reaction (PCR) products containing 5S rDNA repeats from *O. chrysurus* were used as probes for the chromosome mapping of 5S rDNA. DNA was extracted from muscle (Sambrook & Russel, 2001) and the 5S rDNA repeats were generated by PCR with the primers 5SA (5'TAC GCC CGA TCT CGT CCG ATC3') and 5SB (5'CAG GCT GGT ATG GCC GTA AGC3') according to Martins & Galetti Jr. (1999).

The 18S rDNA and 5S rDNA probes were labeled by PCR with biotin-14-dATP for the single-FISH experiments. In the case of *R. aurorubens*, a double-FISH with both probes was carried out by labeling the 18S rDNA with 11-dUTP digoxigenin and 5S rDNA with biotin-14-dATP. Biotin signals were detected and amplified by a two-round application of Avidin-FITC/biotinilated Anti-avidin. Digoxigenin signals were not amplified and were detected by Anti-digoxigenin/rhodamine. Chromosomes were counter-stained with Propidium Iodide (50 µg/ml) or DAPI diluted in Antifade.

The mitotic figures were photographed using a Motic B400 microscope equipped with a Moticam 5000C digital camera. FISH metaphases were photographed with a photomicroscope Olympus BX61 equipped with a DP70 digital camera.

Results

The counts of diploid metaphasic cells revealed a modal diploid number of 2n = 48 uniarmed chromosomes (NF = 48) in both species. However, in *R. aurorubens* (Fig. 1a) a single subtelocentric chromosome pair, the smallest of the chromosome complement, is present among the acrocentrics, while in *O. chrysurus* (Fig. 1b) all chromosomes are acrocentric. The chromosomes exhibit very small differences in size, thus, the precise classification of chromosomes in homologous

Table 1. Karyological studies in Lutjanidae (FN = Fundamental Number of chromosome arms; a = acrocentrics; m = metacentrics; M = male; F = female). *chromosome pair number as explicitly reported by the authors or as deduced from figures in the original papers; ** na = not available.

Species	2 n	Karyotype	FN	C-bands	Ag-NORs number	FISH-NORs number	Main NOR-bearing chromosome pair size (classification*)	FISH-5S rDNA number	5S rDNA-bearing chromosome pair size (classification)	References
Lutjanus alexandrei	48	48a	48	+	2	na**	large (2)	na	na	Rocha & Molina, 2008
L. analis	48	48a	48	+	2	na	large (5)	na	na	Rocha & Molina, 2008
L. analis	48	48a	48	+	2	2	large (6)	2	medium (9)	Nirchio *et al*., 2008
L. argentimaculatus	48	48a	48	na	na	na	na	na	na	Raghunat & Prasad, 1980
L. cyanopterus	48	48a	48	+	2	na	large (2)	na	na	Rocha & Molina, 2008
L. griseus	48	48a	48	+	2-4	2-6	large (6)	2	medium (9)	Nirchio *et al*., 2008
L. jocu	48	48a	48	+	2-4	na	large (2)	na	na	Rocha & Molina, 2008
L. kasmira	48	48a	48	na	na	na	na	na	na	Choudhury *et al*., 1979
L. kasmira	48	48a	48	+	2	na	large (6)	na	na	Ueno & Takai, 2008
L. quinquelineatus F	48	48a	48	+	2	na	large (6)	na	na	Ueno & Takai, 2008
M	47	1m+46a								
L. russellii	48	48a	48	+	2	na	large (6)	na	na	Ueno & Ojima, 1992
L. sanguineus	48	48a	48	na	na	na	na	na	na	Rishi, 1973
L. synagris Cytotype I	48	48a	48	+	2	2	small (24)	2	medium (9)	Nirchio *et al*., 2008
Cytotype II	47	1m+46a								
L. synagris	48	48a	48	+	2	na	small (23)	na	na	Rocha & Molina, 2008
Ocyurus chrysurus	48	48a	48	+	2	na	large (2)	na	na	Rocha & Molina, 2008
Ocyurus chrysurus	48	48a	48	+	2	2	large (6)	2	medium (9)	Present study
Rhomboplites aurorubens	48	2st+46a	48	+	2	2	small (24)	2	small (24)	Present study

pairs is not possible, with the exception of a large chromosome pair (numbered 6) in *O. chrysurus*, which shows a conspicuous secondary constriction, and chromosome pair number 24 in both species, clearly the smallest of the chromosome complement.

