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A First Chromosomal Characterization of Black - Spotted Pufferfish, Arothron nigropunctatus (Tetraodontifrom ; Tetraodonidae)

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Abstract-The present study aims to analyze concerned karyotype and idiogram of black - spotted pufferfish (Arothron nigropunctatus Bloch & Schneider, 1801). Chromosome was prepared from kidney tissues of fish reared at Phuket Marine Biological Center, Phuket province. The mitotic chromosomes were harvested by the colchicine - hypotonic - fixation - air drying method. Conventional staining and Ag - NOR banding techniques were applied to stain the chromosomes. The results showed that A. nigropunctatus has diploid (2n)=38, the fundamental number (NF) was 72. The types of chromosomes are two large metacentric, ten medium metacentric, eight medium submetacentric, two small metacentric, 12 small submetacentric, and four small telocentric chromosomes. This is the first report of marker chromosome; found that NORs located at the short arm region of the submetacentric chromosome. No cytologically distinguishable sex chromosome was observed. The karyotype formula could be deduced as follows:

$$2n\ (38) = L^{m}_{2} + M^{m}_{10} + M^{sm}_{8} + S^{m}_{2} + S^{sm}_{12} + S^{t}_{4}$$

Keywords: Arothron nigropunctatus, Chromosome, Karyotype

1. Introduction

The order Tetraodontiformes represent one of the most peculiar radiations of teleost fishes. Tetraodontiform fishes comprises approximately 412 species, divided into ten families as follows: Triacanthodidae including 23 species in 11 genera, Triacanthidae comprising seven species in four genera, Balistidae composing of 37 species in 12 genera, Monacanthidae composing of 102 species in 27 genera, Aracanidae composing of 13 species in six genera, Ostraciidae composing of 22 species in five genera, Triodontidae monotypic, Tetraodontidae composing of 184 species in 27 genera, Diodontidae comprising 18 species in seven genera, and Molidae comprising five species in three genera (Matsuura, 2015).

Although half of the tetraodontiform fishes distributed along the reef - associated (Alfaro *et al.*, 2007), the ten recognized families inhabit a wide variety of habitats. In addition, family Tetraodontidae is found predominantly on coral reefs, sea grasses, and other tropical, shallow - water environments (Santini *et al.*, 2013; Oliveira *et al.*, 2006). Marine pufferfish of the family Tetraodontidae is well known to possess a potent neurotoxin, tetrodotoxin (TTX) in their viscera, spines on the body, and the four frontal teeth fused (Santos, 1992; Sabrah *et al.*, 2006).

Most marine fishes studied have a diploid complement of 48 acrocentric chromosomes (Sola *et al.*, 1981; Klinkhardt *et al.*, 1995; Brum, 1996). However, in some taxa, close species have been reported showing changes in chromosome number and formula (Caputo *et al.*, 1997; Corrêa and Galetti Jr., 1997) and different sex chromosome systems (Morescalchi *et al.*, 1992; Gomes *et al.*, 1994; Cano *et al.*, 1996). Moreover, some species show supernumerary chromosomes (Pauls *et al.*, 1996). In family Tetraodontidae, the diploid numbers ranged from 28 to 46 chromosomes and most of them (30 species) have the fundamental numbers ranged from 36 to 78 (Arai, 2011).

In the present study is the first cytogenetic study on A. nigropunctatus (Figure 1) accomplished with the Ag - NOR staining technique. Our results provide new cytogenetic information for further study on taxonomy and evolutionary relationship. Moreover, we provide basic and useful information for the conservation, breeding and chromosome evolution study of this fish.

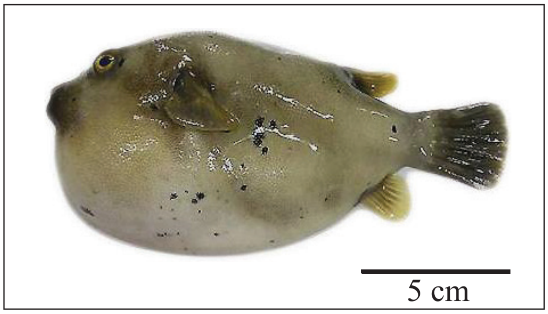

Figure 1. General characteristic of the black - spotted pufferfish (Arothron nigropunctatus).

