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Chromosomal Analysis and NORs Polymorphism of Bagarius SUCHUS (Siluriformes: Sisoridae) by Conventional Banding and FISH Techniques

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Abstract

In the present study, conventional staining and NORs banding as well as Fluorescence in situ hybridization (FISH) using the 18S rDNA and telomeric (TTAGGG)n probes were applied to stain the chromosomes of crocodile catfish, Bagariussuchus (Siluriformes, Sisoridae) from the Chao Praya River, Thailand. Kidney cells of 6 male and 6 female crocodile catfishes were used as a sample. The mitotic chromosome preparations were done directly from kidney cells. The results showed that the diploid chromosome number of B. suchus was 2n=56, the fundamental numbers (NF) were 102 in both male and female. The karyotype comprises 17m+17sm+12a+10t. The nucleolar organizer regions (NORs) were detected by Ag-NORs banding and 18S rDNA probe mapping. The 18S rDNA are terminally located on the short arm adjacent to the telomere of the single pair of the 1st chromosome pair whereas NOR-bearing chromosome is only one chromosome of the 1st chromosome pair (1a 1b, polymorphic characteristic) at the subtelomeric region of the short arm. Moreover, FISH with telomeric probe showed hybridization signals on each telomere of all chromosomes and interstitial telomeric sites were not detected. There were variations in signals of FISH and their position in the karyotype along with variation in DNA sequences. These markers are useful for future discrimination of population of closely related species and their polymorphism.
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Keywords: Bagariussuchus, Chromosome, Conventional staining, Ag-NORs banding, Fluorescence in situ hybridization.

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Introduction

The order Siluriformescomprises 37 recognized families of catfish that are widely distributed and highly diversified in freshwaters (Sullivan et al., 2006).The catfishes of the family Sisoridaeare also the most widely distributed occurring throughout nearly the whole of South and Southeast Asia, from Iran and Turkey in the west (Coad, 1981; Coad &Delmastro, 1985). They contain 22 genera and approximately 168 species (Ferraris, 2007), with new species being discovered frequently (Kottelat, 1983; Mo & Chu, 1986; Ding et al., 1991; Zhou & Chu, 1992). In Thailand, six genera and 18 species were described (Vidthayanon et al., 1997).

Cytogenetic studies in many organisms are quit scarce, in which only conventional technique reported to determine chromosome number and karyotype composition has been performed. Structure, number, and morphology of a nucleolar organizer region (NOR) may be specific to populations, species and subspecies. NOR is frequently used to compare variations, as well as to identify and explain specifications. Changes in chromosome number and structure can alter the number and structure of NOR. Robertsonian translocations may cause losses of NOR. Species which have limited gene exchange due to geographical isolation have elevated karyotype and NOR variety. Therefore, different karyotypes are found even in smalland isolated populations of these species. The use of NORs in explaining kinships depends on a large extent on the uniformity of this characteristic and on the degree of variety within a taxon (Yüksel&Gaffaroğlu, 2008). Very little known concerning its karyological features have been widely accessed by classical methods, and advances in molecular cytogenetics based in Fluorescence *in situ* hybridization (FISH) experiments have resulted in improved chromosomal mapping of large number of sequences and permitted the study of chromosomal variation.

Accordingly, the goal of this work is finding of NOR polymorphism and chromosomal analysis of the *B.suchus* from Thailand byusing different staining methods and FISH technique to provide cytotaxonomic information for the understanding of the chromosomal mapping of the Sisoridae family.

