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Karyotyping and Molecular Sexing in *Brycinus nurse* (Characiformes, Alestidae) from Nile River in Egypt

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ABSTRACT

The present study aimed to investigate the karyotype and molecular sex differences in *Brycinus nurse*. Analysis of 430 metaphases spreads from 12 specimens revealed that the diploid chromosome number is 44 chromosomes and the proposed karyotype formula is n = 3 M + 3 SM + 16 A, FN = 56. No morphologically differentiated heteromorphic sex chromosomes were observed. On the other hand, 29 primers of three molecular markers (RAPD, SCoT and ISSR) were tested to differentiate between males and females of *B. nurse*. From all tested primers only two primers (OPI-18 and SCoT-18) successfully generated female specific bands in the bulked DNA samples which further confirmed using individual DNA samples. According to molecular analysis result in this study it seems that females of *B. nurse* are the heterogametic gender (ZW) and males of *B. nurse* are the homogametic gender (ZZ). In the future, SCoT marker can be used as suitable and powerful marker in genetic analysis studies in fish.

INTRODUCTION

The family of Alestidae includes ecologically and morphologically various fishes allocated in 19 genera with about 117 species exclusively found in fresh water in Africa (Nelson *et al.,* 2016 and Fricke *et al.,* 2019). It was formerly included as a subfamily of the Characidae and later was classified as a separated family named Alestidae (Zamba and Vreven, 2008).

Despite several studies investigated the relatedness among Alestidae members or between Alestidae and some other Characiformes families using morphological and molecular analysis, phylogenetic relationships and the monophyly for the studied fish groups are still not precisely determined and debated (Arroyave and Stiassny, 2011; Oliveira *et al.,* 2011; Arroyave *et al.,* 2013 and Betancur *et al.,* 2019). However, inclusion of karyological data analysis in this kind of studies would provide valuable features to solve the taxonomic identification problems (Gabriela *et al.,* 2013). The big obstacle to achieve that is the lack of karyotypic data for several Characiformes groups including Alestidae which impairs the comparative analysis among them at the chromosomal level (Carvalho *et al.,* 2017).

On the other hand, sex chromosomes in fishes play a significant role in evolution and speciation processes (Kitano and Peichel, 2012) and its identification provides valuable information about the sex determination system. However it is difficult to distinguish between the sex chromosomes and autosomes of almost majority fish species based only on their size and shape (De Rosa *et al.,* 2017). Interestingly, in the past few years, many molecular markers have been developed to investigate sex-specific molecular markers in fishes and many other species such as random amplified polymorphic DNA (RAPD) (Durna, 2009; Xia *et al.,* 2011; Silva *et al.,* 2012 and Al-Qurainy *et al.,* 2018), start codon targeted (SCoT) (Mohamed and Sami, 2015) and inter simple sequence repeat (ISSR) (Wuertz *et al.,* 2006 and Adhikari *et al.,* 2014). Identification of sex specific DNA markers in fishes is very useful for investigating sex determination system, identifying sex chromosomes and sex-related genes. Also it could serve in hatchery management (Durna, 2009) and determining the effect of environmental factors on sex differentiation (De Rosa *et al.,* 2017).

In Egypt about nine species of Alestidae family are extant including *B. nurse*. This species is locally distributed in southern region of Lake Nasser and Upper Egyptian Nile and consider one of the marketable fishes of family Alestidae and used in salted fish industry (Bishai and Khalil, 1997). To date, no information is available about the karyotype or sex determination system in this species or the majority species of Alestidae family. So, the present study aimed to analyze the chromosome feature and study the molecular sex differences between males and females of *B. nurse* using RAPD, SCoT and ISSR markers.

MATERIALS AND METHODS

Specimens collection:

A total of 34 live adult specimens of *B. nurse* (17 males and 17 females) were collected from River Nile at Assiut city, Egypt. The collected specimens were transferred to the laboratory for analysis where 12 specimens (6 males and 6 females) were used for cytogenetic analysis and 22 specimens (11 males and 11 females) were used for molecular analysis.

