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Cytogenetics of Two *Onychostoma* Species in Taiwan by Ag-NOR and 18S rDNA Profiles

Chiao-Chuan Han[1,2], Tsair-Bor Yen[3], Nian-Cih Chen[4], and Mei-Chen Tseng[4,*]

[1]*National Museum of Marine Biology and Aquarium, Pingtung 944, Taiwan. E-mail: hancc@nmmba.gov.tw*

[2]*Graduate Institute of Marine Biology, National Dong Hwa University, Hualien 974, Taiwan*

[3]*Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, Pingtung 912, Taiwan. E-mail: tbyen@mail.npust.edu.tw*

[4]*Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung 912, Taiwan*

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Chiao-Chuan Han, Tsair-Bor Yen, Nian-Cih Chen, and Mei-Chen Tseng (2017) Both *Onychostoma barbatulum* and *O. alticorpus* are primary freshwater fish in Taiwan. The former has been developed as an aquaculture species with high economic value, while the latter is a native endemic species in Taiwan. Understanding the cytogenetic information of these two species is necessary for their selected breeding, recovery, and management. In this study, Giemsa staining, silver-binding nucleolar organizer region (Ag-NOR), C-banding, and fluorescence *in situ* hybridization (FISH) with 18S ribosomal (r)DNA probes were used to analyze the cytogenetic characteristics. Results of Giemsa staining showed that the two *Onychostoma* species shared the same number of chromosomes, 2n = 50. Respective karyotype formulas of the female and male were 10 m + 22 sm + 10 st + 8 t and 11 m + 22 sm + 10 st + 7 t in *O. barbatulum*, and 14 m + 18 sm + 8 st + 10 t and 15 m + 18 sm + 8 st + 9 t in *O. alticorpus*. Karyotypes of both species showed a pair of heteromorphic chromosomes in male fish. Their sex determination should be the XX/XY system. Two pairs of Ag-NORs were found in *O. barbatulum*, but only one pair occurred in *O. alticorpus*. C-banding areas were observed on centromeres or telomeres of some chromosomes. FISH revealed different cytogenetic characters between these two species. The above cytogenetic information will contribute to species identification, population recovery, and advantages for breeding and management in the future.

Key words: Endemic species, Fluorescence *in situ* hybridization, Karyotype, Sex determination, Species identification.

BACKGROUND

It is known that there are 23 *Onychostoma* species that are mainly found in eastern Asia, among which *O. barbatulum* and *O. alticorpus* are distributed on the main island of Taiwan. Both species mostly reside in rivers north of the Kaoping River in southwestern Taiwan and north of the Taimali River in eastern Taiwan (Tzeng 1986; Shen 1993). They dwell in rivers with good water quality. Algae attached to stones are their staple food; moreover, they also take small invertebrates. They were recorded breeding in January to July (Chang 1993). *Onychostoma barbatulum* has been developed as an aquaculture species, and females have a higher growth rate. Artificial breeding and selection efforts were carried out by Tseng et al. (2017). *Onychostoma alticorpus* is an endemic species in Taiwan. Because of depletion of wild fish resources by serious pollution of rivers and overexploitation, it is considered to require recovery efforts and management (Kottelat 1996; Jang-Liawn 2008).

Both species have similar body shapes before reaching a length of about 7 cm. Consequently, it is difficult to distinguish young individuals of

Chiao-Chuan Han and Tsair-Bor Yen contributed equally to this work.

*Correspondence: Tel: +886-8-7703202 ext. 6227. E-mail: mctseng@mail.npust.edu.tw

these two species by external morphological traits. It should be noted that in exploring genetic differences between the two species, the possibility of hybridization of the two species cannot be ruled out. The development of cytogenetics with modern staining and microscopic techniques has resulted in a better understanding of chromosomal structures and provides a useful tool to study species evolution and efficiently characterize species (Rossi et al. 1997; Eichler and Sankoff 2003; Foresti de Almeida-Toledo et al. 2007). The cytogenetics of *Onychostoma* are currently not fully known, and only a few basic studies have been published simply comprising the chromosome numbers and karyotypes of several species (Table 1). Moreover, the cytogenetics of *Onychostoma* hybrids have never been studied.

In general, numbers of chromosomes (2n) in fish range 16~446 (Yu and Yu 1990; Hallerman 2003). The majority of fish chromosome numbers are 2n = 48, which is considered to be an ancestral characteristic of bony fishes (Leggatt and Iwama 2003; Galetti et al. 2006). Nevertheless, numbers of chromosomes in the Cyprinidae range 42~446, among which *Acheilognathus gracilis* has the least and *Diptycus dipogon* the greatest numbers (Hong and Zhou 1985; Yu and Yu 1990). Numbers of chromosomes of 2n = 50 in *Onychostoma lini*, *O. simum*, *O. gerlachi*, and *O. elongatum* were described in previous reports (Gui et al. 1986; Li et al. 1986; Dai 2013; Han et al. 2015), whereas intraspecific and interspecific diversities in numbers of chromosomes in *O. barbatulum* and *O. alticorpus* still need to be explored.

In the past, the cytogenetics of more than 1700 fishes were examined (Arkhipchuk 1995), but sex chromosomes were identified in only 176 species (10.4% of those). The reason that sex chromosomes are just found in a few fishes may be due to chromosomes of fish being too small to distinguish, or microscopic resolution being insufficient to examine them clearly (Devlin and Nagahama 2002). Sex determination systems of fish are divided into XX/XY and ZZ/ZW types, for which the heteromorphic chromosome respectively exists in the male and female (de Oliveira et al. 2007; Diniz et al. 2008). Therefore, to elucidate the sex determination systems of *O. barbatulum* and *O. alticorpus*, karyotypes of females and males of both species were analyzed in this study.