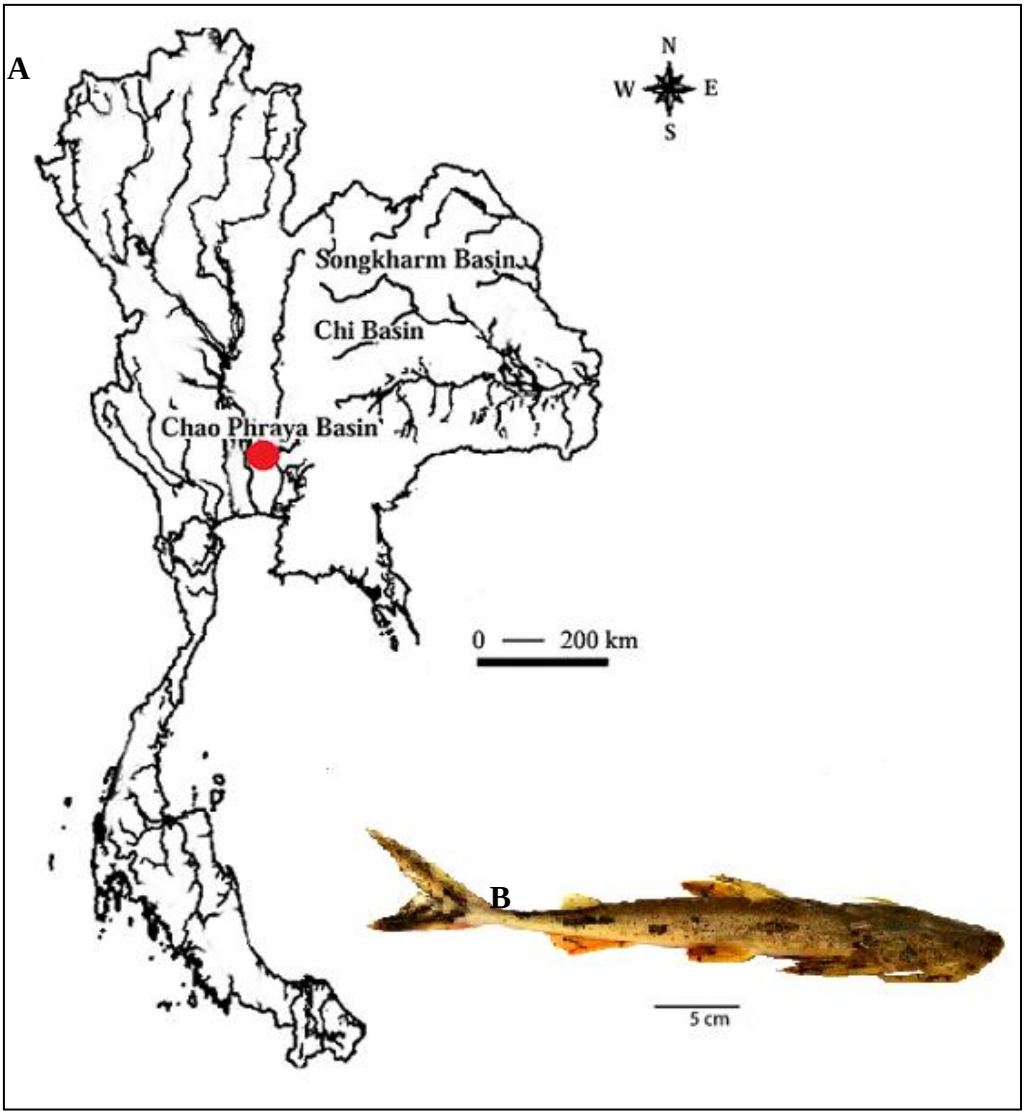

Figure 1:Collection sites of *Bagariussuchus*. Map of Thailand indicating the collection sites (A) andan individual of the *Bagariussuchus*(B).

Review Of Literature

Cytogenetics of the family Sisoridae is scarcely studied. In the genus Bagarius, three species were studied includingB. suchus, B. bagarius and B. yarrelliby Rangsiruji et al. (2007), using conventional staining method. The results showed that all of them display the same 2n (56 chromosomes). Their respective NF were 88, 82and 90. The karyotypes comprise 16m+16sm+4st+20a, 16m+10sm+2st+28a and 14m+20sm+6st+16a, respectively. Moreover, some species in other genera in this family (12 reports) have 2n in the range of 36-62 chromosomes and NF ranges between 66 to 104 (review in Arai, 2011) Table 2, and molecular cytogenetics techniques have never been applied on these species.In Thailand, there were few molecular cytogenetic studies accomplished by using FISH technique. Up to date, there are few reports on Thai catfish using FISH technique i.e. Supiwong et al. (2013)