Cytogenetic analysis:

Mitotic chromosomes were obtained from intestine and kidney tissues. Briefly, the specimens were injected intraperitoneally with 0.1% colchicine at a dose of 20 ul/g body weight for about three hours. The hypotonization was made in 0.075 M KCl for 30 minutes at room temperature. Then tissues fixation was made in cold and freshly prepared 3:1 (methanol: acetic acid) for 24 hours. The slides were prepared according to the method of solid tissues technique (Kligerman and bloom, 1977) and stained with 10% Giemsa for 20 minutes. Chromosomes images were captured under an Olympus BH-2 microscope (Japan) using CCD camera. Chromosome types were determined according to the description of Levan *et al.,* (1964) and classified into: metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A). The fundamental number (NF) or number of chromosome arms was determined as following: M and SM chromosomes were considered as biarmed and those of ST and A chromosomes were considered as uniarmed.

Molecular analysis:

For each examined specimen, about 1 cm^2 from the caudal fin was cut and stored in absolute ethanol at -20 C^O until used. Genomic DNA was isolated from the caudal fin tissues according to the standard protocol described by Muhammad *et al*., (2016). The concentration and quality of the genomic DNA were checked by spectrophotometer and agarose gel electrophoresis, respectively. Equal amount of genomic DNA isolated from five specimens were mixed to prepare the bulked DNA sample for each gender.

RAPD was performed according to the procedures described by Williams *et al.,* (1990), SCoT was carried out as previously described by Collard and Mackill, (2009) and ISSR was done according to Zhigileva *et al.,* (2013). Nine decameric RAPD primers (Operon Technologies Inc., USA),

ten ISSR primers (Bioneer, Inc., South Korea) and ten SCoT primers (Bioneer, Inc., Korea) were randomly selected and used in the present study to investigate the molecular sex differences between males and females of *B. nurse* (Table 1). PCR products of RAPD and SCoT were separated on 1.5% agarose gel, while those of ISSR was separated on 2 % agarose gel, then stained with ethidium bromide and visualized by UV transilluminator, and only the sharp bands were considered for analysis. Interestingly some primers could successfully generate possible female specific bands after screening the bulked DNA samples, and to validate that, these primers were used further with individual DNA samples from a new group of specimens (6 males and 6 females) which allowing to increase the number of individuals tested to increase the accuracy. In brief, by this way when the specific band is present in the bulked and individual DNA samples from females and when this specific band is absent in the bulked and individual DNA samples from males it means that this band could be used as a specific molecular marker to distinguish females.

Table 1. List of RAPD, SCoT and ISSR primers codes and sequences used for molecular analysis.

RAPD Primers			SCoT Primers		ISSR Primers	
No.	**Code**	**Sequence (5′–3′)**	**Code**	**Sequence (5′–3′)**	**Code**	**Sequence (5′–3′)**
1	OPA-02	TGCCGAGCTG	SCOT 01	CAACAATGGCTACCACCA	UBC 807	$(AG)_8T$
2	OPA-03	AGTCAGCCAC	SCOT 02	CAACAATGGCTACCACCC	UBC 808	$(AG)_8C$
3	OPA-05	AGGGGTCTTG	SCOT 16	ACCATGGCTACCACCGAC	UBC 810	$(GA)_8T$
4	OPA-09	GGGTAACGCC	SCOT 18	ACCATGGCTACCACCGCC	UBC 811	$(GA)_8C$
5	OPA-11	CAATCGCCGT	SCOT 22	AACCATGGCTACCACCAC	UBC 812	$(GA)_8A$
6	OPE-04	GTGACATGCC	SCOT 28	CCATGGCTACCACCGCCA	UBC 814	$(CT)_8A$
7	OPI-18	TGCCCAGCCT	SCOT 32	CCATGGCTACCACCGCCT	UBC 815	$(CT)_8G$
8	OPW-05	GGCGGATAAG	SCOT 34	ACCATGGCTACCACCGCA	UBC 840	$(GA)_8YT$
9	OPY-04	GGCTGCAATG	SCOT 35	CATGGCTACCACCGGCCC	UBC 846	$(CA)_8C$
10	--	------	SCOT 36	GCAACAATGGCTACCACC	UBC 873	$(GACA)_4$