In addition to chromosome numbers and karyotypes, some cytogenetic traits of both *Onychostoma* species still need to be carefully explored. In general, it is difficult to identify paired chromosomes before staining. Hence, many banding techniques of chromosomes have been developed using acid-base, heat, salt, enzyme, or dye treatments. Due to different DNA and protein compositions of each pair of chromosomes, these techniques show different banding patterns for distinguishing chromosomes. The C-banding technique can exhibit positions of constitutive heterochromatin, which plays an important role in the karyotype diversity of fish (Kavalco et al. 2004). The silver-staining nuclear organizer region (Ag-NOR) is the position of the major transcriptional activity of ribosomal (r)DNA (Reeder 1990). NOR patterns of the Salmonidae and Cyprinidae were reported to be polymorphic (Phillips and Ihssen 1985; Takai and Ojima 1992; Pendás et al. 1994; Castro et al. 2001). For example, the NORs of *Leuciscus* were interspecifically polymorphic, and those of *Notropis chrysocephalus* and *Chondrostoma lusitanicum* were intraspecifically polymorphic (Gold and Zoch 1990; Rodrigues and Collares-Pereira 1999; Boron et al. 2009).

rRNA is the most abundant RNA in cells. Transcriptions of 18S, 5.8S, and 28S rRNAs are

Table 1. List of karyotypic studies on six *Onychostoma* species from 1986 to 2017

Species	Karyotype	2n	NF	NORs	Reference
O. barbatulum	♀ 10 m + 22 sm + 10 st + 8 t ♂ 11 m + 22 sm + 10 st + 7 t	50	82 83	2 pairs	Han et al. 2015 this study
O. alticorpus	♀ 14 m + 18 sm + 8 st + 10 t ♂ 15 m + 18 sm + 8 st + 9 t	50	82 83	1 pair	Han et al. 2015 this study
O. lini	12 m + 8 sm + 4 st + 26 t	50	70	-	Dai 2013
O. simum	10 m + 16 sm + 16 st + 8 t	50	76	-	Li et al. 1986
O. gerlachi	12 m + 12 sm + 14 st + 12 t	50	74	-	Gui et al. 1986
O. elongatum	12 m + 12 sm + 14 st + 12 t	50	74	-	Gui et al. 1986

2n, chromosome number; NF, fundamental arm number; NORs, nucleolus organizer regions; -, unknown; m: metacentric; sm: submetacentric; st: subtelocentric; t: telocentric.

produced from 45S rDNA by RNA polymerase I (Doudna and Rath 2002). Therefore, the 18S rDNA gene is often used as a specific probe to locate the 45S rDNA region in cytogenetic studies (Gross et al. 2010). It is usually found at one or several different chromosomal loci with a tandem repeated arrangement in higher eukaryotes; nevertheless, the gene is often available in cytotaxonomic studies by fluorescence *in situ* hybridization (FISH) (Gornung et al. 1997; Nakajima et al. 2012; Maneechot et al. 2016).

Both *Onychostoma* species have similar morphological traits. Therefore, the cytogenetic information will provide a useful tool for classification and hybrid identification. The aims of this study were to compare cytogenetic characters of these two species by Ag-NOR, C-banding, and FISH analyses using an 18S rDNA probe and provide genetic information for recovery of native populations and selected breeding in aquaculture farms in the future. In addition, a hybrid was also examined in the study.

MATERIALS AND METHODS

Sampling

Onychostoma barbatulum and *O. alticorpus* were collected from Nanzixian Stream (22.15°N, 120.42°E) in southwestern Taiwan and were maintained in a 2-ton fiberglass-reinforced plastic (FRP) tank. Karyotype analyses of *O. barbatulum* and *O. alticorpus* were each performed on 15~18 random specimens. The probability of an interspecific hybrid was examined by experimental artificial breeding of a female *O. barbatulum* and male *O. alticorpus*.

Feeding

Some individuals of both species were fed in a 2-ton FRP tank until the chromosome preparation. Eight *O. barbatulum* and eight *O. alticorpus* individuals were separately quarantined in a 2-ton FRP tank for 2 weeks. After quarantine, healthy adults were moved to breeding tanks (0.5 ton each) for the hybridization test. The tanks were equipped with temperature control, filtration systems, and life support to maintain a good life quality (21.5°C for *O. barbatulum*; 23°C for *O. alticorpus*; dissolved oxygen (DO) of > 7.5 mg/L; and pH of 7.0~8.0). Each breeding tank contained one male and three female individuals. Fish were fed twice a day. Twelve hours of light and darkness were controlled by a timer setting. In terms of water quality, NH_4^+ (< 0.04 mg/L) and NO_2^- (< 0.2 mg/L) were monitored weekly.

Artificial hybridization

When the fish showed sand-stirring behavior, ova of *O. barbatulum* and sperm of *O. alticorpus* were stripped by gently pressing the abdomen. Fertilized eggs were evenly dispersed in two air-supplied tanks (40 L) under a rearing temperature of 21.5°C. One week after the eggs hatched, the fry were fed with freshly hatched brine shrimp for 3 weeks. Afterward, the proportion of commercial fish powder was gradually increased in the feed until it completely replaced the brine shrimp. The temperature was also gradually raised to 23°C. After 3 months, fish were transferred to an FRP tank (2 tons, with a water temperature of 23~25°C) for 1 year of rearing until the chromosome preparation.

Chromosome preparation

A mixture containing minimal essential medium (Eagle's), 15% fetal bovine serum, and 0.0002% colchicine was filtered through an Acrodisc syringe filter with pore size of 0.45 μm (Pall, Ann Arbor, MI, USA) for sterilization. The sterilized mixture was dispensed to 15-ml centrifuge tubes and stored in a -80°C freezer for further experiments. The stored mixture was allowed to equilibrate to room temperature before use. After the fish were cooled on ice as anesthesia, their head kidneys and renal tissues were excised, cut into small pieces, and cultured with the prepared mixture in centrifuge tubes. Chromosome slides were prepared following procedures of Han et al. (2015).

Giemsa staining

Slides of the two species and their hybrid were further stained with 5% Giemsa (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, rinsed with distilled water, air-dried at room temperature, and finally mounted with gum arabic (Ledley et al. 1972).

Ag-NOR analysis

Slides of the two *Onychostoma* species were completed by silver staining; 2% (w/v) gelatin

was prepared by thoroughly mixing 1 mL formic acid, 2 g gelatin, and 99 mL double-distilled (dd) H_2O on a hot plate at 40~50°C. The solution was preserved in a dark glass bottle. A 50% silver nitrate solution was prepared by dissolving 5 g crystalline silver nitrate in 10 mL ddH_2O. This solution was stored in a dark glass bottle at 4°C. Three drops of 2% gelatin and four drops of the 50% silver nitrate solution were added to the slide and covered with a cover glass. The slide was further placed on a 65°C hot plate for 2~3 min until it turned brown, rinsed with ddH_2O, and air-dried prior to being stained with Giemsa for 30 s (Dracopoli et al. 2001). The slide was observed at 1000× under a light microscope (Leica DM 2500 Microsystems, Wetzlar, Germany) with an oil lens.