2. Materials and Methods

2.1 Chromosome Preparation

Four male and female of A. nigropunctatus were obtained from farming the Institute of Phuket Costal Fisheries Research and Development and Phuket Marine Biological Center, in Phuket Province, Thailand. The 0.01% colchicine (1 mL/100 g body weight) was injected into the abdominal cavity and left for one hour. Chromosomes were prepared from the kidney cells by the squash technique. Kidney tissues were cut into small pieces then mixed with hypotonic solution (0.075 M KCl). After discarding all large pieces of tissues, 7 mL of cell sediments were transferred to a 15 - mL centrifuge tube and incubated for 45 min. Hypotonic solution was discarded from the supernatant after centrifugation at 1,200 rpm for 8 min. Cells were fixed in a fresh cool fixative (3 absolute methanol: 1 glacial acetic acid) to which up to 7 mL were gradually added before being centrifuged again at 1,200 rpm for 8 min, at which time the supernatant was discarded. The fixation was repeated until the supernatant was cleared and the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean by a plastic pipette followed by air - dry technique (Ditcharoen *et al.*, 2019; Supiwong *et al.*, 2019).

2.2 Chromosome Staining

The slide was conventionally stained with 20% stock Giemsa's solution for 15 min. Ag - NOR banding was conducted by adding four drops of 50% silver nitrate and two drops of 2% gelatin on slides, in order. The slides were covered with cover glasses and incubated at 60ºC for 7 min. After that, the slides were soaked in distilled water until the cover glasses were separated (Howell & Black, 1980).

2.3 Chromosome Checking, Karyotyping and Idiograming

Standardized karyotype and idiogram of this fish were constructed. Chromosome checking was performed on mitotic metaphase cells under a light microscope. The frequencies of chromosome number per cell were counted. The maximum frequency of chromosome number per cell is the diploid chromosome number of this fish. Ten cells of four male and female with clearly observable and well - spread chromosome were selected for karyotyping. The length of short arm chromosome (Ls) and long arm chromosome (Ll) were measured and calculated to the length of total arm chromosome (LT, LT=Ls+Ll). The relative length (RL), the centromeric index (CI) and standard deviations (S.D.) of RL and CI were calculated. The CI (q/p+q) between 0.50 - 0.59, 0.60 - 0.69, 0.70 - 0.89, and 0.90 - 1.00 were classified as the 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, acrocentric chromosomes, and one to telocentric chromosome. All parameters were used in karyotyping. The idiogram was constructed using a model drawing of karyotype and accomplished by a computer program (Sarasan *et al.*, 2018; Chaiyasan *et al.*, 2018).

3. Results and discussion

The present study revealed that the diploid chromosome number of A. nigropunctatus was 2n=38 and the NF was 72 in male and female. The types of chromosomes are two large metacentric, ten medium metacentric, eight medium submetacentric, two small metacentric, 12 small submetacentric, and eight small telocentric chromosomes (Figure 2). It was in agreement with the previous reports from Japan (Arai & Nagaiwa, 1976 ; Ojima, 1985). We compared with other fishes in the family Tetraodonidae (genus Arothron), A. hispidus, 2n=42 (NF=82) (Natarajan and Subrahmanjan, 1974); A. immaculatus, 2n=42 (NF=68) (Choudhury *et al.*, 1982) ; A. immaculatus, 2n=42 (NF=72) (Arai & Nagaiwa, 1976) ; A. leopardus, 2n=40 (NF=68) (Choudhury *et al.*, 1982) and A. reticularis, 2n=42 (NF=68) (Choudhury *et al.*, 1982) (Table 1).

Sex chromosome systems of Tetraodontidae have been described by male heterogamety, others by female heterogamety and others no cytologically distinguishable sex chromosome (Sá - Gabriel and Molina, 2004). Present study, no cytologically distinguishable sex chromosome was observed. It may be possible that the fish's sex - chromosomes are at the initiation of differentiation. The results is inconsistent with the report of Ojima (1985) that proposed functionally differentiated sex chromosomes, the multiple sex systems derive from the translocations. In the $X_1X_1X_2X_2/X_1X_2Y$ sex system, the diploid value of females presented one chromosome more than males. The origin of this system may be linked to the existence of centric fusions (Robertsonian rearrangements).