Y = (C, T)

RESULTS AND DISCUSSION

Results

A total of 430 metaphases spreads from six males and six females with at least 30 metaphases spreads from each specimen were examined. The count of chromosomes in the examined metaphase spreads ranged from 41 (4.65%) to 45 (2.56 %) with the majority of cells with 44 chromosomes (79.77 %) as illustrated in Table 2. Karyotypic arrangement revealed three pairs of metacentric chromosomes, three pairs of submetacentric chromosomes, sixteen pairs of acrocentric chromosomes and FN was 56 (Figure 1). No morphologically differentiated heteromorphic sex chromosomes were detected where both males and females show the same diploid chromosome number (2n = 44) with no morphological differences between each chromosomes pairs. The proposed karyotype formula for *B. nurse* is n = 3 M + 3 SM + 16 A, FN=56.

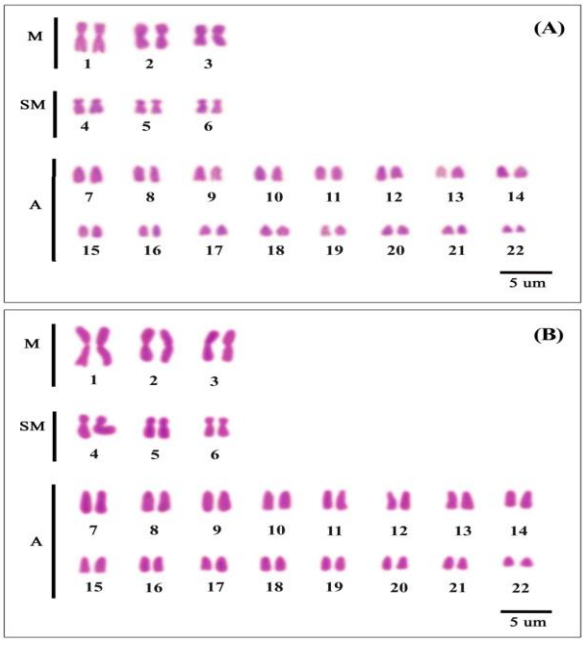

Figure 1. Karyograms of male (A) and female (B) of *B. nurse*.

All 29 primers tested successfully generated number of bands with DNA samples from males and females. The total number of generated bands by RAPD primers was 75 bands, where primer OPE-04 produced the highest number of bands (13 bands) and primer OPA-11 produced the lowest number of bands (4 bands). Among nine tested RAPD primers only one primer OPI-18 successfully generated one possible female specific band (660 pb) with the bulk DNA sample. Interestingly, when this primer was used further with individual DNA samples from males and females, it generated the same specific band in all individual DNA samples from the females only (Figure 2). In addition, the total number of generated bands by SCoT primers was 99 bands, where primer SCoT-18 produced the highest number of bands (18 bands) and primers SCoT-02 and SCoT-16 produced the lowest number of bands (3 bands). Out of ten SCoT primers tested only two primers (SCoT-16 and Scot-18) could generate possible female specific bands (1150 bp and 720 bp, respectively) with the bulk DNA samples. However, when these two primers were used further with the individual DNA samples from males and females only one primer (SCoT-18) produced female specific band (Figure 3) which was generated in all female individuals tested and disappeared in all male individuals tested. Otherwise, the other primer (SCoT-16) which generated a polymorphic band in the bulked DNA samples from females when used further with the individual DNA samples from males and females could generate this polymorphic band in one male out of six males tested and two female out of six females tested individually. It appears that this band is not sex specific and may result from the variation between the individuals. On the other hand, the total number of generated bands by ISSR primers was 53 bands, where primer UBC-815 produced the highest number of bands (9 bands) and primers UBC-807, UBC-814, UBC-846 and UBC-873 produced the lowest number of bands (3 bands). All of the ten ISSR primers tested failed to generate any sex-specific band (Figure 4).

Table 2. Diploid chromosome number distribution in *B. nurse*.