which demonstrated the nine classes of microsatellite repeats on the chromosomes of hi fin Mystus, Mystusbocourti(family Bagridae). The U2 snRNA, 5S and 18S rDNA were presented in only one chromosomepair but none of them presented in a syntenic position. Microsatellites $(CA)_{15}$ and $(GA)_{15}$ showed hybridization signals at subtelomeric regions of all chromosomeswith a stronger accumulation into one specific chromosomal pair. FISH with the telomeric probe revealed hybridization signals on each telomere of all chromosomes and interstitial telomeric sites (ITS) were not detected. In addition, the retrotransposable elements Rex1, 3 and 6 were generally spread throughout the genome.Moreover, the report ofSupiwong et al. (2014) showed the distributions in same family of nine species, i.e., Hemibagrusfilamentus; H. nemurus; H. wyckioides; Mystusatrifasciatus; M. multiradiatus; M. mysticetus; M. bocourti and Pseudomystussiamensis. Two classes of microsatellites; $(CA)_{15}$,$(GA)_{15}$ and one transposable element (TE); Rex1 were mapped by fluorescence in situ hybridization. In all species the microsatellites are abundantly distributed in all chromosomes, usually in the telomeric regions. The retrotransposable element Rex1 is widely distributed over the whole genome including heterochromatin and euchromatin, but with an unexpected accumulation in one chromosome pair in some species.

Research Methods

Biological Material and Chromosome preparation

The specimens of both sexes' crocodile catfish,*B.suchus*(6 males and 6 females) were collected from the Chao Phraya River (Fig. 1),using accidental sampling method by hook. The fishwere transferred to laboratory aquaria and were kept under standard conditions for seven days prior to the experiments. The experiments followed ethical protocols, and anesthesia with clove oil was administered prior to sacrificing the animals to minimize suffering. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method (Chen &Ebeling, 1968; Nanda et al., 1995). The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science, KhonKaen University.

Giemsa's staining, Ag-NORs banding and karyotype

The chromosomes were conventionally stained with 20% Giemsa's solution for 30 minutes (Rooney, 2001). Ag-NOR banding, drops of each 50% silver nitrate and 2% gelatin were added on slides, respectively. Then it was sealed with cover glasses and incubated at 60°Cfor5 minutes.After that it was soaked indistilled water until the cover glasses were separated (Howell & Black, 1980). Approximately 30 metaphase spreads were analyzed per specimen to confirm the diploid chromosome number and karyotype structure. Metaphases were photographed under Olympus Bx50 microscope (Olympus Corporation, Ishikawa, Japan). The chromosomes were measured and the centromere index (CI), relative length (RL), and centromere ratio (CR) were calculated.Idiograming is the diagram of chromosomal karyotype of haploid set which includes autosomes and sex-chromosome. The data of average chromosomal length, chromosome type and the position of centromere were used for idiograming construction. To construct idiogram, the 30 metaphase cells from conventional staining were used in karyotyping and then all chromosomes were measured for individual

length of both short arm and long arm by vernier calipers. A graph of an average length of each chromosome pair was plotted using Microsoft Word.

Chromosome probes and FISH technique

The 18S rDNA probe was direct labeled with Spectrum Orange-dUTP by nick translation according to the manufacture's recommendations (Roche, Mannheim, Germany). Fluorescence *in situ* hybridization (FISH) was performed under high stringency conditions on mitotic chromosome spreads (Pinkel et al., 1986). The metaphase chromosome slides were incubated with RNAse (40 µg/ml) for 1.5 h at 37 °C. After denaturation of chromosomal DNA in 70% formamide/ 2×SSC at 70 °C, spreads were incubated in 2×SSC for 4 min at 70 °C. The hybridization mixture (2.5 ng/µl probes, 2 µg/µl salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37 °C in a moist chamber containing 2×SSC. The post hybridization wash was carried out with 1×SSC for 5 min at 65 °C. A final wash was performed at room temperature in 4×SSCT for 5 min. Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector laboratories).

The detection of the telomeric (TTAGGG)*n* repeats was made with the FITC-labeled PNA probe (DAKO, Telomere PNA FISH Kit/FITC, Cat. No. K5325) and performed according to manufacturer's recommendations.

Results

The diploid number (2*n*) of *B. suchus*was 56chromosomesand the fundamental number (NF) was 102 in both sexes (Fig. 2).The karyotpewas composed of17m+17sm+12a+10t.A summary of the results obtained after measuring the chromosomes of 30 complete metaphase plates is presented in Table 1.The analysis of the NORs with the Ag-NOR banding technique sequential to Giemsa's staining, detected that the Ag-positive signal located on the short arm of one chromosome of the 1st chromosome pair (Figs. 3 and 4 A, B).

