



Revista de Biología Tropical

ISSN: 0034-7744

rbt@cariari.ucr.ac.cr

Universidad de Costa Rica

Costa Rica

Nirchio, Mauro; Ehemann, Nicolás; Siccha-Ramirez, Raquel; Ron, Ernesto; Pérez, Julio Eduardo;
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Island, Venezuela
Revista de Biología Tropical, vol. 62, núm. 4, diciembre-, 2014, pp. 1365-1373
Universidad de Costa Rica
San Pedro de Montes de Oca, Costa Rica

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Karyotype of the invasive species *Pterois volitans* (Scorpaeniformes: Scorpaenidae) from Margarita Island, Venezuela

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Received 17-I-2014. Corrected 27-VI-2014. Accepted 29-VII-2014.

Abstract: The genus *Pterois* includes nine valid species, native to the Red Sea and Indian Ocean throughout the Western Pacific. *P. volitans* and *P. miles* are native to the Indo-Pacific, and were introduced into Florida waters as a result of aquarium releases, and have been recently recognized as invaders of the Western Atlantic and Caribbean Sea (Costa Rica to Venezuela). Thus far, cytogenetic studies of the genus *Pterois* only cover basic aspects of three species, including *P. volitans* from Indo-Pacific Ocean. Considering the lack of more detailed information about cytogenetic characteristics of this invasive species, the objective of the present study was to investigate the basic and molecular cytogenetic characteristics of *P. volitans* in Venezuela, and compare the results with those from the original distribution area. For this, the karyotypic characteristics of four lionfish caught in Margarita Island, Venezuela, were investigated by examining metaphase chromosomes by Giemsa staining, C-banding, Ag-NOR, and two-colour-Fluorescent *in situ* hybridization (FISH) for mapping of 18S and 5S ribosomal genes. Comparing the sequences of the 16S gene of the specimens analyzed, with sequences already included in the Genbank, we corroborated that our specimens identified as *P. volitans* are in fact this species, and hence exclude the possibility of a misidentification of *P. miles*. The diploid number was $2n=48$ ($2m+10sm+36a$) with $FN=60$. Chromosomes uniformly decreased in size, making it difficult to clearly identify the homologues except for the only metacentric pair, and the pairs number two, the largest of the submetacentric series. C-banding revealed only three pairs of chromosomes negative for C-band, whereas all remaining chromosomes presented telomeric and some interstitial C-positive blocks. Only two chromosomes were C-banding positive at the pericentromeric regions. Sequential staining revealed Ag-NOR on the tips of the short arms of chromosome pair number two and the FISH assay revealed that 18S rDNA and 5S rDNA genes are co-located on this chromosome pair. The co-localization of 5S rDNA and 45S rDNA is discussed. Both constitutive heterochromatin and NOR location detected in samples examined in this study, differ from those reported for *P. volitans* in previous analysis of specimens collected in Indian Ocean (Java), suggesting the occurrence of chromosome microrearrangements involving heterochromatin during the spread of *P. volitans*. Rev. Biol. Trop. 62 (4): 1365-1373. Epub 2014 December 01.

Key words: karyotype, 18S and 5S ribosomal genes, 16S rRNA mitochondrial gene, molecular identification, invasive species.

The genus *Pterois* (Linnaeus, 1758) (Scorpaeniformes: Scorpaenidae) contains nine valid species, native to the Red Sea and Indian Ocean throughout the Western Pacific (Schultz 1986). Two similar species, the red lionfish *P. volitans* (Linnaeus, 1758), and the devil firefish *P. miles*, have been considered in the past as a synonymy (Beaufort & DeBriggs, 1962; Dor, 1984), but Shultz (1986) demonstrated that they can be distinguished by dorsal- and anal-fin rays counts (*P. volitans* usually has 11 dorsal and seven anal-fin rays while *P. miles*, has generally 10 dorsal and six anal-fin rays). More recently, sequence analyses of mitochondrial genes resolved specimens of *P. miles* and *P. volitans* in distinct monophyletic clades providing molecular evidence that they are different species (Freshwater et al., 2009).