The present study was accomplished by using the Ag - NOR staining technique. The objective of this technique is to determine NORs regions. Which represented the location of genes (loci) that function in ribosome synthesis (18S and 28S ribosomal RNA), and a positive NOR will be detected when these genes are functioning (Sharma *et al.*, 2002). From the result, the short arm region of the submetacentric chromosome pair 9 showed clearly observable NORs (Figure 3). Normally, most fishes have only one pair of small NORs on chromosomes (Mohannath G. *et al.*, 2016). If some fishes have more than two NORs, it may be caused by the translocation between some parts of chromosome which have NOR and another chromosome. Furthermore, NOR usually located close to the telomere of the chromosome. If NOR appears between the centromere and telomere (interstitial NOR), it may be the result of the tandem fusion between this chromosome with NOR and another one. However, it may be caused by the pericentric inversion between two telocentric chromosomes that one chromosome has NOR at the telomere (Galetti Jr. *et al.*, 2000).

The asymmetrical karyotype of A. nigropunctatus and all three types of chromosomes (metacentric, submetacentric and telocentric chromosomes) that we found the important chromosome markers. The idiogram shows the continuous length gradation of the chromosomes. The largest chromosome is approximately twice the size of the smallest chromosomes. The chromosome markers of A. nigropunctatus are chromosome pair 1, the largest metacentric and chromosome pair 19, the smallest telocentric chromosomes. The data of the chromosomal checks on mitotic metaphase cells of the A. nigropunctatus are shown in

(Table 2). (Figure 4) shows the idiogram from conventional staining and Ag - NOR banding techniques. The karyotype formula for A. nigropunctatus is as follows:

$$2n\ (38) = L^{m}_{2} + M^{m}_{10} + M^{sm}_{8} + S^{m}_{2} + S^{sm}_{12} + S^{t}_{4}.$$

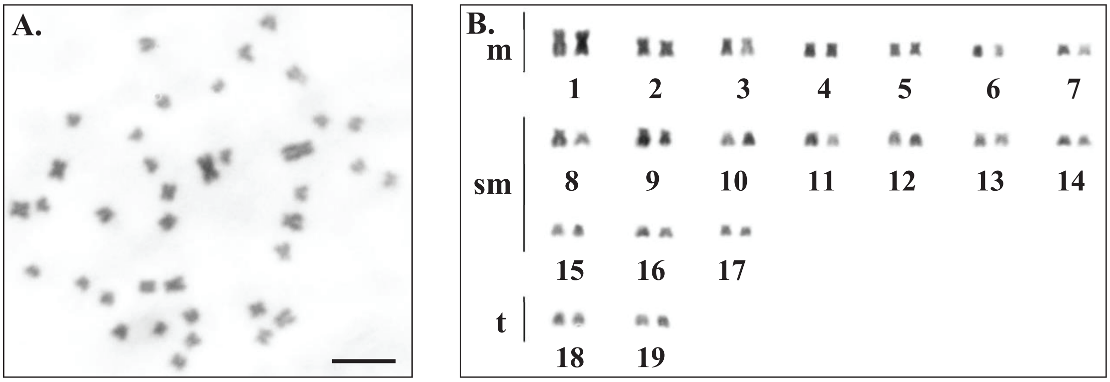

Figure 2. The metaphase chromosome plate (A.) and karyotype (B.) of black - spotted pufferfish (A. nigropunctatus), 2n=38 by conventional staining technique, scale bar indicates 5 micrometers (m = metacentric, sm = submetacentric and t = telocentric chromosomes).