In both species, C-banding showed C-positive blocks of heterochromatin at the pericentromeric regions of all chromosomes (Fig. 2a-b), which were quite conspicuous in one of the largest chromosome pairs of *R. aurorubens* (Fig. 2a). In this latter species, the short arms of the subtelocentric chromosome pair 24 are also C-positive (Fig. 2a).

The analysis of the nucleolus organizer regions with the Ag-NOR technique sequential to Giemsa staining (Fig. 3), detected a maximum of two Ag-positive signals in both species. In *R. aurorubens*, the Ag-positive signals are located along the short arms of chromosome pair 24 (Fig. 3b), which are often heteromorphic and may appear heteropycnotic in Giemsa (Fig. 3a). This heteromorphism is unrelated to sex. In *O. chrysurus*, the Ag-positive signals are located proximally to centromeres (Fig. 3d), on the secondary constriction evident on chromosomes 6 in Giemsa-stained metaphases (Fig. 3c).

FISH with the 18S rDNA probe confirmed the unique location of NORs on the short arms of chromosome pair 24 in *R. aurorubens* (Fig. 4a) and in paracentromeric positions of

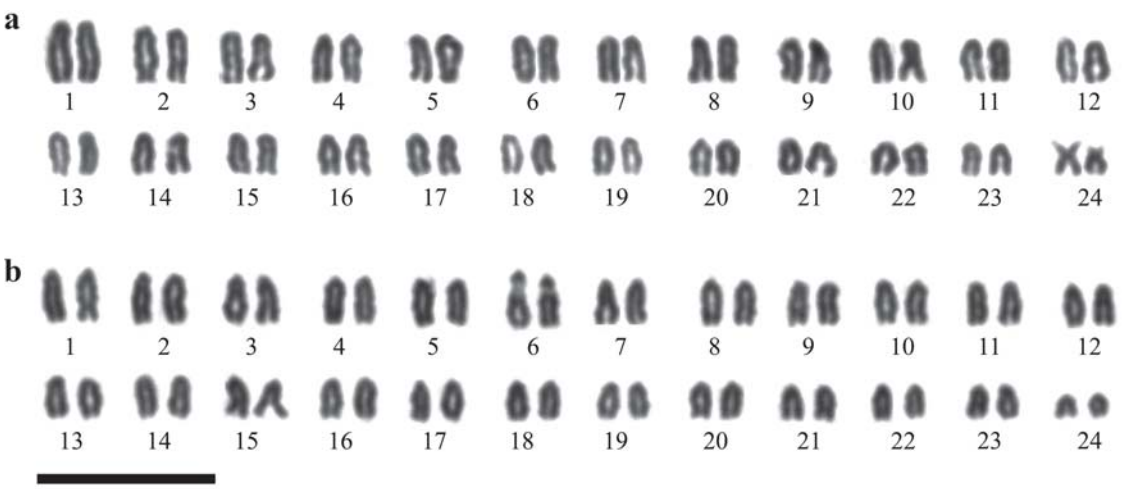

Fig. 1. Giemsa-stained karyotypes of *Rhomboplites aurorubens* (a) and *Ocyurus chrysurus* (b). Bar = 10 µm.

