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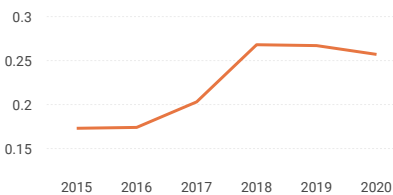
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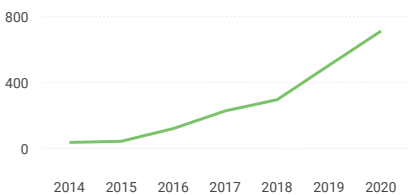
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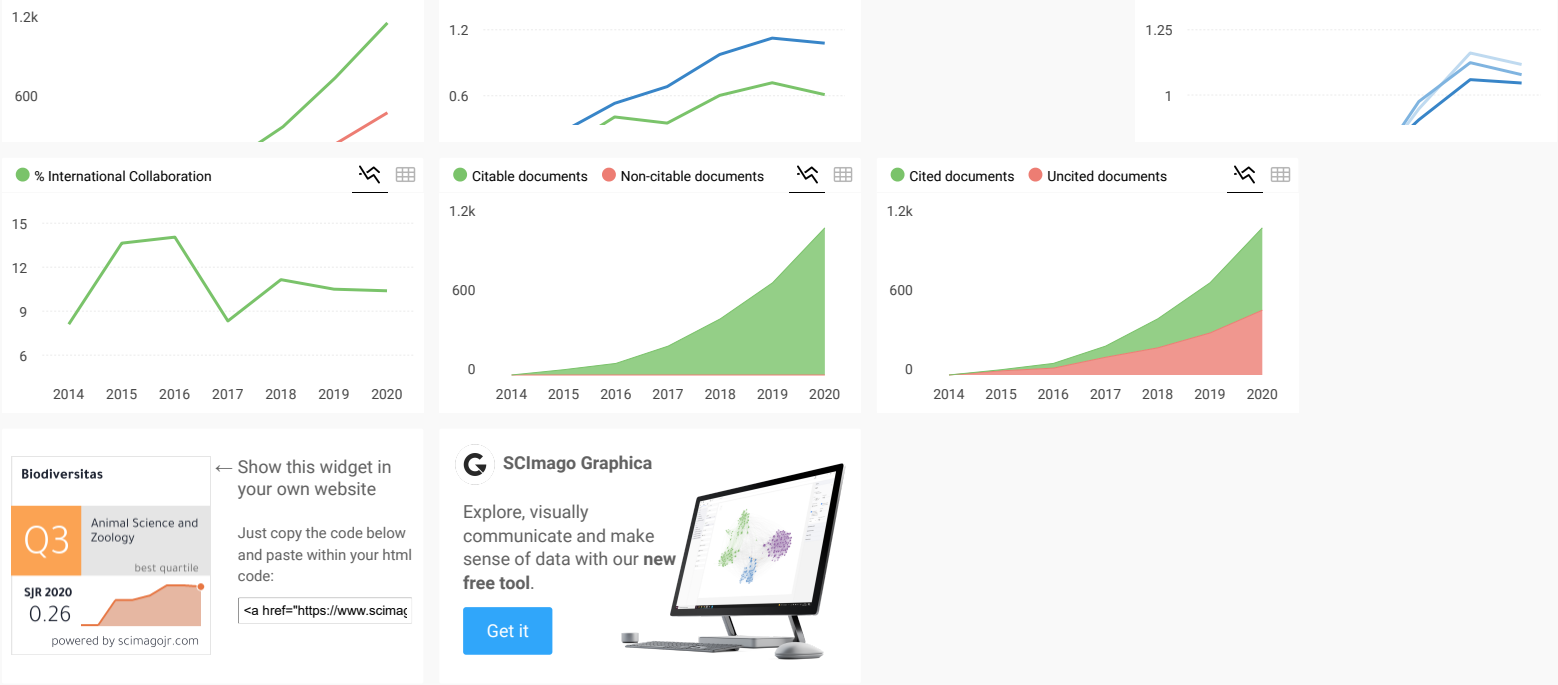
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First chromosome analysis of Thai pufferfish *Pao cochinchinensis* (Steindachner, 1866)