Sex	Number of fishes examined	Diploid chromosome number					Total number of examined cells
		41	42	43	44	45	
Male	6	10	11	15	171	6	213
Female	6	10	13	17	172	5	217
Total	12	20	24	32	343	11	430
%		4.65 %	5.58 %	7.44 %	79.77 %	2.56 %	100 %

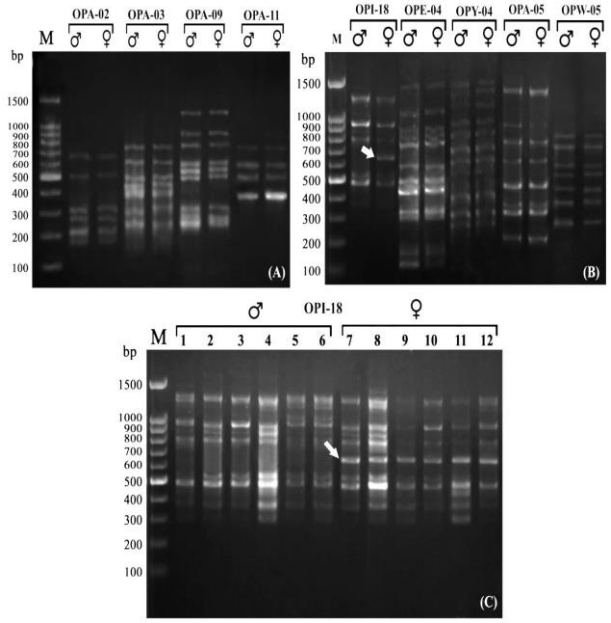

Figure 2. Banding profile pattern of males and females of *B. nurse* generated by primers of RAPD marker; (A) Primers OPA-02, OPA-03, OPA-09 and OPA-11 from bulk samples, (B) Primers OPI-18, OPE-4, OPY-04, OPA-05 and OPW-05 from bulk samples and (C) Primer OPI-18 from individual samples.

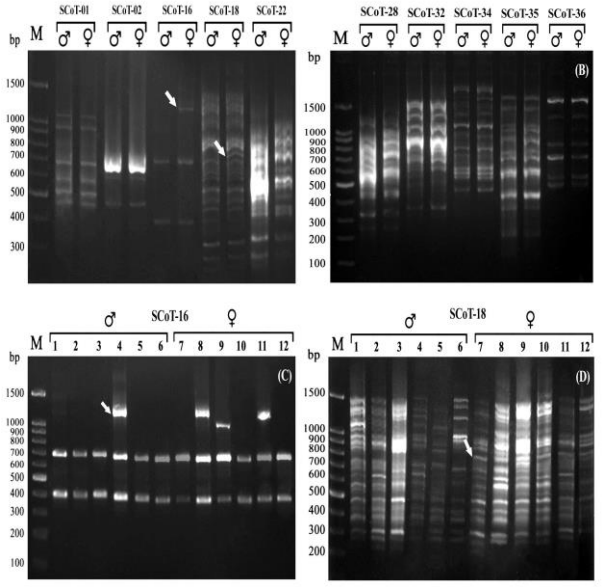

Figure 3. Banding profile pattern of males and females of *B. nurse* generated by primers of SCoT marker; (A) Primers SCoT-01, SCoT-02, SCoT-16, SCoT-18 and SCoT-22 from bulk samples, (B) Primers SCoT-28, SCoT-32, SCoT-34, SCoT-35 and SCoT-36 from bulk samples, (C) and (D) Primers SCoT-16 and SCoT-18 from individual samples, respectively.