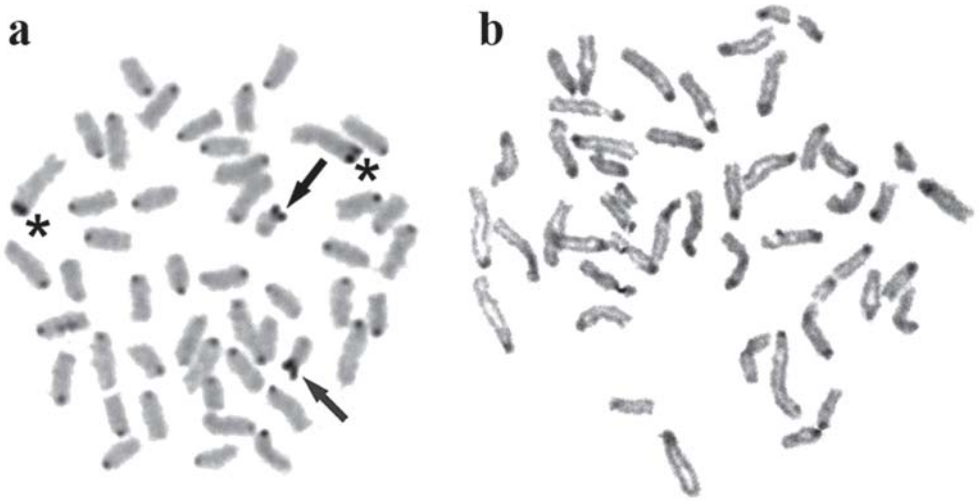

Fig. 2. C-banded metaphases of *Rhomboplites aurorubens* (a) and *Ocyurus chrysurus* (b). Arrows indicate chromosome pair 24. Asterisks indicate a large chromosome pair with conspicuous C-positive blocks.