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Abstract. Pissaparn M, Phimphan S, Chaiyasan P, Tanoamtong A, Liehr T, Suwannapoom C, Reungsing M, Supiwong W. 2020. First chromosome analysis of Thai pufferfish *Pao cochinchinensis* (Steindachner, 1866). *Biodiversitas* 21: 4309-4316. Here first analysis of chromosomes and nucleolar organizer region (NOR) pattern in pufferfish *Pao cochinchinensis* (Steindachner, 1866) was undertaken. Chromosomal preparations were obtained from kidney of *P. cochinchinensis* from Chi River basin in Thailand. Chromosomal characteristics were analyzed by Giemsa staining, Ag-NOR banding as well as fluorescence in situ hybridization (FISH) using microsatellites d(CA)₁₅ and d(CGG)₁₀ probes. *P. cochinchinensis* had $2n = 40$ with the fundamental number (NF) 74, both in male and female. The karyotype exhibited 12 metacentric (m), 10 submetacentric (sm), 12 acrocentric (a) and 6 telocentric (t) chromosomes. No differentiated heteromorphic sex chromosomes were observed. NORs were located on short arms adjacent to telomere of the metacentric chromosome pair 4, which coincide with signals of d(CGG)₁₀ probe. FISH with d(CGG)₁₀ sequences were also displayed at the telomeres of most other chromosomes, whereas d(CA)₁₅ repeats highly accumulated throughout almost all entire chromosomes except for centromeric regions. The results of conventional Giemsa staining presented the differentiation even the same genus. The localization of NORs on one pair of chromosomes only is a common characteristic found in many fish groups as well as other vertebrates. Mapping of two distinct microsatellites demonstrated the remarkable chromosomal diversification that characterizes evolution in the genus *Pao*. Both, conventional and molecular cytogenetics are excellent tools to study, and better understand chromosomal evolution, as well as to uncover biodiversity among fishes.

Keywords: Chromosome, FISH, karyotype, Nucleolar Organizer Region, *Pao cochinchinensis*

Abbreviations: $2n$: diploid chromosome number; NF: fundamental number or number of chromosome arm; FISH: fluorescence in situ hybridization technique; NOR: nucleolar organizer region; m: metacentric chromosome; sm: submetacentric chromosome; a: acrocentric chromosome; t: telocentric chromosome

INTRODUCTION

Up to the present, there are approximately 191 valid species of pufferfish in the list of the catalog of fishes (Eschmeyer et al. 2017). *Pao* Kottelat, 2013 is a genus of the Tetraodontidae family and is the most common freshwater pufferfish, except for *P. leiurus* (Bleeker, 1850) which also occurs in brackish water (Kottelat 2013). *Pao* species are found in Southeast Asia and have been placed in the *Tetraodon* since 2013 (Kottelat 2013). Up to date, there are 15 recognized species in the genus *Pao* (Eschmeyer et al. 2017). *P. cochinchinensis* is part of the genus *Pao*, which contains four other species, i.e. *P. cambodgiensis* (Chabanaud, 1923), *P. leirus*, *P. turgidus* (Kottelat 2000) and *P. hilgendorfi* (Popta, 1905). As they all hail from the same geographical area and share very similar physical characteristics, they are hard to distinguish

from each other. Still, *P. cochinchinensis* has a clear red spot situated towards the rear of the fish, enabling its indubitable identification (Figure 1). All *Pao* species are ambush predators in nature (Kottelat 2013). Concerning their commercial value, pufferfishes are often less expensive than other fishes on the local markets, as they contain inconsistent levels of toxins, depending on fish and season.

Pufferfishes have the smallest chromosomes so far described in vertebrates, hampering high-resolution cytogenetic analyses, so far (Myers 2008). Only 25 species in Tetraodontidae with one species of the genus *Poa* were cytogenetic analyzed (Arai 2011; Table 1) so far. The $2n$ range from 28 in *Canthigaster coronata* (Vaillant & Sauvage, 1875) (Arai 2011) to 46 chromosomes in the genus *Sphoeroides* anonymous, 1798 (Brum et al. 1995, Brum 2000, Sá-Gabriel and Molina 2005, Alves et al.

2008). Shape, size, and number of chromosomes of each species are always fixed, so knowledge about the karyotypes can be used for studies in taxonomy, diversity, kinship, and evolution, even though in certain circumstances also variations have been reported (Hartati et al. 2017). To identify intra- and inter-specific variants karyotyping combined with molecular cytogenetic analyses are the methods best suited here. This kind of approach has played an important role in the precise characterization of structure of genomes (Cioffi and Bertollo 2012), and checking the ploidy status in *Acipenser mikadoi* (Zhou et al. 2013). Recently, molecular cytogenetic studies using fluorescence in situ hybridization (FISH) for mapping repetitive DNA sequences have provided important contributions to the characterization of biodiversity and evolution of divergent fish groups (Cioffi et al. 2018).