P. volitans and *P. miles* are native to the Indo-Pacific and were introduced into Florida waters as a result of aquarium releases (Courtenay, 1995; Whitfield et al., 2002; Semmens et al., 2004), and are recognized as recent invaders of the Western Atlantic and Caribbean Sea (Costa Rica to Venezuela), with hundreds of sightings documented after the first lionfish was reported in May 2009 off the coast of South America (González et al., 2009; Lasso-Alcalá & Posada, 2010). Although their populations are expanding rapidly throughout North-West Atlantic and the Caribbean Sea (Whitfield et al., 2007), 93% of the Atlantic samples of lionfish consist of *P. volitans* and only 7% correspond to *P. miles* (Hamner et al., 2007).

In response to their proliferation throughout the Caribbean, the International Coral Reef Initiative (ICRI) set up an *ad-hoc* committee, held a workshop in order to develop a region-wide strategy for lionfish control and management that requires achieving all possible information specifically with their impact, reproduction and genetics (<http://www.icriforum.org/icri-meetings/icri-regional-lionfish-workshop>).

As long as lionfish invasion continues, genetic research will become a powerful tool for assessing pathways of dispersion, populations divergence, expression of invasion-driving phenotypes, and possibly detection of new

introduction events. Thus, the development of high-resolution lionfish genetic markers will allow a more throughout investigation of this invasion, providing a better understanding of both invasion genetics and marine phylogeography (Morris & Green, 2012).

This paper presents a molecular and cytogenetic characterization of lionfish collected at Margarita Island, Venezuela, reporting the chromosome number and formula, constitutive heterochromatin distribution by C-banding, and the locations of ribosomal genes by Ag-staining and fluorescent *in situ* hybridization (FISH). It will also include the description of co-localization of the major and minor ribosomal genes, and information about sequences of the mitochondrial gene 16S, with the aim to contribute to achieve genetic information on this invasive species.

MATERIALS AND METHODS

Study site: Four specimens of lionfish collected at Margarita Island (10°57'7"N - 64°9'50"W), Venezuela, were analyzed. All the specimens were identified as *Pterois volitans* according to Shultz (1986), and vouchers were deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes, UNESP, Botucatu, São Paulo, Brazil (LBP13291), and Laboratorio de Citogenética de Peces of Escuela de Ciencias Aplicadas Del Mar, Universidad de Oriente, Nueva Esparta, Venezuela (ECAM-880, ECAM- 901, ECAM-949).

Molecular analysis: In order to molecularly corroborate the taxonomic identification of the specimens of lionfish here studied, a fragment (542 bp) of 16S rRNA mitochondrial gene was amplified and sequenced for a subset of two specimens and compared with the same gene fragment corresponding to *P. volitans*, *P. mombasae*, *P. milles*, *P. radiata* and *P. antennata* retrieved from the Genbank. For this molecular analysis, total DNA was obtained from small fragments of muscular tissue, applying the methodology of extraction in saline solution described by Aljanabi et al.

(1997). A partial sequence of about 542 bp of the gene 16S rRNA was amplified using the primers 16S-F 5' ACG CCT GTT TAT CAA AAA CAT 3' and 16S-R 5' CCG GTC TGA ACT CAG ATC ACG T 3' (Kocher et al. 1989). Amplified DNA segments were visualized in 1% agarose gel, using Blue Green Loading Dye I (LGC biotechnology). PCR products were purified through ExoSap-IT® (USB Corporation) and sequenced using BigDye Terminator v 3.1 (Applied Biosystems) according to the manufacturer's instructions in a ABI3130 (Perking-Elmer) sequencer.

Chromosome analysis: Chromosome preparations were obtained from kidney cells as described in Nirchio & Oliveira (2006). Kidney cells suspensions were obtained from fishes injected intramuscularly with yeast glucose solution for mitosis stimulation 24 hours before injecting colchicine (Lee & Elder, 1980). Silver-stained nucleolus organizer regions (Ag-NORs) were obtained as described by Howell & Black (1980). C-bands were obtained following the method of Sumner (1972).