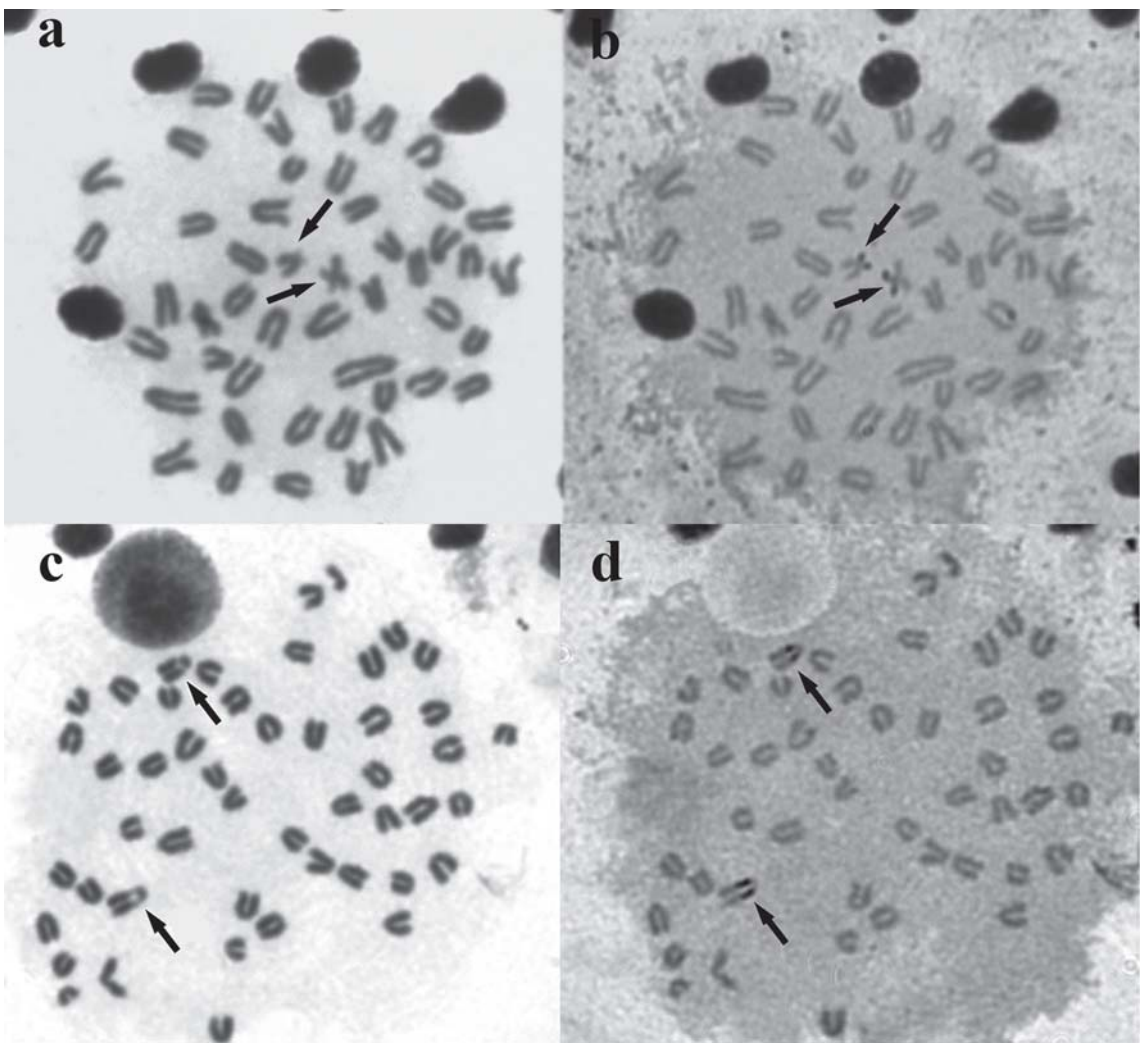

Fig. 3. Metaphases of *Rhomboplites aurorubens* (a-b) and *Ocyurus chrysurus* (c-d) sequentially stained with Giemsa (left) and $AgNO_3$ (right). Arrows indicate the NOR-bearing chromosomes.

chromosome pair number 6 in *O. chrysurus* (Fig. 4c). FISH with 5S rDNA produced one hybridization signal close to the centromere of the smallest subtelocentric chromosome pair in *R. aurorubens* (Fig. 4b) and the centromere of a medium-sized acrocentric chromosome pair in *O. chrysurus* (Fig. 4d). Thus, the double FISH (Fig. 4a-b) shows that both ribosomal gene clusters are located on the same chromosome pair in *R. aurorubens*.

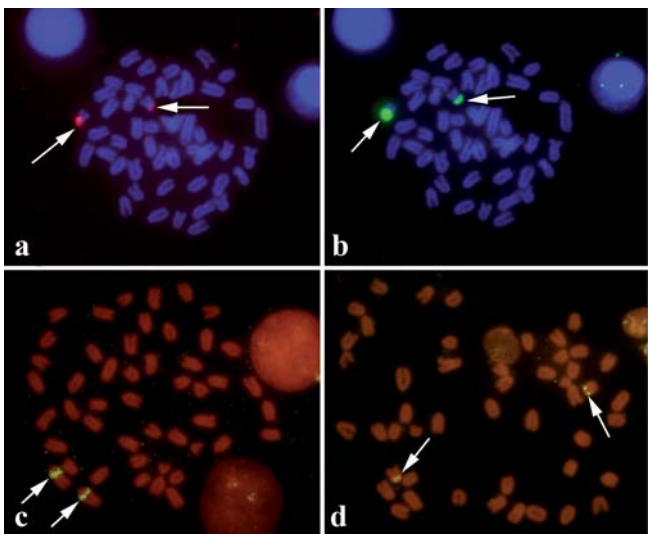

Fig. 4. Metaphases of *Rhomboplites aurorubens* (a-b) and *Ocyurus chrysurus* (c-d) after FISH with 18S rDNA (left) and with 5S rDNA (right). Arrows indicate the NOR bearing chromosomes. Arrowheads indicate the 5S rDNA bearing chromosomes.

Discussion

By adding the chromosome complement of *R. aurorubens*, reported in this study, to the Lutjanidae database, the number of the species of the family so far cytogenetically analysed rises to 13 (Table 1), out of the approximately 105 recognized species (Nelson, 2006).