However, this kind of study is scarcely applied in Thai freshwater fish. Exceptions are studies in Bagrid catfishes (Supiwong et al. 2013, 2014a,b), Channids (Cioffi et al. 2015), three *Clarias* species (Maneechot et al. 2016), *Toxotes chatareus* (Hamilton, 1822) (Supiwong et al. 2017), two cyprinid fishes (Saenjundaeng et al. 2018), Asian swamp eel, *Monopterus albus* (Zuiew, 1793) (Supiwong et al. 2019) and silurid catfishes (Ditcharoen et al. 2019). The present study is the first report on chromosomal characteristics in *P. cochinchinensis* using conventional staining, Ag-NOR banding, and FISH technique, being the basis for further study by advanced genetics and for future comparative approaches concerning systematics and evolutionary relationships of buffer-fishes.

Table 1. Yet available cytogenetic reports on the family Tetraodontidae

Species	2n	NF	Karyotype	NORs	Locality	Reference
<i>Arothron hispidus</i>	42	-	-	-	India	Arai (2011)
<i>A. immaculatus</i>	42	68	12m+14sm+16st/a	-	India	Arai (2011)
<i>A. manilensis</i>	42	72	14m+16sm+12st	-	Japan	Arai (2011)
<i>A. meleagris</i>	38	-	-	-	Japan	Arai (2011)
<i>A. reticularis</i>	42	68	12m+14sm+16st/a	-	India	Arai (2011)
<i>Canthigaster coronata</i>	28	36	6m+2sm+20st/a	-	Japan	Arai (2011)
<i>C. rivulate</i>	34	44	4m+6sm+10st+14a	-	Japan	Arai (2011)
<i>C. patoca</i>	40	70	14m+16sm+10st/a	-	Japan	Arai (2011)
<i>Dichomyctere fluviatilis</i>	42	64	2m+4sm+2st+34a	-	India	Arai (2011)
	42	48	2m+4sm+2st+34a	-	S.Asia	Arai (2011)
<i>D. nigroviridis</i>	42	62	20m/sm+22st	-	S.Asia	Hardie and Hebert (2003)
<i>Legoccephalus inermis</i>	44	46	2m+42a	-	India	Arai (2011)
<i>L. laevigatus</i>	46	-	-	-	Brazil	Sá-Gabriel and Molina (2005)
<i>L. lunaris</i>	44	68	10m+14sm+20st/a	-	India	Hardie and Hebert (2003)
*<i>Pao cochinchinensis</i>	40	74	12m+10sm+12a+6t	2	Thailand	*Present study
<i>P. palemnangensis</i>	42	-	-	-	S.Asia	Arai (2011)
<i>Sphoeroides greeleyi</i>	46	70	24m/sm+22st/a	2	Brazil	Brum et al. (1995)
<i>S. spengleri</i>	46	64	18m/sm+28st/a	-	Brazil	Brum et al. (1995)
	46	66	20m/sm+26st/a	-	Brazil	Alves et al. (2008)
<i>S. testudineus</i>	46	68	18m+4sm+6st+18a	2	Brazil	Sá-Gabriel and Molina (2005)
<i>S. tyleri</i>	46	60	14m/sm+32st/a	-	Brazil	Brum (2000)
<i>Takifugu chrysops</i>	44	64	6m+14sm+24st/a	-	Japan	Arai (2011)
<i>T. niphobles</i>	44	64	24m/sm+24st/a	-	Japan	Arai (2011)
	44	64	4m+16sm+24st/a	-	Japan	Arai (2011)
<i>T. pardalis</i>	44	-	-	-	Japan	Arai (2011)
	44	66	6m+16sm+22st/a	-	Japan	Arai (2011)
<i>T. poecilonotus</i>	44	66	12m+10sm+22st/a	-	Japan	Arai (2011)
	44	66	-	-	Japan	Arai (2011)
	44	66	12m+8sm+24a	-	China	Arai (2011)
<i>T. rubripes</i>	44	66	10m+12sm+22st/a	-	Japan	Arai (2011)
	44	64	20m/sm+24st/a	-	China	Arai (2011)
	44	62	12m+6sm+26a	-	China	Arai (2011)
<i>T. vermicularis</i>	44	66	8m+14sm+22st/a	-	Japan	Arai (2011)
<i>T. xanthopterus</i>	44	66	8m+14sm+22st/a	-	Japan	Arai (2011)
<i>Tetraodon cutcutia</i>	42	70	16m+12sm+4st+10a	-	India	Arai (2011)