The 18S rDNA showed hybridization signals at the short arm adjacent to telomere of the 1st chromosome pair (Fig. 5 C). FISH with telomeric sequences (TTAGGG)*n*were detected the hybridization signals on each telomeric of all chromosomes, and interstitial telomeric sites were not found (Fig. 5 D).

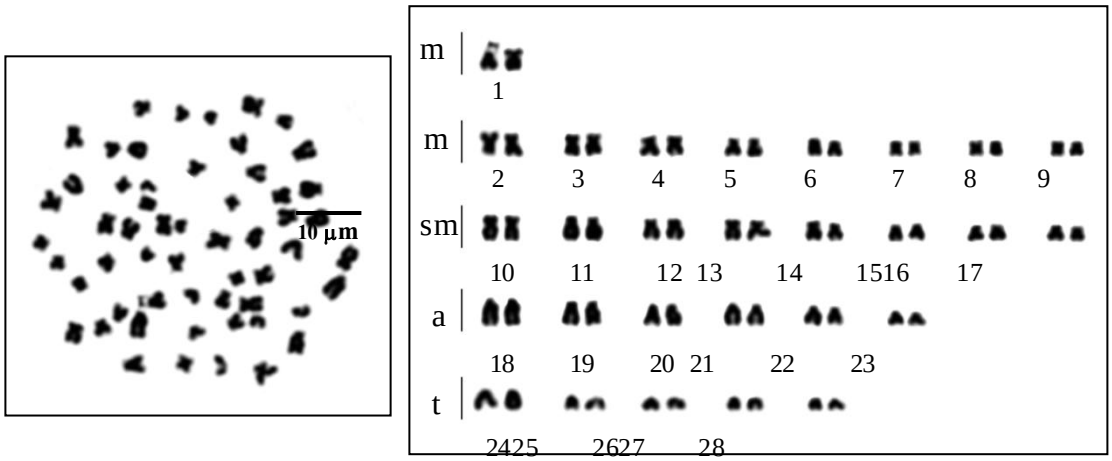

Figure2:Metaphase chromosome plate and karyotype of the crocodile catfish (*Bagariussuchus*), 2*n*=56 by conventional staining.

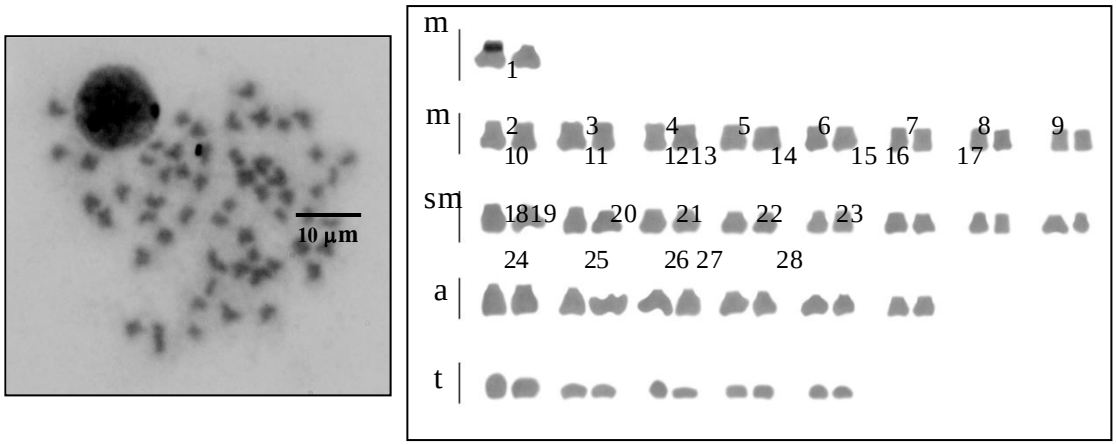

Figure 3:Metaphase chromosome plate and karyotype of the crocodile catfish (*Bagariussuchus*), 2*n*=56 by Ag-NORbanding**.**

Table1. *Mean length of short arm chromosome (Ls); length of long arm chromosome (Ll); length of total arm chromosome (LT); relative length (RL); centromeric index (CI) and standard deviation (SD) of RL and CI from 30 metaphase chromosome plates ofthe crocodile catfish,Bagariussuchus, 2n=56.*