C-Banding analysis

Slides of the two *Onychostoma* species with chromosome samples were maintained at 60°C overnight, and transferred into 0.2 N HCl at room temperature for 5~15 min. Afterward, a sample was rinsed with ddH_2O, air-dried, incubated in 5% $Ba(OH)_2$ for 0.5~5 min at 50°C, and rinsed with ddH_2O. The sample was further rinsed with 2× SSC (0.3 M NaCl and 0.03 M sodium citrate, at pH 7.0) for 90 min at 60°C, washed with ddH_2O, air-dried, and stained with Giemsa for 90 min (Fujiwara et al. 1998). The stained sample was observed at 1000× under a light microscope (Leica DM 2500 Microsystems) with an oil lens.

18S rDNA subcloning and analysis

DNA was extracted from 5~10 mg of muscle tissue of one specimen of both *O. barbatulum* and *O. alticorpus* using a Puregene Core kit A (Qiagen Sciences, Germantown, MD, USA). The 18S rDNA of the two species was amplified by polymerase chain reactions (PCRs) using 18S forward and reverse primers (White et al. 1990). Amplification was performed in a Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The reaction solution consisted of approximately 50 ng genomic DNA, 50 pmol each of the forward and reverse primers, 2.5 mM dNTP, 0.1 mM $MgCl_2$, 10× buffer, and 2 U *Taq* polymerase (Takara Shuzo, Shiga, Japan) brought up to 50 μL with sterile water. The PCR program included one cycle of 4 min at 94°C, 35 cycles of 30 s at 94°C, 1 min at 54°C, and 1 min at 72°C, followed by a single further extension of 5 min at 72°C. We evaluated 10 μL of the product on a 0.8% agarose gel to check the PCR success and confirm the product sizes. The remaining PCR products were run on 0.8% agarose gels and purified using a DNA Clean/Extraction kit (GeneMark, Taichung, Taiwan). Purified DNA was subcloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into the *Escherichia coli* JM109 strain. Plasmid DNA was isolated using a mini plasmid kit (Geneaid, Taichung, Taiwan). Two clones from *O. barbatulum* and *O. alticorpus* were sequenced on an Applied Biosystems (ABI, Foster City, CA, USA) automated DNA sequencer ABI3730x1 using a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA). T7 or SP6 primer was used in the sequencing reaction, and the PCR cycle parameters for sequencing were 35 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C. The 18S rDNA sequences were checked using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov). The difference between the two 18S rDNA sequences was estimated by MEGA software (Tamura et al. 2007).

FISH using 18S rDNA probes

Labeled 18S rDNA probes of the two species were generated using a PCR DIG probe synthesis kit (Roche, Mannheim, Germany). The reaction solution contained 10× buffer, 4 mM $MgCl_2$, 200 μM dNTP, 1 μM 18S rDNA primers, 50 ng plasmid DNA, and 2 U *Taq* polymerase, and was brought up to 100 μL with sterile water. DIG probes were purified by ethanol precipitation.

Chromosome slides were processed with 100 μg/mL RNase A in 2× SSC buffer at 37°C for 1 h, and washed thrice in 2× SSC for 5 min each. Slides were then quickly immersed in a cold series of ethanol solutions (70%, 95%, and 100%) to dehydrate the chromatin. After air-drying, chromosome spreads were done by exposing chromosomes to 0.005% pepsin (Roche) in 10 mM HCl at 37°C for 10 min to remove residual proteins, and then washed in phosphate-buffered saline (PBS). Slides were then quickly dehydrated through a cold ethanol series and air-dried. Chromosomes were denatured at 80°C for 5 min in hybridization buffer (2× SSC, 10% dextran sulfate, and 50% deionized formamide). All slides were placed on ice for 3~5 min prior to the addition of 35 μL hybridization buffer containing 50 ng labeling probe. Hybridization occurred at 37°C for 12~16 h. Post-hybridization washes were carried out at

42°C for 15 min in 1× SSC with 50% deionized formamide, followed by 0.1× SSC at 60°C for 5 min thrice, and then rinsed thrice in PBS buffer with 0.2% Tween 20 at 37°C for 5 min each. Anti-digoxigenin-rhodamine Fab fragments were diluted to 1:200 using TNB buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% blocking reagent). An antibody solution (100 μL) was added to the slide at 37°C for 30 min and then washed for 5 min thrice in TNT buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) before being transferred to room temperature. The antibody solution at 100 μL was added to the slide and incubated at 37°C for 30 min, and was washed thrice in TNT buffer at room temperature for 5 min. The slide was immersed in a 70%~100% ethanol series to dehydrate the chromatin. Chromosomes were counterstained with 0.05 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) and mounted in a 1,4-diazabicyclo [2.2.2] octane (DABCO) anti-fading solution (50% glycerol and 2% DABCO in PBS).

Chromosome observation

Chromosomes were observed using an optical microscope at 1000× with an oil lens. Digital images of the chromosomes were recorded and analyzed with a chromosome band analytical system (BandView 5.5, Applied Spectral Imaging, Migdal HaEmek, Israel). Chromosomes were classified according to relative lengths of their arms, and every chromosome could be identified into one of four groups (m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric). Each group was characterized by a defined amplitude of arm ratios (m: 1~1.7, sm: 1.7~3, st: 3~7, t: 7~∞) as described by Levan et al. (1964). In the FISH experiments, all slides were observed on a Leica DMLB fluorescence microscope (Leica Microsystems Wetzlar) equipped with a cooled CCD camera.

RESULTS

Numbers of chromosomes in the two *Onychostoma* species ranged *ca*. 40~50, and they mostly shared the same number, 2n = 50 (Tables 2, 3). The great majority of chromosomes were metacentric and submetacentric. The karyotype formulae of female and male *O. barbatulum* were 10 m + 22 sm + 10 st + 8 t and 11 m + 22 sm + 10 st + 7 t, respectively. The fundamental arm numbers (FNs) were 82 and 83. The karyotype formulae of the female and male *O. alticorpus* were 14 m + 18 sm + 8 st + 10 t and 15 m + 18 sm + 8 st + 9 t, respectively; the (FNs) were 82 and 83. Although obvious differences in the karyotypes were present between these two species, both species shared a pair of larger sm chromosomes. In addition, males of *O. barbatulum* and *O. alticorpus* had a pair of heteromorphic chromosomes which indicated that the sex determination system should be XX/XY (Fig. 1). Two pairs of NORs were located on telomeres of the no. 1 and 3 metacentric chromosomes in *O. barbatulum*, and only one pair of NORs was located on telomeres of the no. 2 metacentric chromosome in *O. alticorpus*, showing that there was a significant difference in the number of NORs between these two species (Fig. 2). However, there was no intraspecific difference in the number or positions of the Ag-NORs in males and females of these two species. While C-banding staining areas were distributed in the centromeres or telomeres of several chromosomes, no significant differences were observed between these two species (Fig. 3).