Note: 2n: diploid chromosome number, NF: fundamental number (number of chromosome arm), m: metacentric, sm: submetacentric, a: acrocentric, t: telocentric, NORs: nucleolar organizer regions, S.: Southeast, and -: not available



Figure 1. General morphological characteristics of pufferfish, *Pao cochinchinensis*. Bar = 2 cm

MATERIALS AND METHODS

Sample collection

Four male and four female specimens of *P. cochinchinensis* were obtained from the Chi River, Roi-Et Province, Northeast of Thailand. The fishes were transferred to laboratory aquaria and kept under standard conditions for three days before the experiments.

Chromosome preparation

Chromosomes were prepared in vivo (Supiwong et al. 2012a, 2013b, Kasiroek et al. 2017) as follows. Colchicine solution (0.05%, 1 ml/100 g body weight) was injected into the fish's intramuscular and/or its abdominal cavity and then left for 1 h. The kidney tissues were cut into small pieces, and then squash mixed with 0.075 M KCl. After discarding all large pieces of tissue, 8 ml of cell sediments were transferred to a centrifuge tube and incubated for 25–35 min at room temperature. The KCl was discarded from the supernatant after centrifugation at 1,200 rpm for 8 min. Cells were fixed in fresh, cool fixative)3 methanol: 1 glacial acetic acid(.

Chromosome staining

Conventional staining by 20% Giemsa's solution for 30 min was carried out following Supiwong et al. (2012b, Getlekha et al. 2017) and Ag-NOR banding was conducted by adding 4 drops of 50% silver nitrate solution and 2 drops of 2% gelatin solution on slides with spread metaphases, respectively. The slides were then sealed with coverslips and incubated at 60°C for 5 min. After that, slides were soaked in distilled water until the coverslips were separated. The slide was stained with 20% Giemsa's solution for 1 min (Sangpakdee et al. 2017, Chaiyasan et al. 2018).

Chromosome characterization

Chromosome counting was performed on mitotic metaphase cells under a light microscope. Twenty clearly

observable and well-spread metaphase plates of male and female, each was selected and documented. The length of short chromosome arm (Ls) and long chromosome arm (Ll) were measured and calculated to the length of total chromosome (LT, $LT=Ls+Ll$). The relative length (RL), the centromeric index (CI), and standard deviation (S.D.) of RL and CI were estimated. The CI (q/p+)(between 0.50–0.59, 0.60–0.69, 0.70–0.89, and 0.90–1.00 are described as metacentric, submetacentric, acrocentric, and telocentric chromosomes, respectively. The fundamental number (number of chromosome arm, NF) was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosome. All parameters were used in karyotyping and idiogramming (Tanomtong et al. 2014, Choo-seangjaew et al. 2017).

FISH technique

The use of microsatellites d(CA)₁₅, and d(CGG)₁₀ probes described by Kubat et al. (2008) followed here with slight modifications. These sequences were directly labeled with Cy3 at 5' terminal during synthesis by Sigma (St. Louis, MO, USA). FISH was performed under high stringency conditions on mitotic chromosome spreads (Cioffi et al. 2011). 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 µL probes and 8 µL hybridization buffer) 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 (Supiwong et al. 2017 2019). Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector laboratories), and analyzed in an epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

RESULTS AND DISCUSSION

Chromosome number, fundamental number, and karyotype of *Pao cochinchinensis*

The chromosome number in *P. cochinchinensis* was $2n=40$. The NF was 74 in both males and females (Figure 2A). There were four types of chromosomes in this species including metacentric, submetacentric, acrocentric, and telocentric chromosomes. Three sizes of chromosomes including large, medium, and small can be distinguished. The details of the autosome types and sizes are presented as follows: eight large metacentrics, six large submetacentric, four large acrocentrics, four medium metacentrics, four medium submetacentric, four medium acrocentric, four small acrocentric and six small telocentric chromosomes (Table 2). No differentiated heteromorphic sex chromosomes were observed. The karyotype formula for *P. cochinchinensis* could be deduced as:

$$2n \text{ (diploid)} 40 = L^m_8 + M^m_4 + L^{sm}_6 + M^{sm}_4 + L^a_4 + M^a_4 + S^a_4 + S^t_6$$