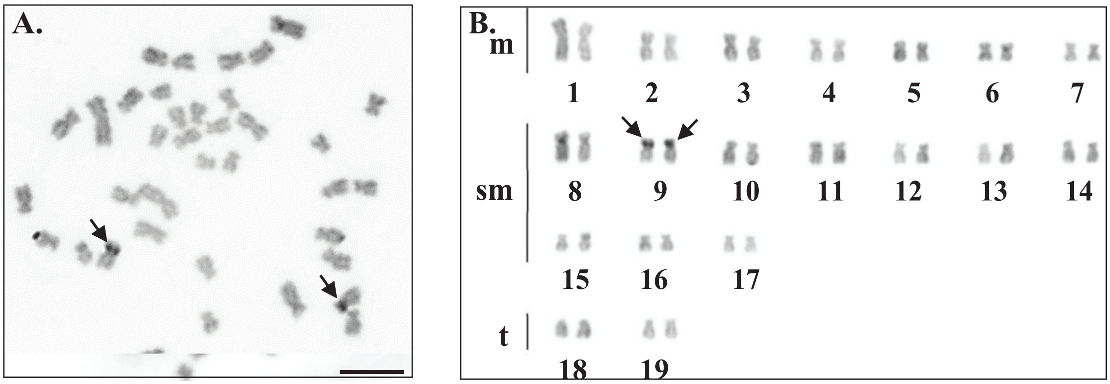

Figure 3. The metaphase chromosome plate (A.) and karyotype (B.) of black - spotted pufferfish (A. nigropunctatus), 2n=38 by Ag - NOR banding technique, scale bars indicates 5 micrometers. The arrows indicate nuclear organizer regions, NORs (m = metacentric, sm = submetacentric and t = telocentric chromosomes).

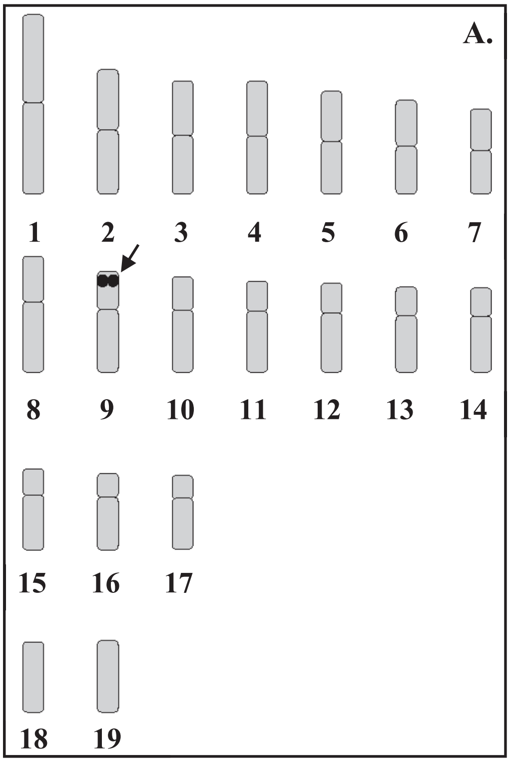

Figure 4. Idiogram showing lengths and shapes of chromosomes of the black - spotted pufferfish (A. nigropunctatus), 2n=38 (A.). The arrow indicates NOR - bearing chromosome pair 9.

Table 1. Review of cytogenetic publications in the genus Arothron (Tatraodonidae).

A. nigropunctatus	**2n**	**Chromosome formula**	**NF**	**Reference**
Arothron hispidus	42	36sm + 6t	78	Natarajan and Subrahmanjhan (1974)
A. immaculatus	42	14m + 16sm + 12t	72	Arai and Nagaiwa (1976)
	42	12m + 14sm + 16t	68	Choudhury *et al.* (1982)
A. leopardus	40	14m + 14sm + 12t	68	Choudhury *et al.* (1982)
A. nigropunctatus	38	14m + 20sm + 4t	72	Present study
A. reticularis	42	12m + 14sm + 16t	68	Choudhury *et al.* (1982)

Remarks: 2n = diploid chromosome number, NF = fundamental number, m = metacentric, sm = submetacentrics and t = telocentric chromosomes.

Table 2. Mean length of short arm chromosome (Ls), length long arm chromosome (Ll), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 10 metaphase cells of the black - spotted pufferfish (A. nigropunctatus), 2n=38.