The 5S and 18S rDNA sites were identified by FISH according to the method proposed by Pinkel et al. (1986). Probes used to localize the 45S rDNA sites were obtained from total DNA of *Characidium zebra* using the primers 18S F (5' CCG TGA TGG CTC CTT TTG AT 3') and 18S R (5' CCG AGG ACC TCA CTA AAC CA 3'). These probes were labeled with Digoxigenin-11-dUTP (Roche Applied Science) by PCR and the hybridization signal detection was performed using anti-digoxigenin-rhodamine (Roche Applied Science). 5S rDNA was mapped using a probe obtained by PCR from the total DNA of *Synbranchus marmoratus* using the primers 5S F (5' GCC CGA TAC CGT CCG ATC TCT 3') and 5S R (5' CAG GCT GCC ATG GGT GTA ACG 3'). These probes were labeled with Biotin-16-dUTP (Roche Applied Science) by PCR and the hybridization signal detection was performed using conjugated Avidin-Fluorescein.

The slides were incubated with RNase (40µg/mL) at 37°C for one hour. After that,

DNA denaturation in formamide 70%/2xSSC at 70°C for two minutes was performed and the chromosome preparations were dehydrated in an ethanol series (70, 85, and 100%). After drying, the hybridization mix (100ng probes, 10mg/mL dextran sulfate, 2xSSC, and 50% formamide in a 30µL final volume) was pipetted onto the samples, which were then incubated overnight at 37°C in a humid chamber. After hybridization, the slides were washed 2X for 10 minutes in 15% formamide solution/0.2xSSC pH 7.0 at 42°C, followed by 3X washes in 0.1xSSC at 60°C for five minutes each; the slides were then incubated in 5% NFDM/4xSSC buffer for 15 minutes and washed with Tween 0.5%/4xSSC for five minutes at room temperature with shaking. Probe detection was done with 0.07% avidin-FITC (Sigma) conjugated with NFDM/4xSSC buffer for 30 minutes, followed by signal amplification using 2.5% antividin-biotin conjugated with NFDM/4xSSC buffer for 30 minutes. Treatments with avidin-FITC and antividin-biotin were accomplished at 37°C in a humid chamber. After each step of signal detection, the slides were washed 3X with 0.5%/4xSSC Tween for five minutes at room temperature. Chromosomes were counterstained with antifade/DAPI.

The mitotic figures were photographed using a Motic B410 microscope equipped with a Motic Moticam 5000C digital camera. Chromosomes were classified according to the arm ratio criteria (Levan et al., 1964). FISH metaphases were photographed with an Olympus BX61 photomicroscope equipped with a DP70 digital camera. Images were digitally processed with ADOBE® PHOTOSHOP CS6 Extended®.

RESULTS

The alignment of two sequences (542pb) of the 16S rRNA mitochondrial gene corresponding to samples identified as *Pterois volitans* and 12 sequences retrieved from Genbank, corresponding to the species *P. volitans*, *P. mombasae*, *P. miles*, *P. radiata* and *P. antennata*, allowed detecting the existence of 519

conserved sites, 23 variable sites, 20 parsimony informative sites and three sites with unique mutations. Figure 1 shows the phylogenetic trees constructed using the methods of maximum likelihood and maximum parsimony, both presenting identical topology and demonstrating the existence of two monophyletic lineages supported by high bootstrap values: one consisting of the species *P. mombasae*, *P. antennata* and *P. radiata* and another consisting of *P. volitans* and *P. miles*. In all specimens of *P. volitans* here examined the diploid number was $2n=48$ ($2m+10sm+36a$) with $FN=60$ (Table 1, Fig. 2). Chromosomes uniformly decreased in size, making it difficult to clearly identify the homologues except for the only metacentric pair number one, and the pair number two, the largest of the submetacentric

series. Figure 3 (A, B) shows a sequentially stained plate that allowed the identification of the NOR-bearing chromosomes.