Chromosome markers of *P. cochinchinensis*

The determination of chromosome marker for this species was firstly obtained by using the Ag-NOR banding technique. These regions can be detected by silver nitrate staining, if they are active during the interphase prior to mitosis since they specifically stain a set of acidic proteins related to ribosomal synthesis process; this technique

actually reveals active NORs but cannot detect inactive rDNA associated to NORs (Supiwong et al. 2014a). The regions adjacent to the telomere of the short arms of the metacentric chromosome pair 4 exhibited active Ag-NOR signals (Figure 2B).

Patterns of d(CA)₁₅ and d(CGG)₁₀ microsatellite repeats in *Pao cochinchinensis*

The results of mapping of microsatellite repeats on the chromosomes of *P. cochinchinensis* showed that d(CGG)₁₀ microsatellite repeat signals are highly accumulated at the telomeric regions of several chromosome pairs including the regions coincident with the NOR position (chromosome pair 4), and the interstitial positions of some other pairs (Figure 2.C). However, the patterns of d(CA)₁₅ microsatellite repeats are more accumulated than those of d(CGG)₁₀ microsatellite repeats. The d(CA)₁₅ microsatellite repeats signals are observed on all chromosome pairs. These signals are distributed throughout the whole chromosomes except for centromeric regions of most chromosome pairs whereas, in some pairs, they were found on the whole chromosome (Figures 2.D).

Idiograms of *Pao cochinchinensis* chromosomes

In Figure 3 all previous results are summarized and idiograms of *P. cochinchinensis* are provided.

Table 2. Mean length of short arm chromosomes (Ls), length long arm chromosomes (Ll), length total arm chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of CI, RL from 20 metaphases of *Pao cochinchinensis*, $2n = 40$.

Pair	Ls	Ll	LT	CI±SD	RL±SD	Size	Type
1	0.87	0.89	1.76	0.503±0.135	0.081±0.016	Large	metacentric
2	0.80	0.81	1.61	0.502±0.004	0.074±0.008	Large	metacentric
3	0.70	0.72	1.42	0.504±0.005	0.065±0.005	Large	metacentric
4*	0.61	0.63	1.24	0.505±0.009	0.056±0.005	Large	metacentric
5	0.52	0.53	1.05	0.504±0.007	0.049±0.005	Medium	metacentric
6	0.47	0.48	0.95	0.502±0.004	0.043±0.005	Medium	metacentric
7	0.55	0.98	1.53	0.636±0.025	0.070±0.007	Large	submetacentric
8	0.48	0.87	1.35	0.641±0.030	0.062±0.006	Large	submetacentric
9	0.43	0.76	1.19	0.635±0.034	0.054±0.004	Large	submetacentric
10	0.37	0.68	1.05	0.642±0.038	0.048±0.006	Medium	submetacentric
11	0.34	0.62	0.96	0.645±0.054	0.043±0.005	Medium	submetacentric
12	0.34	0.99	1.33	0.744±0.029	0.061±0.007	Large	acrocentric
13	0.29	0.83	1.12	0.739±0.038	0.051±0.004	Large	acrocentric
14	0.26	0.75	1.01	0.741±0.034	0.046±0.004	Medium	acrocentric
15	0.24	0.70	0.94	0.745±0.044	0.042±0.003	Medium	acrocentric
16	0.21	0.65	0.86	0.759±0.052	0.038±0.003	Small	acrocentric
17	0.17	0.58	0.75	0.773±0.047	0.033±0.004	Small	acrocentric
18	0.00	0.67	0.67	1.000±0.000	0.030±0.006	Small	telocentric
19	0.00	0.58	0.58	1.000±0.000	0.025±0.006	Small	telocentric
20	0.00	0.45	0.45	1.000±0.000	0.020±0.004	Small	telocentric