Chromosome pair	**Ls**	**Ll**	**LT**	**RL±SD**	**CI±SD**	**Size**	**Type**
1a*	0.758	1.024	1.782	0.047±0.004	0.575±0.012	L	m
1b	0.522	0.953	1.474	0.039±0.003	0.646±0.027	L	sm
2	0.758	0.950	1.708	0.045±0.002	0.556±0.017	L	m
3	0.697	0.894	1.590	0.042±0.001	0.562±0.012	L	m
4	0.661	0.838	1.499	0.039±0.003	0.559±0.022	L	m

5	0.635	0.783	1.418	0.037±0.004	0.552±0.021	L	m
6	0.574	0.739	1.313	0.034±0.003	0.563±0.026	M	m
7	0.564	0.705	1.269	0.033±0.003	0.556±0.017	M	m
8	0.547	0.655	1.202	0.031±0.003	0.545±0.016	M	m
9	0.503	0.614	1.117	0.029±0.002	0.550±0.013	M	m
10	0.551	1.012	1.563	0.041±0.002	0.648±0.025	L	sm
11	0.496	0.954	1.450	0.038±0.002	0.658±0.031	L	sm
12	0.512	0.883	1.395	0.037±0.002	0.633±0.022	L	sm
13	0.486	0.856	1.342	0.035±0.001	0.638±0.024	L	sm
14	0.472	0.826	1.298	0.034±0.001	0.636±0.023	M	sm
15	0.460	0.746	1.206	0.032±0.001	0.619±0.016	M	sm
16	0.405	0.737	1.142	0.030±0.001	0.645±0.026	M	sm
17	0.378	0.714	1.092	0.029±0.001	0.654±0.022	M	sm
18	0.410	1.353	1.763	0.046±0.001	0.767±0.026	L	a
19	0.358	1.213	1.571	0.041±0.002	0.772±0.044	L	a
20	0.365	1.060	1.425	0.037±0.002	0.744±0.034	L	a
21	0.347	0.987	1.334	0.035±0.002	0.740±0.025	L	a
22	0.318	0.906	1.223	0.032±0.002	0.740±0.026	M	a
23	0.291	0.802	1.093	0.029±0.001	0.734±0.024	M	a
24	0.000	1.323	1.323	0.035±0.002	1.000±0.000	L	t
25	0.000	0.988	0.988	0.026±0.001	1.000±0.000	M	t
26	0.000	0.930	0.930	0.024±0.001	1.000±0.000	M	t
27	0.000	0.874	0.874	0.023±0.001	1.000±0.000	S	t
28	0.000	0.811	0.811	0.021±0.001	1.000±0.000	S	t

Remarks: *= NOR-bearing chromosome (satellite chromosomes), m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome,L = large, M = medium and S = small.

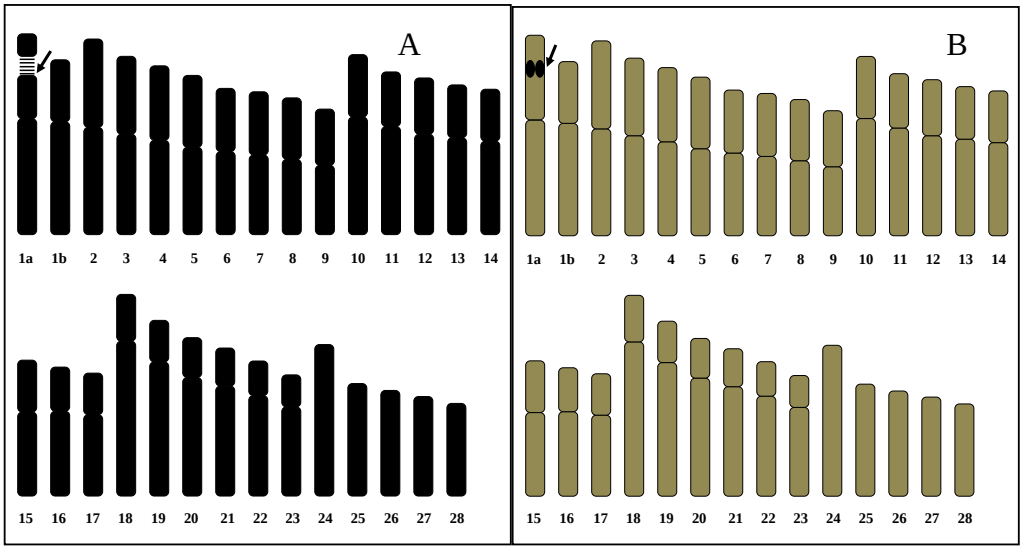

Figure 4.:Idiogram showing lengths and shapes of chromosomes of the crocodile catfish(*Bagariussuchus*), 2*n*=56, by conventional staining (A) and Ag-NOR banding (B). Arrows indicate nucleolar organizer region (NOR).