Most of the chromosomes showed telomeric and/or interstitial C-positive blocks. In addition two chromosomes showed C-positive bands in the pericentromeric regions (Fig. 4).

Chromosomal mapping of 5S and 45S rDNA performed by multi-colour FISH using biotin-labeled and digoxigenin-labeled probes is shown in figure 5. In simultaneous hybridization Biotin-16-dUTP-labelled 5S rDNA probe was detected as a yellow signal (Fig. 5B) whereas Digoxigenin-11-dUTP-labeled 18S rDNA was detected as a red signal (Fig. 5C) and revealed the co-localization of 5S and 18S rRNA genes in the chromosome pair number 2.

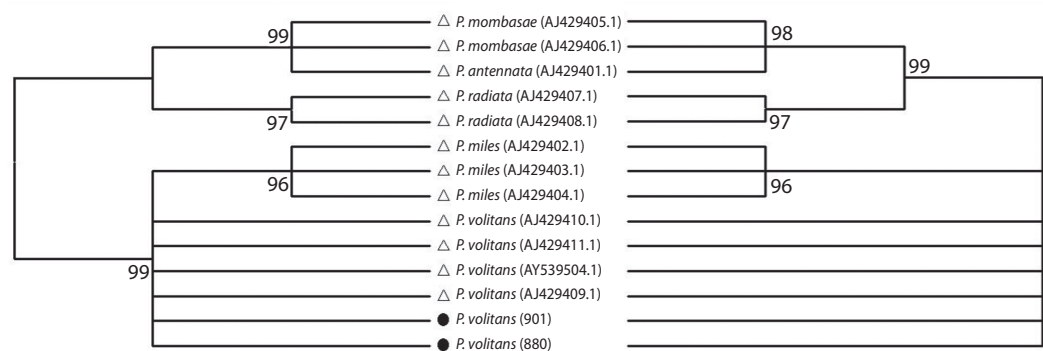


Fig. 1. Phylogenetic trees constructed using maximum likelihood (left) and maximum parsimony (right) methods. Black circles correspond to the specimens used in the present study.

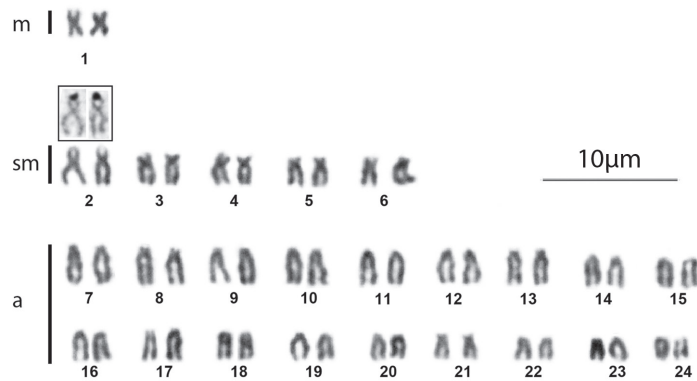


Fig. 2. Giemsa-stained karyotype of *Pterois volitans*.

TABLE 1
Chromosome data for the *Pterois lunulata*, *P. radiata* and *P. volitans*

Species	2n	Karyotype	NF1/NF2	Ag-NORs	18S-rDNA	5S-rDNA	C-bands	Locality	Ref.
<i>Pterois lunulata</i>	48	2m+12st+34a	52/58	2	2		+	Indian Ocean	1
<i>P. lunulata</i>	48	2m+10sm+18st+18a	60/78	-	-	-	-	Japan	2
<i>P. radiata</i>	48	4m+8st+36a	52/60	2	2	-	+	Indian Ocean	1
<i>P. volitans</i>	48	2m+10st+36a	50/60	2	2	-	+	Indian Ocean	1
<i>P. volitans</i>	48	2m+10sm+36a	60	2	2	2	+	Venezuela	3

NF1 shows fundamental arm number, when only m and sm are counted as two-armed. **NF2** shows fundamental arm number, when m, sm, and st are counted as bi-armed. [References: 1- Caputo et al. (2003); 2- Nishikawa et al. (1977); 3- present paper].