Note: *=NOR-bearing chromosome

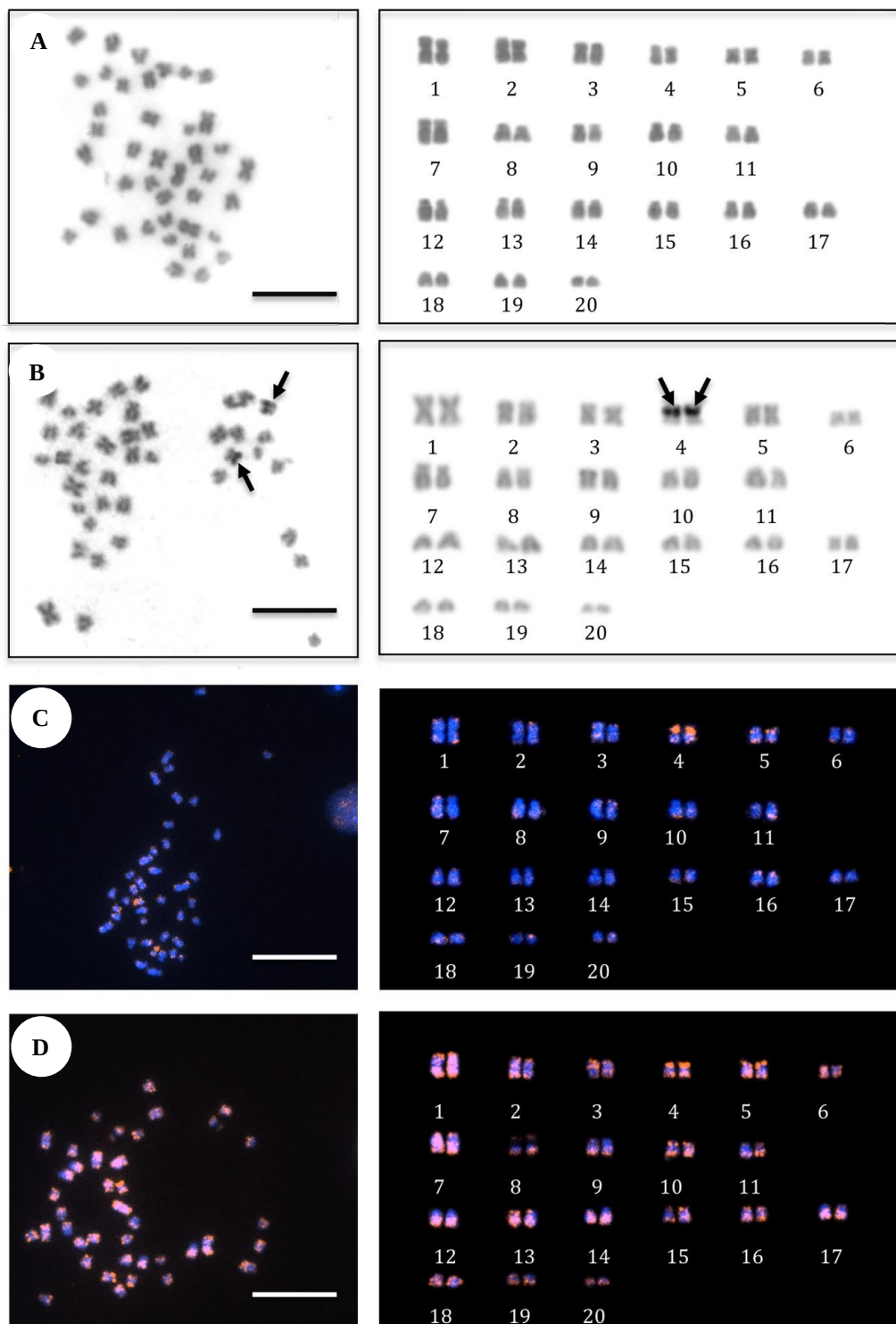


Figure 2. Metaphases and karyotypes of the pufferfish, *Pao cochinchinensis*, $2n=40$ by conventional staining technique (A), Ag-NOR banding technique (B), FISH with d(CA)₁₅ probe (C) and FISH with d(CGG)₁₀ probe (D). The arrows indicate the nucleolar organizer regions/NORs of the 4th chromosome pair and scale bars indicate 5 µm