The 48 all-acrocentrics karyotype here reported for the examined specimens of *O. chrysurus* is consistent with data reported by Rocha & Molina (2008) for specimens from the Brazilian north-eastern coast. This karyotype is shared by 10 out of the 13 species so far analyzed (Table 1). The second species reported in this paper, *R. aurorubens* shows instead a karyotype characterized by the presence of a subtelocentric chromosome pair, which is a novelty among the so far studied species of the family. The remaining two species, *Lutjanus quinquelineatus* (Ueno & Takai, 2008) and *L. synagris* (Nirchio *et al*., 2008), show Robertsonian rearrangements (Table 1), so that a reduction of the diploid number to 47 and a karyotype composed by 46 acrocentric chromosomes and one metacentric chromosome has been observed in males of *L. quinquelineatus* (Ueno & Takai, 2008), and in two out of the 21 examined specimens of *L. synagris* (Nirchio *et al*., 2008). Thus, in the former species, the Robertsonian rearrangement is related to the presence of a chromosomal sex determination mechanism, while in the latter it is apparently unrelated to sex and reflects an intra-specific chromosome polymorphism. It is worth noting that specimens of *L. synagris* from Brazil (Rocha & Molina, 2008) do not show any biarmed chromosome.

The pattern of the heterochromatin distribution observed in *O. chrysurus* and *R. aurorubens* confirms that a limited presence of heterochromatic blocks at the centromeres of all chromosomes is a general characteristic of Lutjanidae, as this pattern is shared by all the 11 species investigated in this sense (Table 1), including those species for which more than one population has been studied, such as *L. analis*, *L. synagris* (Nirchio *et al*., 2008; Rocha & Molina, 2008) and *O. chrysurus* (Rocha & Molina, 2008; this paper). This evidence suggests that heterochromatinization processes have not played an important role in the karyotypic evolution of Lutjanidae.

Regarding the number and location of the major ribosomal genes, in *O. chrysurus* and *R. aurorubens*, the silver staining, which generally detects those NORs which are active in the preceding interphase (Hubbel, 1985; Jimènez *et al*., 1988; Sánchez-Pina *et al*., 1984), produced results which overlap with those obtained by FISH with 18S rDNA, *i.e*., two NOR sites were detected in both species, which show, however, different locations, on different chromosome pairs. It is useful to consider the obtained results together with the whole data set on nucleolar organizer regions in the family, mostly obtained by silver staining, in 11 species (Table 1). Most (nine) of the species show a single chromosome pair coding for major ribosomal genes. The remaining two species, *L. griseus* and *L. jocu*, show other variable NOR sites in addition to the main species-specific NOR-bearing chromosome pair.

The precise classification of chromosome pairs is difficult due to the small differences in chromosome size and to the absence of banding. However, in spite of this, at least three different main karyomorphs can be identified with certainty, which are summarized in Fig. 5. A group of seven species shows a large NOR-bearing chromosome pair and the NORs are located in a paracentromeric position. Different authors, in different studies (Table 1) have classified this chromosome pair as number 2, 5 or 6. However, in all species, in the Giemsa-stained metaphases this chromosome pair shows a large secondary constriction, corresponding to the NOR site, which may affect chromosome size and therefore its classification. Thus, pursuing a parsimonious criterion, this pair might be considered homeologous in this group of species (Fig. 5a). Two species, *L. synagris* and *R. aurorubens*, show NORs on the smallest chromosome pair, but their location is clearly different, representing therefore the two remaining karyomorphs. In *L. synagris* (Fig. 5b), NORs are located in a paracentromeric position of chromosome pair

Fig. 5. Idiograms reporting the major and minor ribosomal gene locations summarizing the three main karyomorphs (a-c) identified in the species of Lutjanidae investigated so far. See text for details. Solid circles represent locations of 18S rDNA. Grey bars represent locations of 5S rDNA.

24 (Nirchio et *al*., 2008; numbered 23 by Rocha & Molina, 2008), whereas in *R. aurorubens* (Fig. 5c) NORs are located on the short arms of this same chromosome pair, which however is, differently from the other Lutjanidae species, the only one made by subtelocentrics in the chromosome complement.

Considering that the karyomorph of Fig. 5a is the most common in Lutjanidae and widespread in Perciformes, it could be tentatively assumed that this might reflect the plesiomorphic condition in the family from which karyotypes with additional NORs (*L. griseus*, Nirchio *et al*., 2008; *L. jocu*, Rocha & Molina, 2008) or with different locations of NORs (*L. synagris*, Fig. 5b, Nirchio *et al*., 2008; *R. aurorubens*, Fig. 5c, present paper) derived.