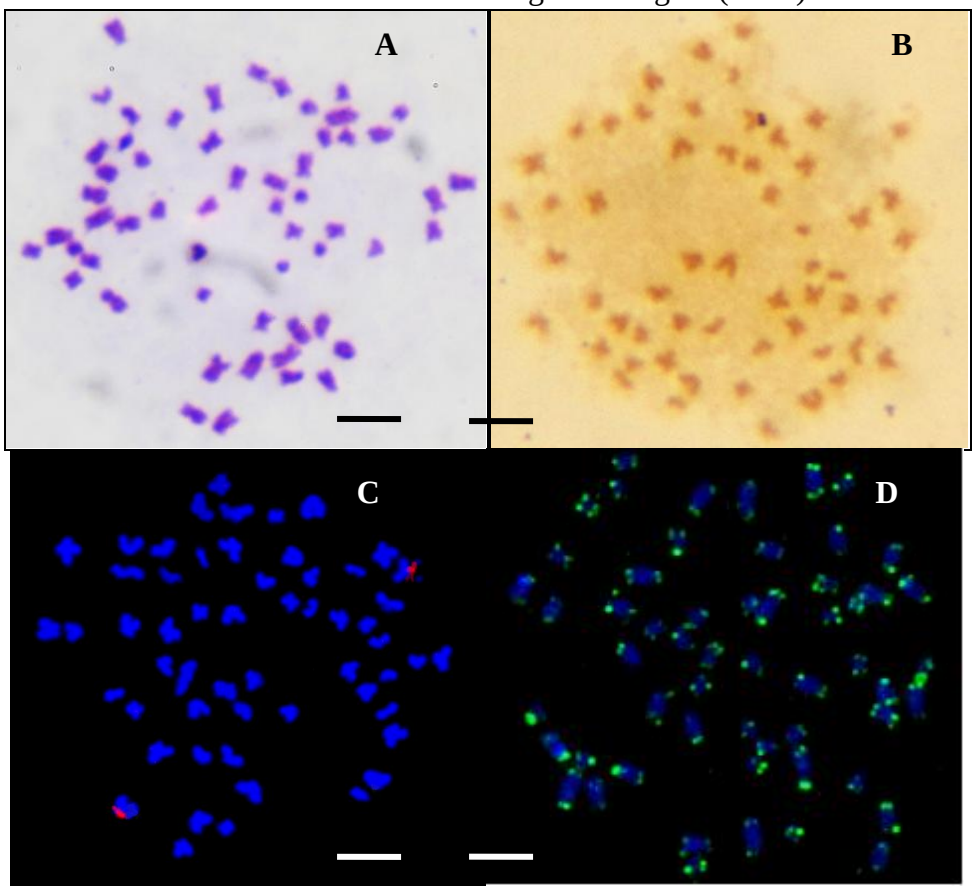

Figure 5:Chromosomal analysis of the *Bagariussuchus* chromosomes. Hybridization of metaphase chromosomes Conventional staining (A), Ag-NORs banding (B), 18S rDNA probe (C) and Telomeric probe (D). Scale bars indicate 5 µm.

Discussion & Conclusions

Discussion

The *B.suchus*had 2*n*=56 which is in accordance with the previous study conducted by Rangsiruji et al. (2007). Such 2*n* is also same as the other species of the genus *Bagarius*(Table 2). However, the NF was 102 and karyotype composed of17m+17sm+12a/st+10t/a chromosomes, which differ from the previous study ofRangsiruji et al. (2007) that reported the karyotype of *B. suchus* consisting of 16m+16sm+4a/st+20t/a chromosomes and NF=88.This fact suggests that some pericentric inversions have occurred in the karyotype differentiation of this species. In fact, the occurrence of chromosomal rearrangements has been considered a relatively common evolutionary mechanism inside the Sisoridae family (reviewed inArai 2011).