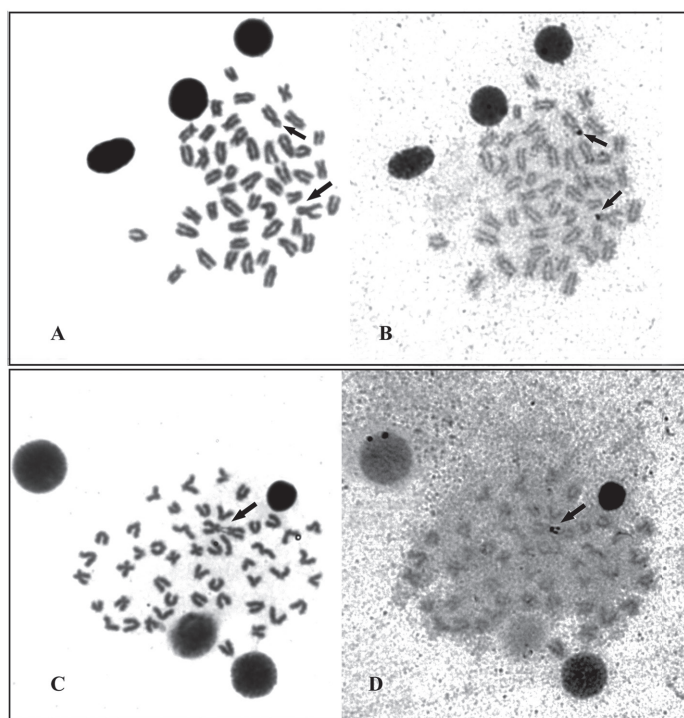


Fig. 3. Sequential Giemsa (A, C) and AgNO₃-stained (B,D) plate of *Pterois volitans*. Plates C, D show chromosome association. Arrows indicate the NOR-bearing chromosome.

DISCUSSION

The overall mean genetic distance between the sequences here analyzed was $d=0.0166$, which reveals that nucleotide differences are small, even amongst taxonomic units recognized as different species ($d=0.0122$ between *P. volitans* and *P. miles*; $d=0.0121$ between *P.*

radiata and *P. mombasae*; $d=0.0131$ between *P. radiata* and *P. antennata*; $d=0.0028$ between *P. mombasae* and *P. antennata*). However, the comparisons of the 16S gene sequences of the specimens analyzed in the present study with sequences already included in GenBank, corroborate that the specimens studied here belong to a clade that includes exclusively other

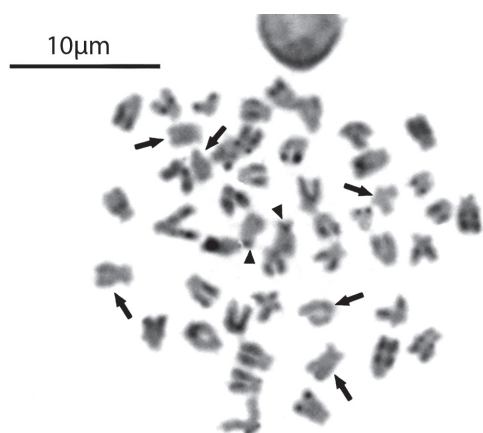


Fig. 4. C-banded metaphase of *Pterois volitans*. Arrows indicate negative chromosomes for C-banding. Arrowheads indicate chromosomes with pericentromeric heterochromatin.

from subjective attribution of small chromosomes to sm or st categories, rather than representing real polymorphisms (Arai, 2011). Thus it is likely that differences in the karyotype of *P. volitans* between this study and published data by Caputo et al. (2003) are due to differences in classification of few chromosome pairs.

However differences between Indian Ocean and Venezuelan *P. volitans* samples were detected both in NORs location and C-bands. Indeed Caputo et al. (2003) reported the presence of terminal-centromeric located Ag-NORs on the short arms of a pair of subtelocentric elements, while samples examined in this study have the Ag-NORs located on the short arm of the largest submetacentric chromosome pairs classified as number two.