18S rDNA sequences were subcloned from *O. barbatulum* and *O. alticorpus* and were respectively 1844 and 1842 bp in length. Respective ratios of G+C in *O. barbatulum* and *O. alticorpus* were 56.3% and 56.6%. In total, 13 different nucleotides and two insertions/deletions were observed between these two sequences (Fig. 4). When two sets of 18S rDNA probes were individually hybridized to chromosomes of these two species using the FISH technique, an 18S rDNA locus was located on telomeres of the no. 10 submetacentric chromosome in both *O. barbatulum* and *O. alticorpus* (Fig. 5).

All fertilized eggs of the hybrids had a very low hatching rate (< 1%). Most surviving individuals had a body shape or eye deformity (Fig. 6). The number of chromosomes in the hybrid was 75 and was triploid (Fig. 7).

DISCUSSION

Among 435 species of the Cyprinidae, chromosome numbers of 282 species were identified as being 2n = 50, those of 70 species were 2n = 48; and those of 52 species were 100 or 150. Therefore, 2n = 50 is the most common character of the Cyprinidae (Buth et al. 1991; Klinkhardt et al. 1995; Sola and Gornung 2001; Ueda et al. 2001). The same results were

obtained for both *O. barbatulum* and *O. alticorpus*. Chromosomes of the Cyprinidae are generally characterized by a high fundamental arm number (FN); that is, there are more metacentric (m) and submetacentric (sm) chromosomes in the karyotype. So far, the study of the cytogenetics in *Onychostoma* fishes has been insufficient. The karyotypic formulae and FN of *O. simum* (Sauvage and Dabry de Thiersant 1874), *O. elongatum* (Pellegrin and Chevey 1934), *O. lini* (Wu 1939), and *O. gerlachi* (Peters 1881) are listed in table 1. Among these species, *O. lini* has the highest number of t chromosomes, because it is very difficult to distinguish between st and t chromosomes. In this study, two *Onychostoma* species also had similar characteristics to Cyprinidae, with both of their FNs being > 80. *Onychostoma barbatulum* consisted of 10 or 11

Table 2. Diploid chromosome counts of 18 *Onychostoma barbatulum* specimens

Specimen number	Sex	Number of cells analyzed	Diploid counts											
			≤ 40	41	42	43	44	45	46	47	48	49	50	> 50
1	♀	55	1	2		1		1		2	2	5	41	
2	♀	53		1	1	1		1	2	3	2	7	35	
3	♂	50					1			1	1	5	42	
4	♀	59	1							1		6	51	
5	♂	43			1			1		2	1	2	36	
6	♀	26								1		3	22	
7	♀	34				1				2	1	1	29	
8	♀	35							1			6	28	
9	♂	34			1	1		1		1		3	27	
10	♂	29											29	
11	♀	30							1		2	2	25	
12	♂	30								1	1	3	25	
13	♂	30						1			3	6	20	1
14	♀	30	1							3		3	23	
15	♂	30									2	1	27	
16	♂	28	1		1	1	1						24	
17	♂	21								1	3		17	
18	♂	26	2						1	1	2	1	19	

Table 3. Diploid chromosome counts of 15 *Onychostoma alticorpus* specimens

Specimen number	Sex	Number of cells analyzed	Diploid counts										
			≤ 40	41	42	43	44	45	46	47	48	49	50
1	♀	31	1						1	2	3	1	23
2	♀	31	1			1	1	2	1	1	2		22
3	♀	36						1	2	3		2	28
4	♂	34								3	1		30
5	♂	38				1			2	2	3	1	29
6	♂	34			1					3	1	1	28
7	♂	31					1	2		1		3	24
8	♀	34			1	2			2		2		27
9	♀	33				1				1	1	2	28
10	♀	33	1		1					3	3		25
11	♂	32							2	3	3	2	22
12	♂	34				1	1		2	3	1	1	25
13	♀	33					1	1	2		2		27
14	♂	36							1		1	3	31
15	♂	38	1					1			2	2	32

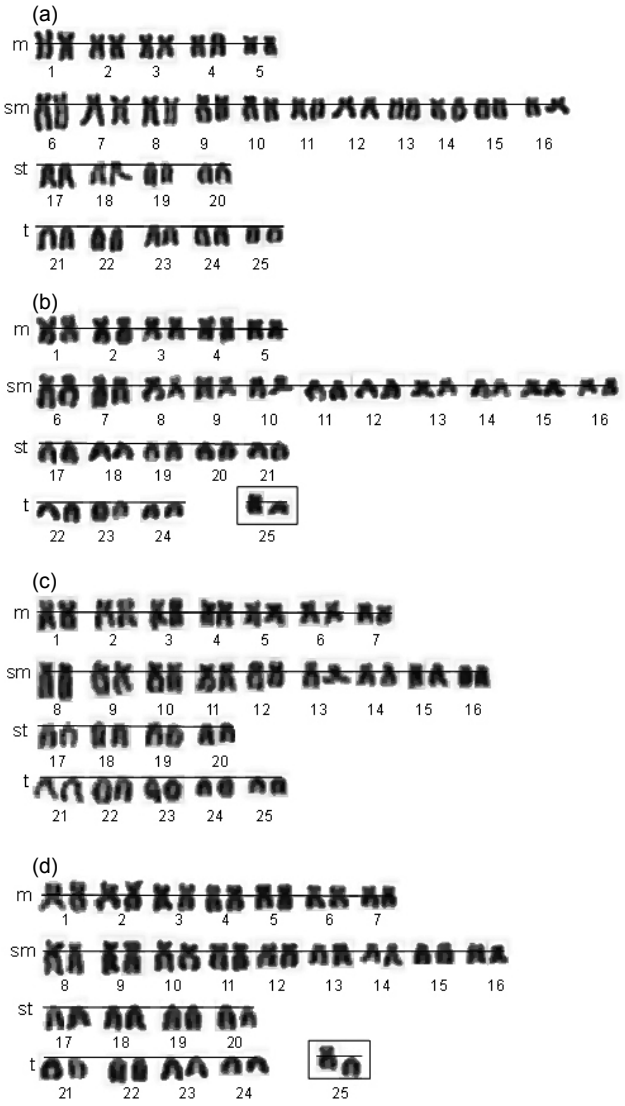

Fig. 1. Karyotypes of (a) female and (b) male *Onychostoma barbatulum* and (c) female and (d) male *O. alticorpus*.