Chro.pair	Ls	Ll	LT	RL±SD	CI±SD	Chro.size	Chro.type
1	0.98	1.03	2.01	0.097±0.007	0.511±0.005	Large	Metacentric
2	0.68	0.71	1.39	0.067±0.004	0.513±0.008	Medium	Metacentric
3	0.61	0.65	1.27	0.061±0.005	0.515±0.015	Medium	Metacentric
4	0.62	0.65	1.26	0.061±0.004	0.511±0.012	Medium	Metacentric
5	0.56	0.59	1.15	0.056±0.004	0.514±0.008	Medium	Metacentric
6	0.52	0.53	1.05	0.051±0.005	0.509±0.005	Medium	Metacentric
7	0.46	0.49	0.95	0.046±0.003	0.516±0.015	Small	Metacentric
8	0.52	0.79	1.31	0.062±0.005	0.605±0.021	Medium	Submetacentric
9*	0.43	0.70	1.13	0.055±0.003	0.622±0.022	Medium	Submetacentric
10	0.37	0.70	1.07	0.052±0.003	0.651±0.018	Medium	Submetacentric
11	0.34	0.68	1.02	0.049±0.002	0.671±0.025	Medium	Submetacentric
12	0.34	0.66	1.00	0.048±0.004	0.662±0.025	Small	Submetacentric
13	0.32	0.63	0.95	0.046±0.005	0.665±0.021	Small	Submetacentric
14	0.32	0.63	0.94	0.045±0.003	0.666±0.033	Small	Submetacentric
15	0.30	0.61	0.91	0.044±0.004	0.676±0.023	Small	Submetacentric
16	0.27	0.59	0.86	0.042±0.004	0.687±0.024	Small	Submetacentric
17	0.27	0.57	0.83	0.041±0.003	0.684±0.037	Small	Submetacentric
18	0.00	0.80	0.80	0.039±0.004	1.000±0.000	Small	Telocentric
19	0.00	0.80	0.80	0.039±0.003	1.000±0.000	Small	Telocentric

Remarks: * = NOR - bearing chromosome and Chro. = chromosome.

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5. References

Alfaro, M.E., Santini, F. & Brock, C.D. (2007). Do reefs drive diversification in marine teleosts? Evidence from the pufferfishes and their allies (order Tetraodontiformes). *Evolution 61*, 2104-2126.

Arai, R. (2011). *Fish karyotypes: A check list*. Spring Tokyo, Japan.

Arai, R. & Nagaiwa, K. (1976). Chromosomes of tetraodontiform fishes from Japan. *Bulletin of the National Museum of Nature and Science, Series A 2* (1), 59-72.

Brum, M.J.I. (1996). Cytogenetic studies of Brazilian marine fish. *Brazilian Journal of Genetics, 19*, 421-427.

Cano, J., Pretel, A., Melendez, S., Garcia, F., Caputo, V., Fenocchio, A.S. & Bertollo, L.A.C. (1996). Determination of early stages of sex chromosome differentiation in the sea bass Dicentrarchus labrax L. (Pisces: Perciformes). *Cytobios, 87* (348), 45-59.

Caputo, V., Marchegiani, F., Sorice, M. & Olmo, E. (1997). Heterochromatin heterogeneity and chromosome variability in four species of gobiid fishes (Perciformes: Gobiidae). *Cytogenetics and Cell Genetics, 79* (3-4), 266-271.

Chaiyasan, P., Supiwong, W., Saenjundaeng, P., Seetapan, K., Pinmongkhonkul, S. & Tanomtong, A. (2018). A report on classical cytogenetics of hihgfin barb fish, Cyclocheilichthys armatus (Cypriniformes, Cyprinidae). *Cytologia, 83* (2), 149-154.

Choudhury, R.C., Prasad, R. & Das, C.C. (1982). Karyologycal studies in five tetraodontiform fishes from Indian Ocean. *Copeia, 1982*, 728-732.

Corrêa, M.M.O. & Galetti Jr., P.M. (1997). Chromosomal diversity in Scorpaenidae (Teleostei, Scorpaeniformes): Cytogenetic studies in Scorpaena brasiliensis and Scorpaena isthmensis from the Coast of Rio de Janeiro, Brazil. *Cytologia, 62* (4), 397-404.

Ditcharoen, S., Bertollo, L.A.C., Ráb, P., Hnátková, E., Molina, W.F., Liehr, T., Tanomtong, A., Triantaphyllidis, C., Ozouf - Costaz, C., Tongnunui, S., Pengseng, P., Supiwong, W., Aroutiounian, R. & Cioffi, M.B. (2019). Genomic organization of repetitive DNA elements and extensive karyotype diversity of silurid cafishes (Teleostei: Siluriformes): A comparative cytogenetic approach. *International Journal of Molecular Sciences 20: Article Number 3545*.