Table2.*Cytogenetic Publications Of The FamilySisoridae.*

Species	**2*n***	**NF**	**Karyotype**	**Ag-NORs**	**Locality**	**Reference**
Bagariussuchus	56	88	16m+16sm+4st+20a	-	Thailand	Rangsiruji et al. (2007)
	56	102	17m+17sm+12a+10t	1a	Thailand	The present study
B. bagarius	56	82	16m+10sm+2st+28a	-	Thailand	Rangsiruji et al.
B. yarrelli	56	90	14m+20sm+6st+16a	-	Thailand	(2007)
Euchioglanisdavidi	36	50	8m+6sm+22st/a	-	China	Li et al. (1981)
E.kishinouyei	50	70	14m+6sm+30st/a	-	China	Li et al. (1981)
Gagatacenia	46	66	4m+8Sm+8st+26a	-	India	Mishra (1998)
Glyptosternonreticulatum	42	-	-	-	India	Rishi et al. (1998)
Glyptothoraxfokiensis	52	104	20m+18sm+14st	-	China	Yu et al. (1989)
G. telchitta	56	102	18m+26sm+2st+10a	-	India	Khuda-Bukhsh et al. (1986)
G. glyptothoraxtrilineatus	52	-	18m+24sm+10a	-	India	Khuda-Bukhshet al. (1995)
	62	90	16m+12sm+2st+32a		Thailand	Rangsiruji et al. (2007)
Gogangraviridescens	42	-	14m+20sm+8a	-	India	Khuda-Bukhsh et al. (1995)
	48	86	12m+22sm+4st+10a	-	India	Sharma &Tripathi (1981)
Pseudecheneissulcata	52	-	8m+14sm+30st/a	-	India	Rishi et al. (1998)
	48	86	12m+22sm+4st+10a	-	India	Sharma&Tripathi (1981)

Remarks:2n = diploid number, NF = fundamental number, m = metacentric, sm = submetacentric, st= subtelocentric, a = acrocentric,t = telocentric, NORs = nucleolar organizer regions and - = not available.

The analysis of the NORs with the Ag-NOR banding sequential to Giemsa's staining, detected the Ag-positive signals at the short arm of only one chromosome of the 1st metacentric chromosomepair.This is the first study of NOR bearing chromosome in the family Sisoridae. The NORs are effective cytotaxonomic markers in family Sisoridae and allowed us to distinguish most of the analyzed species, in which the ribosomal sites were similarly located on the same chromosomal pair (chromosome pair 1).

The present study showed that a polymorphism of chromosome is only one chromosome of the 1st chromosome pair (1a 1b). This is in agreement with several previous reports on the finding inMoenkhausiasanctaefilomenae (Foresti et al., 1989), Aphaniusfasciatus (Vitturi et al., 1995), Leporinusfriderici (Galetti et al., 1995), Salmotrutta (Castro et al., 1996),Salvelinus alpines (Reed & Phillips, 1997), Chondrostomalusitanicum (Rodrigues &Collares-Pereira, 1996, Collares-Pereira &Ráb, 1999), Hopliasmalabaricus (Born &Bertollo, 2000), Oedalechiluslabeo (Rossi et al., 2000), Astyanaxscabripinnis(Mantovani,2000; Marco-Ferro et al.,2001;Soza et al., 2001), A. altiparanae (Pacheco et al., 2001;Mantovani et al., 2005), Bryconamericusaff. exodon (Paintner-Marques et al., 2002), Apareiodonaffinis (Jorge &Filho, 2004), Aphaniusfasciatus (Vitturib et al., 2005), Prochiloduslineatus(Gras et al., 2007), B. aff. iheringii (Capistano et al., 2008), and Puntioplitesproctozysron (Supiwong et al., 2012). NORs can be the perfect markers to display wide chromosomal polymorphism within and between species in many groups of fishes. This variety may affect NOR number, its localization on the chromosome, size, and active numbers in each genome. The previous NORs studies showed variations between species, within species, and even between individuals (Galetti et al., 1984; Gold et al., 1993; Castro et al., 1996).