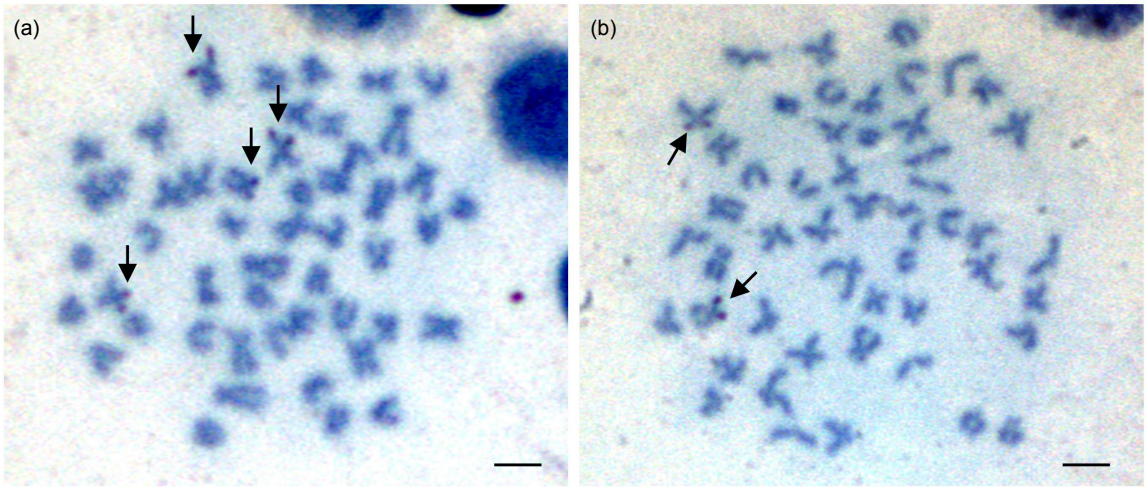

Fig. 2. NOR locations of (a) *Onychostoma barbatulum* and (b) *O. alticorpus* are indicated by arrows. Bars equal 5 μm (1000×).

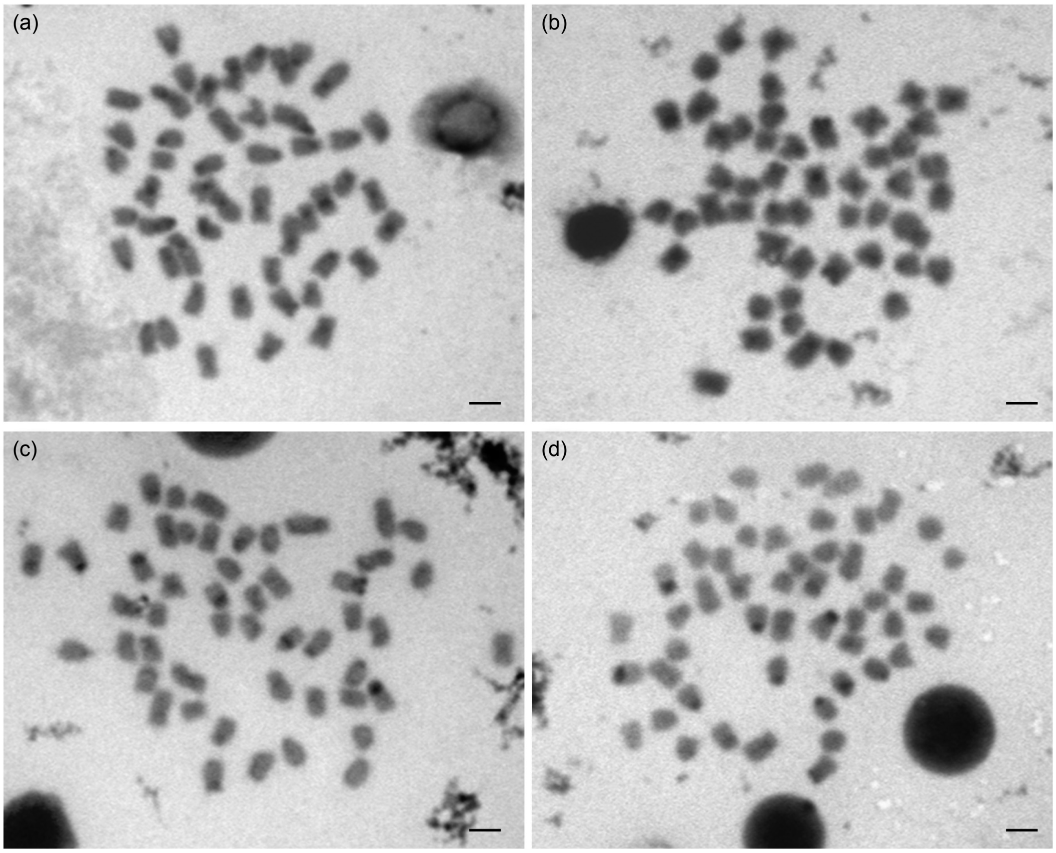

Fig. 3. C-Banded metaphase of *Onychostoma barbatulum* (a: female, b: male) and *O. alticorpus* (c: female, d: male). Darker staining on the chromosomes are where C-positive signals occurred. Bars equal 5 μm (1000×).