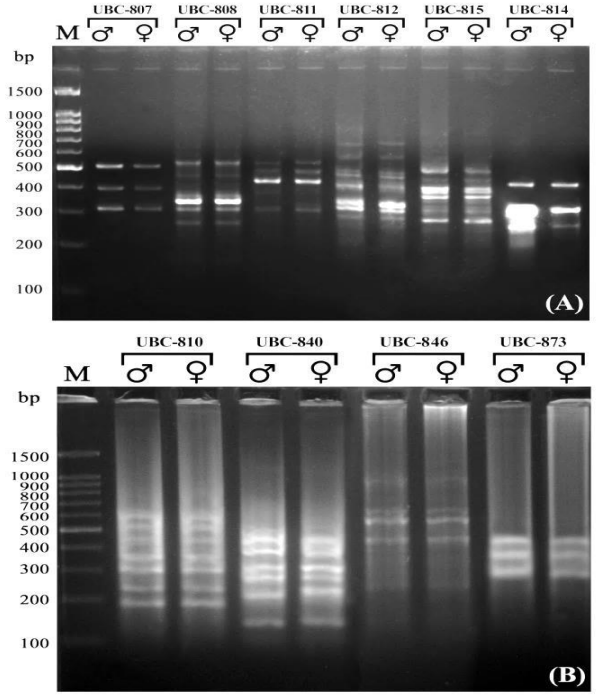

Figure 4. Banding profile pattern of males and females of *B. nurse* generated by primers of ISSR marker; (A) Primers UBC-807, UBC-808, UBC-811, UBC-812, UBC-815 and UBC-814 from bulk samples, (B) Primers UBC-810, UBC-840, UBC-846and UBC-873 from bulk samples.

Discussion

The lack of karyological data and genetic makeup information for several fish groups impair any evolutionary and taxonomic comparative studies at chromosomal and molecular levels and this is the case for family Alestidae. To date, only limited number of species was studied at chromosomal level with no information about sex chromosomes or sex determination related genes in the members of this family.

Herein, to extend the karyological database of family Alestidae, the conventional karyotype of *B. nurse* was investigated for the first time where both males and females showed the same diploid chromosome number (2n = 44) and the same fundamental number (FN = 56). The diploid chromosome number and FN in *B. nurse* presented in the present study are differing from those observed in two species of the same genus namely *Brycinus longipinnis* (2n = 48) (Post, 1965) and *Brycinus macrolepidotus* (2n = 54 and FN = 76) (Krysanov and Golubtsov, 2014). Also, it was different than those of Arnoldichthys spilopterus in genus *Arnoldichthys* in the same family (2n = 56) (Hinegardner and Rosen, 1972). In addition, the diploid chromosome number and FN in *B. nurse* were different than those of Hepsetus odoe (2n = 58 and FN = 96); a member of family Hepsetidae which is very close to family Alestiade (Carvalho *et al.*, 2017). All these species identified in family Alestidae with variable diploid chromosome number ranged from 44 in *B. nurse* to 56 in Arnoldichthys spilopterus are showing variable morphometric

and meristic traits. This variability in fish characteristics which highlighted with karyotype variability is a distinguish mark for many Characiformes groups (Carvalho *et al.,* 2017). It seems that the information about karyological data and its inclusion with the morphological and molecular data will securely improve our knowledge about the relatedness among Alestidae members or even between Alestidae and the other Characiformes families. In the present study, we used Gimesa staining for conventional karyotype analysis which is still the simple, fast and considerable method for identification of fishes chromosomes number and formula, however using banding techniques and molecular cytogenetics tools is required in future studies.

On the other hand, in the present study no numerical or morphological differences were observed between the chromosomes sets of males and females of *B. nurse* suggesting no evidence of morphologically differentiated heteromorphic sex chromosomes and this is the case of Alestidae species which have been mentioned earlier and many other Characiformes species (Scacchetti *et al.,* 2015; Carvalho *et al.,* 2017 and Lourenço de Freitas *et al.,* 2018). However, there are many molecular markers such as RAPD, SCoT and ISSR were developed to differentiate between males and females at molecular level in many different organisms including fish. These molecular markers are valuable especially in species that lack morphologically differentiated heteromorphic sex chromosomes.