As far as the 5S rDNA sites are concerned, by adding the results here reported for *O. chrysurus* and *R. aurorubens*, data are available for only five species (Table 1). In *O. chrysurus*, the minor ribosomal genes show a number and location corresponding to those observed in the other three species that have been investigated so far, *Lutjanus analis*, *L. synagris* and *L. griseus* (Nirchio *et al*., 2008), *i.e*., a single site in a paracentromeric position of a medium-sized acrocentric chromosome pair, which might be homeologous in all of them, numbered as 9 in Table 1 and Fig. 5 (a, b). *Rhomboplites aurorubens* similarly shows a single pair of 5S rDNA bearing chromosomes. However, this pair is certainly different, being the smallest of the chromosome complement, where major ribosomal genes are co-located (Fig. 5c). The syntenic organization of 45S and 5S rDNA *loci* is quite uncommon in vertebrates and in fish in particular (Martins, 2007).

By framing these cytogenetic data within a systematic and phylogenetic context, some considerations concerning the validity of genus *Ocyurus* and the relationships among the Lutjaninae species can be made. There is a long term debate regarding the validity of the genus *Ocyurus*. According to Domeier & Clarke (1992), the morphological characters in *Ocyurus*, which allows the separation of this genus from *Lutjanus*, are merely adaptations to a pelagic lifestyle. On the other hand, the numbers of morphological and meristic similarities among these genera are far greater. In addition, Domeier & Clarke (1992) and Loftus (1992), based on the evidence of natural and laboratory hybrids between *L. synagris* (and likely *L. griseus*) and *O. chrysurus*, claimed that *Ocyurus* probably does not represent a distinct evolutionary lineage from *Lutjanus*. Finally, according to Clarke *et al*. (1997), the resemblance of the larval forms also provides further evidence for the synonymization of the two genera.

Similar evidence, suggesting the synonymization of *Ocyurus* with *Lutjanus*, was obtained using molecular markers. In fact, in a phylogenetic study based on mitochondrial 12S rRNA and cytochrome b genes sequences of 14 species of snappers occurring in western Atlantic, Sarver *et al*. (1996) emphasized that the single most-parsimonious tree obtained from analysis of weighted characters placed *Ocyurus* in a clade with the red snapper group (*Lutjanus campechanus* and *L. vivanus*); whereas the strict consensus of the three most-parsimonious trees from the analysis of unweighted characters placed it in a polytomy with several species of *Lutjanus* and the monotypic genus *Rhomboplites*. In addition to these, recent mitochondrial data (16S rDNA and cytochrome b) obtained by Miller & Cribb (2007), who investigated phylogenetic relationships of Indo-Pacific snappers (27 species), with the inclusion of three western Atlantic snappers, showed that the genus *Lutjanus* is paraphyletic.

In this context, *O. chrysurus* shares the cytogenetic features shown by most of the species of *Lutjanus* from both the Indo-Pacific and western Atlantic Oceans, while *R. aurorubens* shows the most derived features. Therefore, the cytogenetic data here reported, while supporting the classification of *Rhomboplites* as a monotypic genus, do not rule out the inclusion of *Ocyurus chrysurus* into the genus *Lutjanus*.

Considering the more general picture of karyotype evolution in Perciformes, the presence of 48 chromosomes has been regarded as a primitive condition for this fish group (Accioly & Molina, 2008; Galetti Jr. *et al*., 2000, 2006). It can be stated, however, that there can be found both extreme karyotype conservativeness, as for example in the Haemulidae, as well as tendencies for karyotype differentiation, such as in Gobiidae (Molina, 2007). The marine family of Lutjanidae, including the species studied in this work, show a higher level of chromosomal stability, compared to closely related taxa such as Sparidae (Johnson, 1980; Orrell & Carpenter, 2004; Miller & Cribb, 2007), which shows higher levels of chromosomal diversification. Nevertheless, in spite of their morphologically conservative karyotype, when investigated for the finer cytogenetic features, Lutjaninae species were found to have undergone a certain degree of chromosome divergence.

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