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                                                                                                                                         130
O. barbatulum  GTAGTCATAT GCTTGTCTCA AAGATTAAGC CATGCAGGTC TAAGTACACA CGGCCGGTAC AGTGAAACTG CGAATGGCTC ATTAAATCAG TTATGGTCCC TTTGATCGCT CCACCCGGTA CTTGGATAAC
O. alticorpus  GTAGTCATAT GCTTGTCTCA AAGATTAAGC CATGCAGGTC TAAGTACACA CGGCCGGTAC AGTGAAACTG CGAATGGCTC ATTAAATCAG TTATGGTCCC TTTGATCGCT CCACCCGGTA CTTGGATAAC
                                                                                                                                         260
O. barbatulum  TGTGGCAATT CCAGAGCTAA TACATGCAAA CGGGCGCCGA CCTGCCTCCC CCCCGGGGGT GGCGGGGACG CGTGCATTTA TCAGATCCAA AACCCAACCG GCGGCTTGGG CTCCGGCCCC TCCCCGGACC
O. alticorpus  TGTGGCAATT CCAGAGCTAA TACATGCAAA CGGGCGCCGA CCTGCCTCCC CCCCGGGGGG GGCGGGGACG CGTGCATTTA TCAGATCCAA AACCCATCCG GCGGTCGGGG CTCCGGCCCC GCCCCGGTCC
                                                                                                                                         390
O. barbatulum  CTTTGGTGAC TCTAGATAAC CTCGGGCCGA TCGCGCGCCC TCCGCGGCGG CGACGATTCT TTCGAATGTC TGCCCTATCA ACTTTCGATG GTACTTTAGG CGCCTACCAT GGTGACCACG GGTAACGGGG
O. alticorpus  CTTTGGTGAC TCTAGATAAC CTCGGGCCGA TCGCGCGCCC TCCGCGGCGG CGACGATTCT TTCGAATGTC TGCCCTATCA ACTTTCGATG GTACTTTAGG CGCCTACCAT GGTGACCACG GGTAACGGGG
                                                                                                                                         520
O. barbatulum  AATCAGGGTT CGATTCCGGA GAGGGAGCCT GAGAAACGGC TACCACATCC AAGGAAGGCA GCAGGCGCGC AAATTACCCA TTTCCGACTC GGAGAGGTAG TGACGAAAAA TAACAATACA GGTCTCTTTC
O. alticorpus  AATCAGGGTT CGATTCCGGA GAGGGAGCCT GAGAAACGGC TACCACATCC AAGGAAGGCA GCAGGCGCGC AAATTACCCA TTTCCGACTC GGAGAGGTAG TGACGAAAAA TAACAATACA GGTCTCTTTC
                                                                                                                                         650
O. barbatulum  GAGGCCCTGT AATTGGAATG AGCGTATCCT AAACCCATGG GTGAGGACCC ATTGGAGGGC AAGTCTGGTG CCAGCAGCCG CGGTAATTCC AGCTCCAATA GCGTATATTA AAGTTGCTGC AGTTAAAAAG
O. alticorpus  GAGGCCCTGT AATTGGAATG AGCGTATCCT AAACCCATGG GCGAGGACCC ATTGGAGGGC AAGTCTGGTG CCAGCAGCCG CGGTAATTCC AGCTCCAATA GCGTATATTA AAGTTGCTGC AGTTAAAAAG
                                                                                                                                         780
O. barbatulum  CTCGTAGTTG GATCTCGGGA GTGGGCTGGC GGTCCGCCGC GAGGCGAGCC ACCGCCTGTC CCGGACCCTG CCTCCCGGCG CCCCCCGGAT GCCCTTAACT GGGTGTCCGG TCACCTCGGG GCCCGGAGCG
O. alticorpus  CTCGTAGTTG GATCTCGGGA GTGGGCTGGC GGTCCGCCGC GAGGCGAGCC ACCGCCTGTC CCGGACCCTG CCTCCCGGCG CCCCCCGGAT GCCCTTAACT GGGTGTCCGG TCACCTCGGG GCCCGGAGCG
                                                                                                                                         910
O. barbatulum  TTTACTTTGA AAAAATTAGA GTGTTCAAAG CAGGCCGCCC GTCGCCGCTG AATACCGCAG CTAGGAATAA TGGAATAGGA CTCCGGTTCT ATTTTGTGGG TTTCTGGAAC CCGGGGCCAT GATTAAGAGG
O. alticorpus  TTTACTTTGA AAAAATTAGA GTGTTCAAAG CAGGCCGCCC GTCGCCGCTG AATACCGCAG CTAGGAATAA TGGAGTAGGA CTCCGGTTCT ATTTTGTGGG TTTCTGGAAC CCGGGGCCAT GATTAAGAGG
                                                                                                                                        1040
O. barbatulum  GACGGCCCGG GGGCATTCGT ATTGCGCCGC TAGAGGTGAA ATTCTTGGAC CGGCGCAAGA CGGACGAAAG CGAAAGCATT TGCCAAGAAT GTTTTCATTA ATCAAGAACG AAAGTCGGAG GTTCGAAGAC
O. alticorpus  GACGGCC-GG GGGCATTCGT ATTGCGCCGC TAGAGGTGAA ATTCTTGGAC CGGCGCAAGA CGGACGAAAG CGAAAGCATT TGCCAAGAAT GTTTTCATTA ATCAAGAACG AAAGTCGGAG GTTCGAAGAC
                                                                                                                                        1170
O. barbatulum  GACCAGATAC CGTCGTAGTT CCGACCGTAA ACGATGCCAA CCCGCGATCC GGCGGCGTTA TTCCCATGAC CCGCCGGGCA GCGTACGGGA AACCACGAGT CTTTGGGTTC CGGGGGGGAG TATGGTTGCA
O. alticorpus  GATCAGATAC CGTCGTAGTT CCGACCGTAA ACGATGCCGA CCCGCGATCC GGCGGCGTTA TTCCCATGAC CCGCCGGGCA GCGTGCGGGA AACCACGAGT CTTTGGGTTC CGGGGGG-AG TATGGTTGCA
                                                                                                                                        1300
O. barbatulum  AAGCTGAAAC TTAAAGGAAT TGACGGAAGG GCACCACCAG GAGTGGAGCC TGCGGCTTAA TTTGACTCAA CACGGGAAAC CTCACCCGGC CCGGACACGG AAAGGATTGA CAGATTGATA GCTCTTTCTC
O. alticorpus  AAGCTGAAAC TTAAAGGAAT TGACGGAAGG GCACCACCAG GAGTGGAGCC TGCGGCTTAA TTTGACTCAA CACGGGAAAC CTCACCCGGC CCGGACACGG AAAGGATTGA CAGATTGATA GCTCTTTCTC
                                                                                                                                        1430
O. barbatulum  GATTCTGTGG GTGGTGGTGC ATGGCCGTTC TTAGTTGGTG GAGCGATTTG TCTGGTTCAT TCCGATAACG AACGAGACTC CGGCTTGTTA AATAGTTACG CGGCCCCGTG CGGTCGGCGT TCAACTTCTT