Karyotype diversification processes in species are subject to multiple factors, whether intrinsic (genomic or chromosomal particularities) or extrinsic (historic contingencies). Among these, restricted gene flow between populations is an important factor for fixation of karyotype changes. For example, after the occurrence of an inversion, it can be lost in the polymorphic state or, under the proper conditions, spread in the population until it is fixed. Inversions maintain areas of imbalance between alleles in loci within or influenced by these rearrangements, leading to an adaptive condition, primarily along environmental gradients. This could occur, particularly in relation to possible historical expansion and adaptation to new environments (Hoffmann &Rieseberg, 2008).

Ribosomal RNA genes are among the most mapped sequences in fish chromosomes. Accordingly, they can be excellent genetic markers for the comparative genomic studies, evolutionary studies as well as the genetic identification of fish species (Fontana, 2003). In higher eukaryotes, the moderately repetitive ribosomal RNA genes (rDNAs) are arranged in two different families: the nucleolus forming major (45S) and the non-nucleolus forming minor (5S) rDNAs. The major family is composed of the regions coding for 18S, 5.8S and 28S rRNA genes separated by internal transcribed spacers (ITS 1 and ITS 2) and surrounded by non transcribed spacer (NTS) sequences (Long & David, 1980; Pendas et al., 1993). The nucleolar organizer regions (NORs) contain 45S rDNA gene cluster, which has also been studied by means of $AgNO_3$ and CMA_3 staining. The minor family is composed of a highly conserved 120 bp long coding sequences separated by variable non transcribed spacer (NTS). In several fish species, chromosome location of the two rDNA families are usually different

(Martinez et al., 1996; Fujiwara et al., 1998; Sajdak et al., 1998; Martins et al., 2000; Ferro et al., 2001).

The FISH helped simultaneous chromosomal localization of the 18S rDNA on the chromosomes of *B. suchus* and is being reported for the first time. In the present study, NOR signal was observed only one chromosome of the 1st chromosome pair (1a 1b) using silver nitrate staining that stains only transcriptionally active regions, whereas the FISH is able to detect 18S rDNA on both homologous chromosomes pair. Thus, the molecular karyotyping using FISH technique helps precise characterization of this species. Furthermore, the 18S rDNA probe has been considered as an important marker to evidence the karyotypic differentiation, which is not detected by conventional tools, in species considered karyotypically conserved and uniform (Almeida et al., 2010). The heteromorphism of signal intensity observed between homologous chromosomes may be caused by a variety of mechanisms, namely unequal crossing over, transposition, tandem amplification and other rearrangements involving homologous segments causing structural modifications in the NORs (Vicari et al., 2006; Sczepanski et al., 2010).

Telomeric (TTAGGG)*n* sequences are present in the telomeres of vertebrate chromosomes, and the study of these sequences provides insight into the chromosomal rearrangements that have occurred during karyotype evolution of distinct organisms (Cioffi&Bertollo, 2009;Meyne et al., 1989). FISH with the telomeric (TTAGGG)*n*probe revealed hybridization signals on each telomere of all chromosomes and internal transcribed spacers were not observed, which indicates that Robertsonian fusions or chromosomal translocations might be not involved in the karyotypic evolution of*B. suchus*.

In this respect, cytogenetic techniques have been used to characterize populations, species, genera and families, and many of them have proved to be efficient marker in identifying intra and inter-specific banding/staining techniques. They have facilitated accurate chromosome identification and permitted a better understanding of cytogenetics. These finding have revealed the mechanisms involved in the evolutionary processes. Recently, the studies on chromosome structure and evolution were challenged with the introduction of new molecular cytogenetic techniques that enabled taxonomic identification of species with the use of genes or specific genomic segments. This will eventually help in fisheries development through better management of genetic resources (Kumar et al., 2013).

Conclusions

The conclusion of the present study supported the conserved of the diploid chromosome numbers (2*n*) = 56 in *Bagarius* species. However, variation in karyotype has been reported in this family, which is summarized in table 2. It is evident from the frequency distribution (Klinkhardtet al., 1995) that 2*n*=56 is by far the most common diploid chromosome number in catfishes. The variation in karyotypes between species may be due to prevalence of non-robertsonianrearrangements (Kirpichnikov, 1981).There were variations in signals of FISH and their position in the karyotype along with variation inDNA sequences. These markers may be future useful for discrimination of population of closely related species and their polymorphism.Nevertheless, two probes were used in the present study; even so, other probes such as microsatellites should be usedin the further comparative studies. In the same way, others in the family Sisoridae should be studied additionally to explain properly of the chromosomal evolution in this family.

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