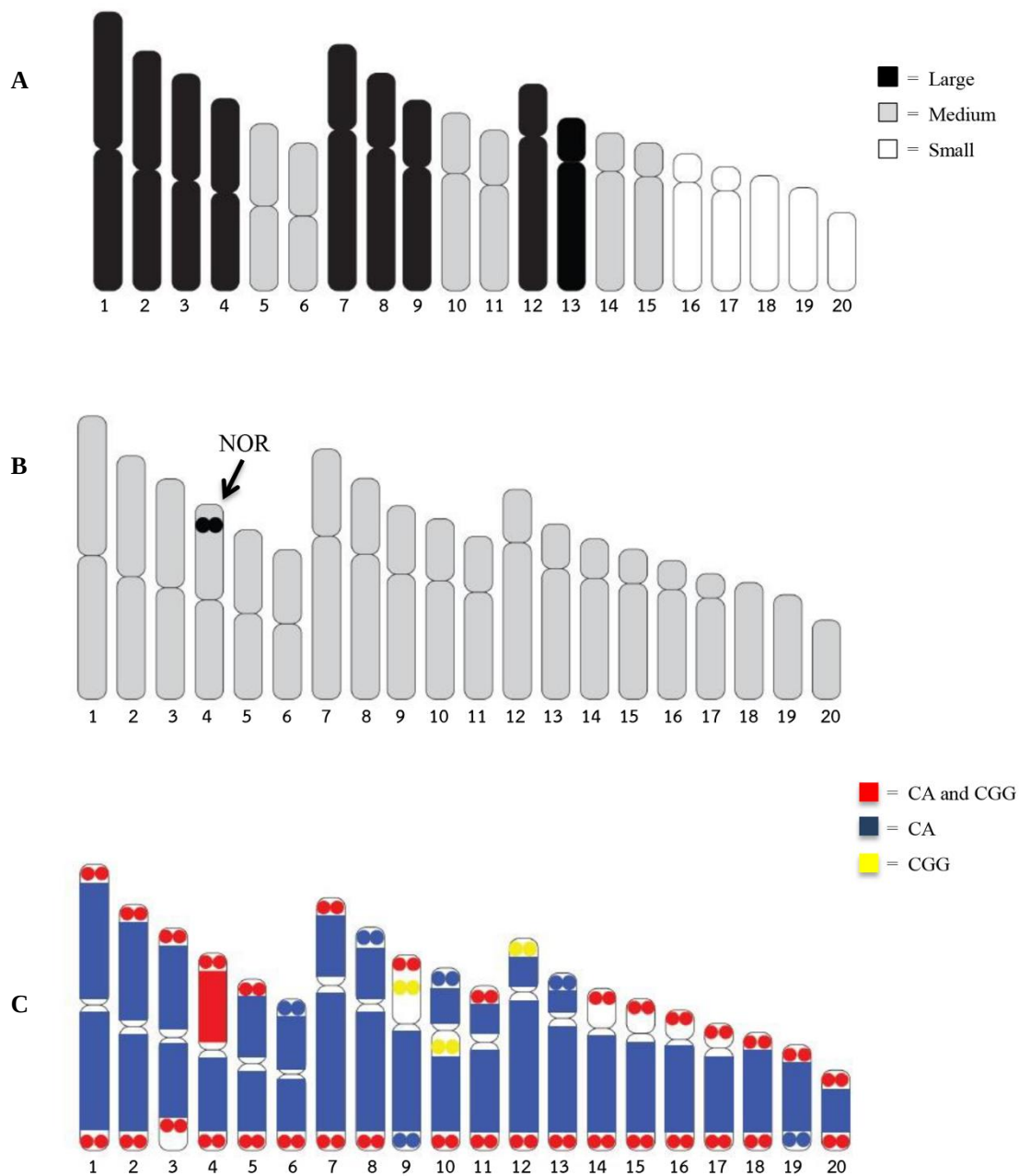


Figure 3. Idiograms showing lengths and shapes of chromosomes of the pufferfish, *Pao cochinchinensis*, 2n=40 by conventional staining technique (A), Ag-NOR banding technique (B) and FISH technique (C). Arrow indicates nucleolar organizer regions/NORs.

Discussion

Present study is the first report on karyotyping of *P. cochinchinensis*. To compare with other species in the family Tetraodontidae, the obtained result exhibited the same 2n as in *Canthigaster patoca* (Arai 2011). However, it is different from *P. palemnangensis* (same genus) which had the 2n as 42 chromosomes (Arai 2011). Moreover, they are different in 2n to other pufferfish species, as shown in Table 1; the most frequently observed 2n in this family is 42 chromosomes (30.77%). Interestingly also NF and karyotype structure were different in all previously studied species: NFs in family Tetraodontidae vary from 36 to 74

and most species in this family have karyotypes consisting of metacentric, submetacentric, acrocentric/subtelocentric chromosomes (Table 1). Accordingly, this fish family displays a high diversity of chromosomes and karyotypes. An idiogram from conventional staining is shown in Figure 3.A. The largest chromosome of *P. cochinchinensis* has about four folds of the size of its smallest.