O. alticorpus  GATTCTGTGG GTGGTGGTGC ATGGCCGTTC TTAGTTGGTG GAGCGATTTG TCTGGTTCAT TCCGATAACG AACGAGACTC CGGCTTGCTA AATAGTTACG CGGCCCCGTG CGGTCGGCGT TCAACTTCTT
                                                                                                                                        1560
O. barbatulum  AGAGGGACAA GTGGCGTTCA GCCACGCGAG ATGGAGCAAT AACAGGTCTG TGATGCCCTT AGATGTCCGG GGCTGCACGC GCGCCACAAT GGGCGGATCA GCGTGTGTCT ACCCTGCGCC GAGAGGCGCG
O. alticorpus  AGAGGGACAA GTGGCGTTCA GCCACGCGAG ATGGAGCAAT AACAGGTCTG TGATGCCCTT AGATGTCCGG GGCTGCACGC GCGCCACAAT GGGCGGATCA GCGTGTGTCT ACCCTGCGCC GAGAGGCGCG
                                                                                                                                        1690
O. barbatulum  GGTAACCCGC TGAACCCCGC TCGTGATCGG GACTGGGGAT TGAAACTATT TCCCATCAAC GAGGAGTTCC CAGTAAGCGC GGGTCATAAG CTCGCGTTGA TTAAGTCCCT GCCCTTTGTA CACACCGCCC
O. alticorpus  GGTAACCCGC TGAACCCCGC TCGTGATCGG GACTGGGGAT TGAAACTATT TCCCATCAAC GAGGAATTCC CAGTAAGCGC GGGTCATAAG CTCGCGTTGA TTAAGTCCCT GCCCTTTGTA CACACCGCCC
                                                                                                                                        1820
O. barbatulum  GTCGCTACTA CCGATTGGAT GGTTTAGTGA GGTCCTCGGA TCGGCCCCGC CGGGGCTCCT CGCGGGCCCT GGCGGAGCGC CGAGAAGACG ATCAAACTTG ACTATCTAGA GGAAGTAAAA GTCGTAACAA
O. alticorpus  GTCGCTACTA CCGATTGGAT GGTTTAGTGA GGTCCTCGGA TCGGCCCCGC CGGGGCTCCT CGCGGGCCCT GGCGGAGCGC CGAGAAGACG ATCAAACTTG ACTATCTAGA GGAAGTAAAA GTCGTAACAA
                                 1844
O. barbatulum  GGTTTCCGTA GGTGAACCTG CGGA
O. alticorpus  GGTTTCCGTA GGTGAACCTG CGGA
```

Fig. 4. Cloned 18S rDNA sequences from *Onychostoma barbatulum* (NCBI accession no. MF598161) and *O. alticorpus* (acc. no. MF598162). Gray highlights are variable sites.

metacentric and 22 submetacentric chromosomes, which differed from *O. alticorpus* which had a larger number of metacentric chromosomes (14 or 15 m). However, *O. barbatulum* and *O. alticorpus* had slightly larger NFs (82~83) than those of the others (70~76). *Onychostoma barbatulum* had two pairs of NORs on the m chromosome, while *O. alticorpus* had one pair of NORs on the m chromosome short arm, illustrating a difference in chromosomal characters between these two species. Buth et al. (1991) reported that 69 species of the Cyprinidae in North America possessed one or two pairs of NORs, and only three species had three pairs of NORs. Klinkhardt et al. (1995) pointed out that nearly 100 species of the Cyprinidae in their study contained only one pair of NORs which was located in telomeres of either the m or sm chromosome short arm. Most of the NOR pairs in the Cyprinidae of North American fishes were polymorphic with 15 or more types (Buth et al. 1991). Similar results were reported by Takai and Ojima (1992). They also found that NORs of more than 30 species of Japanese carp revealed high variations among species. These results from previous studies indicate that the morphological position of NORs can be an effective tool for cytogenetic classification in the Cyprinidae. For example, there are three types of NORs present

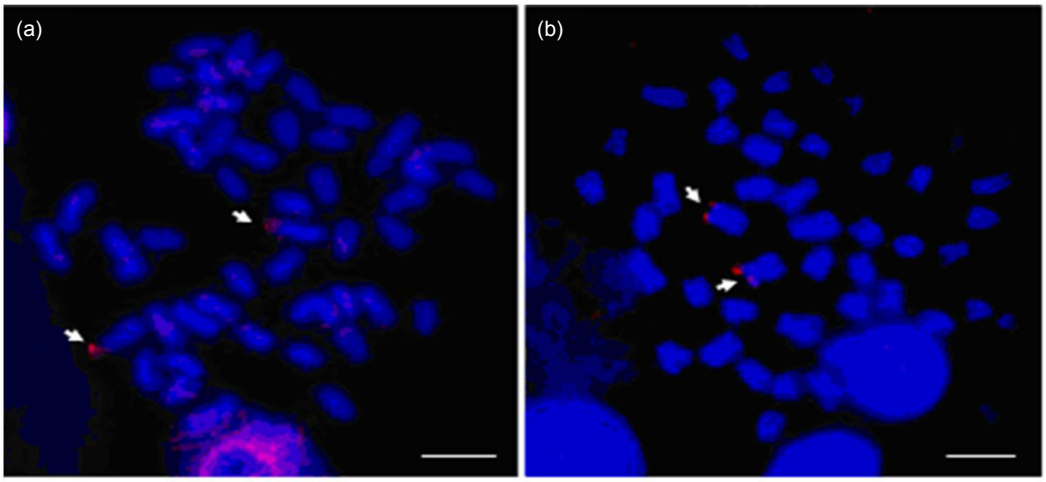

Fig. 5. FISH results of chromosomes of *Onychostoma* species using 18S rDNA probes; (a) *O. barbatulum* and (b) *O. alticorpus*. Bars equal 5 μm (1000×). Arrows indicate FISH signals.

Fig. 6. Body shape and eye deformities in (a) a male and (b) female hybrid of *Onychostoma barbatulum* × *O. alticorpus*. Bars equal 3 cm.

among three species of *Leuciscus*. *Leuciscus leuciscus* has two pairs of NORs located on the long arm of the sm chromosome and the short arm of the st chromosome; *L. idus* revealed a pair of NORs on the long arms of its st chromosome; and *L. cephalus* showed a pair of NORs on the short arms of the st chromosome (Boron et al. 2009). Therefore, positions and numbers of NORs are useful characters for distinguishing these three *Leuciscus* species. Also *O. barbatulum* and *O. alticorpus* had different morphological positions of the NORs, which can be applied in taxonomic studies in the future.

When interspecific karyotype patterns are relatively conservative, C-banding may present differences between species (Martinez et al. 1989; Galetti et al. 1991). This technique was used to identify karyotypic variations within a population of *Astyanax scabripinnis* (Mantovani et al. 2000). C-Banding in these two *Onychostoma* species appeared on either centromeres or telomeres of some chromosomes. It is difficult to discriminate *O. barbatulum* from *O. alticorpus* by C-banding. Such a widespread distribution of C-banding was also observed in *Rhodeus* spp., *Vimba* spp., and *Danio rerio* (Sola and Gornung 2001; Ueda et al. 2001; Rábová et al. 2003). In the past, the C-banding technique was mainly used to identify sex chromosomes based on specific positions of bandings and their chromatin-stained areas in chromosomes (Rudek 1974; Diniz et al. 2008). However, many fish species reveal a very weak C-banding heterochromatin region (Rábová et al. 2003; Boron et al. 2009), and *O. barbatulum* and *O. alticorpus* in this study were no exception.