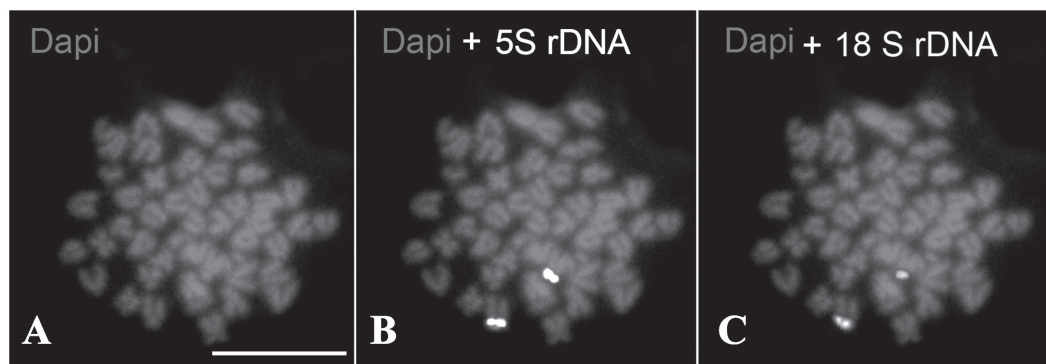


Fig. 5. Fluorescence *in situ* hybridization of 5S rRNA and 18S rRNA genes in *Pterois volitans* chromosomes. (A) DAPI, (B) hybridization signal of 5S rDNA, (C) hybridization signal of 18S rDNA. Note the co-localization of 18S and 5S rDNA. Chromosomes are counterstained with DAPI and the group of images (A, B and C) is the same metaphases submitted to two-colour FISH. Bar=10µm.

sequences of *P. volitans*, and hence excluding the possibility of a misidentification of *P. miles*.

Thus far, cytogenetic studies of the genus *Pterois* only cover three species indicating for *P. volitans* identical diploid number of chromosomes with differing number of arms. Caputo et al. (2003) reported for this species a karyotype, $2n=48$, $2m+10st+36a$, that differs from the karyotype described here as $2n=48$, $2m+10sm+36a$. In some cases, differences in karyotype formula reported by different authors may derive from technical artifacts or

Some metaphase plates showed association by a heteropycnotic tips on the short arm of these chromosome explaining why some interphase cells shows two nucleoli whereas others display only one.

Constitutive heterochromatin was identified only in four homologues in Javanese *P. volitans*, located at centromeric and interstitial positions (Caputo *et al.*, 2003), whereas in the samples studied here almost all chromosomes exhibited C-positive blocks. Indeed only three pairs of chromosomes were negative for

C-banding with all remaining ones presenting telomeric and some interstitial C-positive blocks and, in some cases, interstitials. Only two chromosomes showed pericentromeric heterochromatic regions. Since the presence of interstitial C-heterochromatin would reveal the occurrence of chromosomal rearrangements by pericentric inversions (Zenzes & Voiculescu, 1975), chromosome microrearrangements involving heterochromatin seem to have occurred during the spread of *P. volitans* which is consistent with the significant molecular differentiation of lionfish reported by Betancur et al. (2011) between the Northern (US east coast, the Bahamas and Bermuda) and the Caribbean populations of *P. volitans* of the Western Atlantic.