Chromosomal studies on fishes have been providing new information about karyotypic variability at inter- and intraspecific levels, which can be of great interest to phylogenetic, systematics, and taxonomy (Supiwong et al. 2012b). The single NOR-bearing chromosomes in the

present result are most likely homologous to that observed in *Sphoeroides greeleyi* (Brum et al. 1995) and *S. testudineus* (Sá-Gabriel and Molina 2005). A single NOR-bearing chromosome is a common characteristic found in many fish groups as well as vertebrates (Supiwong et al. 2010, 2012a,b, 2013b, 2015, 2017, Chooaseangjaew et al. 2017, Getlekha et al. 2017; Kasiroek et al. 2017; Phimphan et al. 2017; Sangpakdee et al. 2017; Chaiyasan et al. 2018). NOR mapping is frequently used to compare variations, as well as to identify and explain speciation. However, changes in chromosome number and structure can alter the number, and structure of NOR. Structure, number, and morphology of a NOR may be specific to populations, species, and subspecies. Robertsonian translocations (centric fusion) may cause losses of NORs. Species, which have limited gene exchange due to geographical isolation, have elevated karyotype numbers and NOR variation. 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 (Supiwong et al. 2012b).

In this study microsatellite distributions on chromosomes within the Family Tetraodontidae is done for the first time. Comparisons with other families revealed that the d(CA)₁₅ microsatellite repeats mapping are similar to the nine species of the Bagridae family including *Hemibagrus filamentus*, *H. spilopterus*, *H. wyckii*, *H. wyckioides*, *Mystus atrifasciatus*, *M. multiradiatus*, *M. mysticetus*, *M. bocourti*, and *Pseudomystus siamensis* (Supiwong et al. 2013a, 2014b), *Clarias batrachus* and *C. macrocephalus* (Maneechot et al. 2016), *Toxotes chatareus* (Supiwong et al. 2017). This pattern can be found in non-Thai fish species. For example, the catfishes *Imparfinis schubarti* (Heptapteridae), *Steindachneridion scripta* (Pimilodidae), and *Rineloricaria latirostris* (Loricariidae) present a remarkable accumulation of both d(GA)₁₅ and d(A)₃₀ microsatellites in telomeric regions (Cioffi et al. 2011). However, this pattern is quite different from four *Channa* species, i.e. *Ch. lucius*, *Ch. micropeltes*, *Ch. striata* and *Ch. gachua* (Cioffi et al. 2015), *Cl. gariepinus* (Maneechot et al. 2016) and two cyprinid fishes, *Catlocarpio siamensis* and *Probarbus jullieni* (Saenjundaeng et al. 2018). These fishes have the patterns of d(CA)₁₅ repeats which distributed throughout the chromosome without strong accumulations in specific regions in all chromosomes.

The patterns of d(CGG)₁₀ repeats in *P. cochinchinensis* are similar to *Ch. lucius* (Cioffi et al. 2015) but they differ from other *Channa* that they are distributed throughout the chromosome without high accommodations in the regions related with NOR positions. Moreover, the patterns are also different from *T. chatareus* (Supiwong et al. 2017) and Asian swamp eel, *M. albus* (Supiwong et al. 2019) and the wolf fish (*Hoplias malabaricus*) in which 12 different microsatellite repeats, including d(CA)₁₅, d(GA)₁₅, d(CAA)₁₀, and d(CGG)₁₀ repeats showed strong hybridization signals at subtelomeric and heterochromatic regions of several autosomes, with a varied amount of signal on the sex chromosomes (Cioffi et al. 2011). From the previous studies, it is believed that microsatellites have specific zones as heterochromatin (telomeres, centromeres,

and in the sex chromosomes) of fish genomes (Cioffi and Bertollo 2012). However, microsatellites have also been found in noncentromeric regions, many of them were located either near or within genes (Rao et al. 2010). This phenomenon is also valid for *P. cochinchinensis* in the present study.

The first cytogenetics study in *P. cochinchinensis*, both by classical and molecular cytogenetics, led to results having the potential to contribute substantially to the understanding of chromosomal evolution in Tetraodontidae family.

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