The first molecular marker used in the present study was RAPD marker which has been used extensively in molecular sexing studies in fishes and was able to generate female (Xia *et al.,* 2011 and Silva *et al.,* 2012) or male (Kovacs *et al.,* 2001) specific bands in many fish species but in other fish species it failed to detect any sex-specific markers (Durna, 2009). In the present study, one primer (OPI-18) out of nine RAPD primes tested successfully generated a female specific band (660 bp) in the bulked DNA sample from females specimens which confirmed using individual DNA samples. The second marker was SCoT marker which developed by Collard and Mackill (2009) for genetic analysis and generating gene-targeted markers in plants. However, SCoT was used recently to study genetic diversity in other organisms such as fish (Marie and Allam, 2017) and camel (Al-Soudy *et al.,* 2018). In addition, it was also used as a novel gene targeting marker in sex-determination in date palm (Mohamed and Sami, 2015) with no available reports about using it in molecular sexing studies in animals. Herein SCoT marker was used for the first time to investigate the molecular sex differences in fish and among ten primers tested to differentiate between males and females of *B. nurse* only one primer (SCoT-18) tested successfully generated a female specific band (720 bp) in the bulked DNA sample from females specimens which confirmed using individual DNA samples. The third marker was ISSR which has been proved to be a reliable technique in gender determination in plants (Adhikari *et al.,* 2014 and Sarmah *et al.,* 2017). However ISSR marker failed previously in detecting sex-specific marker in fishes such as sturgeon species (Wuertz *et al.,* 2006), and also in the present study it failed to detect any sex specific marker in *B. nurse*.

Identification of sex determination system in fishes is required when studying any fish group to be used for academic or applied research. According to the molecular analysis results in this study it seems that females are the heterogametic gender (ZW) and males are the homogametic gender (ZZ) and ZZ/ZW system is the supposed system for sex-determination in *B. nurse*. Supporting this idea is the observation that ZZ/ZW sex determination system is the most common system in fish (Smirnov and Trukhina, 2019), furthermore many species of Characiformes exhibit homeologous ZZ/ZW sex chromosomes in different stages of differentiation (Scacchetti *et al.,* 2015). However further studies using banding techniques and molecular cytogenetic analysis are needed to confirm this idea.

Finally, according to our findings in the present study the diploid chromosome number of *B. nurse* is 44 chromosomes and FN is 56, with no morphologically differentiated heteromorphic sex chromosomes. Both RAPD and SCoT were effective markers to discriminate between males and females of *B. nurse* at molecular level and successfully generated female specific bands. This suggesting that the sex determination system in *B. nurse* is ZZ/ZW system. Also, SCoT is a suitable and potent marker to be used in fish genetic analysis studies in the future.

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التوصيف الكروموسومي والفروق الجزيئيه بين الجنسين في سمك الراي من نهر النيل في مصر

السيد عبد المنصف محمد ، نجلاء نجيب ظهري ، جمال إبراهيم أحمد و رأفت فؤاد عبده

قسم الوراثه – كليه الزراعه – جامعه أسيوط – أسيوط - مصر

تهدف الدراسه الحاليه الي التعرف علي التوصيف الكروموسومي والإختلافات الجزيئيه بين الجنسين في سمك الراي (*Brycinus nurse*) .أظهر التحليل الكروموسومي لـ 430 ميتافيز من 12 فرد أن العدد الكروموسومي الثنائي هو 42 كروموسوم وأن الهيئه الكروموسوميه هي: (n = 3 M + 3 SM + 16 A, FN = 56) ولم يلاحظ وجود أي كروموسومات جنس مميزه مورفولوجيا. وعلي الجانب الآخر أظهر كل من: أحد بادئات الـ RAPD التسعه المستخدمه (OPI-18) وكذلك أحد بادئات الـ SCoT العشره المستخدمه (SCoT-18) حزمه واحده متخصصه للإناث. ولم ينجح أي من بادئات واسم الـ ISSR العشره المستخدمه في إظهار أي حزمه متخصصه للجنس. وطبقا لنتائج التحليل الجزيئي لهذه الدراسه فإن الإناث في سمك الراي هي الجنس مختلف الجاميطات (ZW) وأن الذكور هي الجنس متماثل الجاميطات (ZZ). واسم الـ SCoT هو واسم قوي وفعال ويمكن أن يستخدم مستقبلا في دراسات التحليل الوراثي في الأسماك.