Extensive mapping of the 45S and 5S rDNA in fish has revealed a non-association of the two classes of ribosomal genes in fish genomes, with most species having these sequences on different chromosomes (Cioffi & Bertollo, 2012). Co-localization of 5S rDNA and 45S rDNA could facilitate translocations between the 5S and 45S cluster, causing disruptive interference in the structure and function of such genes (Nirchio & Oliveira, 2006) and, therefore, possibly explain why the majority of vertebrates have the 5S and 45S rDNA clusters on different chromosomes (Martinez et al., 1996; Fujiwara et al. 1998; Ferro et al., 2001) this arrangement being evolutionarily advantageous. The presence of clusters of these sequences either very close to or far apart from each other on the same chromosome has already been reported in teleosts including *Salmo salar* (Pendás et al., 1994), *Oncorhynchus mykiss* (Morán et al., 1996), *Acheilognathus tabira* subsp. 1, *Cyprinus carpio* (Inafuku et al., 2000), *Rhomboplites aurorubens* (Nirchio et al., 2009) and *Mugil incilis* (Hett et al., 2011). Although the terminal localization of single major ribosomal genes on the short arm of a pair of chromosomes seems to be a shared feature among the species of lionfish cytogenetically studied until now (Caputo et al., 2003; this paper), reports on co-localization of 18S and 5S ribosomal genes in *P. volitans*, as revealed by the multi-colour

FISH are restricted to this study. Thus, further investigations of 5S and 45S ribosomal genes in other *Pterois* species are required to provide a more throughout analysis of the evolution of these repetitive sequences in the genome of this fish genus.

ACKNOWLEDGMENTS

This work was supported by Consejo de Investigación, Universidad de Oriente, Venezuela, and by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, to CO.

RESUMEN

Cariotipo de la especie invasora *Pterois volitans* (Scorpaeniformes: Scorpaenidae) de Isla Margarita Island, Venezuela. El género *Pterois* contiene nueve especies válidas, nativas del Mar Rojo y el Océano Índico en el Pacífico occidental. *P. volitans* y *P. miles* son nativas del Indo-Pacífico, y fueron introducidas en las aguas de Florida como resultado de la liberación de peces confinados en acuario y han sido reconocidas recientemente como invasoras en el Atlántico Occidental y Mar Caribe (Costa Rica hasta Venezuela). Los estudios citogenéticos realizados hasta ahora en el género *Pterois* cubren solamente aspectos básicos de tres especies que incluyen a *P. volitans* del océano Indo-Pacífico. Debido a la ausencia de información detallada sobre las características cromosómicas de esta especie invasora, el objetivo del presente estudio fue investigar las características citogenéticas en ejemplares de Venezuela mediante técnicas convencionales y moleculares y comparar los resultados con los reportados para el área de distribución original. Para ello, se investigaron las características cariotípicas mediante tinción con Giemsa, bandedo-C, impregnación con Nitrato de Plata (Ag-NOR) e hibridación fluorescente *in situ* (FISH) dual para localizar los genes ribosomales 18S rDNA y 5S rDNA en cuatro ejemplares de pez león capturados en la Isla Margarita, Venezuela. La comparación de secuencias del gen 16S de los especímenes analizados con secuencias ya incluidas en el Genbank permitieron corroborar la identificación de *P. volitans* excluyendo así la posibilidad de una identificación errónea de *P. miles*. El número diploide fue $2n=48$ ($2m+10sm+36a$) con un $FN=60$. Los cromosomas presentaron tamaños que disminuyen de manera uniforme dificultando la identificación de homólogos, excepto el único par metacéntrico y el par cromosómico número 2. El bandedo-C reveló tres pares de cromosomas bandas-C negativos, mientras que los restantes presentaron bloques

bandas-C positivos en posición telomérica y, en algunos casos, intersticial. Sólo dos cromosomas mostraron bandas-C pericentroméricas. La tinción secuencial reveló las Ag-NOR localizadas en los extremos de los brazos cortos del par número dos y el ensayo FISH demostró que los genes 18S rDNA y 5S rDNA se localizan en ese mismo par. Se discute la co-localización de los genes 5S rDNA y 18S rDNA. La distribución de la heterocromatina constitutiva y localización de las NORs en los peces examinados difirió de la reportada para ejemplares de *P. volitans* del Océano Índico (Java), sugiriendo que durante la propagación de *P. volitans* han ocurrido reorganizaciones cromosómicas que involucran la heterocromatina.

Palabras clave: cariotipo, genes ribosomales 18S and 5S, genes mitocondriales 16S rARN, identificación molecular, especies invasoras.

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