Galetti Jr., P.M., Aguilar, C.T. & Molina, W.F. (2000). An overview on marine fish cytogenetics. *Hydrobiologia, 420*, 55-62.

Gomes, V., Phan, V.N. and Passos, M.J.A.C.R. (1994). Karyotypes of three species of marine catfishes from Brazil. *Boletim do Instituto Oceanografico, 42* (1-2), 55-61.

Howell, W.M. & Black, D.A. (1980). Controlled silver - staining of nucleolus organizer regions with a protective colloidal developer: A 1 - step method. *Experientia, 36*, 1014-1015.

Klinkhardt, M., Tesche, M. & Greven, H. (1995). *Database of Fish Chromosome*. Westarp Wissenschaften, Magdeburg.

Matsuura, K. (2015). Taxonomy and systematics of tetraodontiform fishes: A review focusing primarily on progress in the period from 1980 to 2014. *Ichthyological Research, 62*, 72-113.

Mohannath, G., Pontvianne, F. & Pikaard, C.S. (2016). Selective nucleolus organizer inactivation in Arabidopsis is a chromosome positioneffect phenomenon. *Proceedings of the National Academy of Sciences of the United States of America*.

Morescalchi, A., Hureau, J.C., Olmo, E., Ozouf - Costaz, C., Pisano, E. & Stanyon, R. (1992). A multiple sex - chromosome system in Antarctic ice - fishes. *Polar Biology, 11* (8), 655-661.

Natarajan, R. and Subrahanjam, K. (1974). A karyotype study of some teleosts from Portonovo waters. *Proceedings of the Indian Academy of Sciences (B) 70* (pp. 173-196).

Ojima, Y. (1985). Cited in Rishi, K.K. Current status of fish cytogenetics. In: Das, P. and Jhingram, V.G. (Eds.). *Fish Genetics in India*. Today and Tomorrow's Printers and Publishers, India.

Pauls, E., Affonso, P.R.A.M., Netto, M.R. C.B. & Pacheco, M.L. (1996). Supernumerary chromosomes on marine fish Upeneus parvus (Poem 1853, Mullidae) from Atlantic Ocean (chromosomes Supernumerarios en el pez Upeneus parvus (Poey 1853, Mullidae) del Océano Atlántico). *Archivos de Zootecnia, 45* (170), 295-299.

Sá - Gabriel. G.L. & Molina. F.W. (2004). Sex Chromosomes in Stephanolepis hispidus (Monacanthidae, Tetraodontiformes). *Cytologia, 69* (4), 447-452.

Santini, F., Sorenson, L. & Alfaro, E.M. (2013). A new phylogeny of tetraodontiform fishes (Tetraodontiformes, canthomorpha) based on 22 loci. Molecular *Phylogenetics and Evolution, 69*, 177-187.

Sarasan, T., Jantarat, S., Supiwong, W., Yeesin, P., Srisamoot, N. & Tanomtong, A. (2018). Chromosomal analysis of two snakehead fishes, Channa marulius (Hamilton, 1822) and C. marulioides (Bleeker, 1851) (Perciformes: Channidae) in Thailand. *Cytologia, 83* (1), 115-121.

Sharma, O.P., Tripathi, N.K. & Sharma, K.K. (2002). A review of chromosome banding in fishes. In: Sobti, R.C., Obe, G. & Athwal, R.S. (Eds.), *Some Aspects of Chromosome Structure and Functions*. Narosa Publishing House (pp. 109-122).

Sola, L., Cataudella, S. & Capanna, E. (1981). New developments in vertebrate cytotaxonomy. III. Karyology of bony fishes: A review. *Genetica, 54* (3), 285-328.

Supiwong, W., Pinthong, K., Seetapan, K., Saenjundaeng, P., Bertollo, L.A.C., Oliveira, E.A., Yano, C.F., Liehr, T., Phimphan, S., Tanomtong, A. & Cioffi, M.B. (2019). Karyotype diversity and evolutionary trends in the Asian swamp eel Monopterus albus (Synbranchiformes, Synbranchidae): a case of chromosomal speciation?. *BMC Evolutionary Biology, 19*.