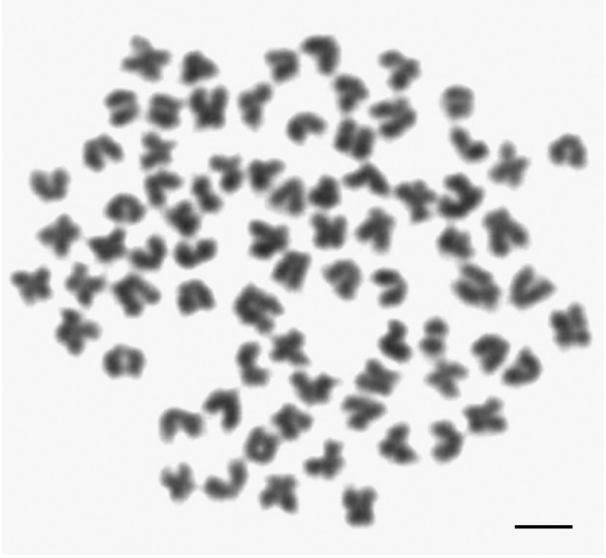

Fig. 7. Triploidy of the hybrid from a female *Onychostoma barbatulum* and male *O. alticorpus*. The chromosome number is 75. The bar equals 5 μm (1000×).

Regarding the FISH results, locations of the 18S rRNA gene in the two species of *Onychostoma* demonstrated interspecific variations in the distributions of these sequences in chromosomes. Nucleoli are nuclear structures organized around active NORs, which are chromosomal regions with tandem-repeated 45S rDNA sequences (Hernandez-Verdun et al. 2002; Hernandez-Verdun 2004). For the reason given above, NOR locations should correspond to 18S rRNA gene sequence locations in chromosomes. In the study of Hatanaka and Galetti (2014) on *Prochilodus argenteus*, complete sequential staining for 18S rDNA FISH and silver nitrate demonstrated that the major 18S hybridization site corresponded to the Ag-NOR. Grassi et al. (2017) also indicated that NOR-positive bands of *Hoplias malabaricus* (Bloch 1794) coincided with results from the FISH technique with 18S rDNA probes. However, a previous study also indicated discordance in numbers between NOR and 18S rRNA gene locations. In *Symphysodon*, chromosomal sites of 18S rDNA and NORs were mainly located in terminal and proximal interstitial regions of the short arms, but discordance in the numbers and positions of NORs and the 18S rRNA gene was observed (Gross et al. 2010). The authors suggested that this could facilitate the transposition to other chromosome pairs through translocation events. Mendes et al. (2011) also described inconsistencies in numbers and positions of NORs and FISH with an 18S rDNA probe in *Hyphessobrycon anisitsi*, *H. luetkenii*, *Deuterodon stigmaturus*, and *Astyanax eigenmanniorum*. In our study, inconsistencies between NORs and FISH in numbers and positions were also observed for *O. barbatulum* as the results revealed two pairs of NORs, but one pair of FISH signals.

There is fairly general agreement that the sex determination system in most Cyprinidae fishes is XX/XY (Rudek 1974; Schartl 2004). Sex heteromorphic chromosomes, *i.e.*, metacentric chromosomes paired with telocentric chromosomes, were found in both *O. barbatulum* and *O. alticorpus*, the sex determination systems of which were the same as most cyprinid fishes. It is reasonable that understanding the genetic sex of fish can benefit their selected breeding. *Onychostoma barbatulum* is now a cultured fish of high economic value in Taiwan. Females have a higher growth rate than males, and all mature

females are larger than 160 mm in body length in wild populations (Chang 1993; Chuang 2003). Tseng et al. (2017) indicated that the female fry ratio of *O. barbatulum* could be increased by controlling the temperature to change the phenotypic sex ratio of progeny. Therefore, to set up whole-feminization technology would be useful to decrease feeding costs. However, to practically execute whole-feminization technology, further studies on growth rates of phenotypic and genetic females are required in the future.

Triploidy events were found in the artificial hybrid of these two species with 3n = 75. This implied that mating may occur between sympatric *O. barbatulum* and *O. alticorpus*. Hybrids may appear in native waters but with rare occurrence due to post-mating isolation which can take place after these two species breed, including a low hatching rate, a low survival rate, body shape deformities, ablepsia, and so on.

CONCLUSIONS

In this study, two *Onychostoma* species showed common genetic characters of having the same number of chromosomes as 2n = 50 and a relatively large first pair of submetacentric chromosomes. Differences between the two species were that *O. barbatulum* contained five pairs, while *O. alticorpus* had seven pairs of metacentric chromosomes. In addition, in the Ag-NOR analysis, it was evident that *O. barbatulum* and *O. alticorpus* respectively had two pairs and one pair of NOR positions. Results also demonstrated that Ag-NOR was a reliable and effective cytogenetic marker for distinguishing *O. barbatulum* and *O. alticorpus*. Weak C-banding was found on centromeres or telomeres of some chromosomes in these two species. The results of 18S rDNA FISH indicated that homological 45S rDNA locus located at no.10 submetacentric chromosome of *O. barbatulum* and *O. alticorpus*. This cytogenetic study can provide valuable background information for subsequent studies on the classification of *Onychostoma* or the Cyprinidae in the future. According to the karyotype analysis of male and female individuals of *O. barbatulum* and *O. alticorpus*, both species have sexual chromosomes, and the sexual determination system is XX/XY. Triploidy events were found in an artificial hybrid of these two species. This implied that hybrids may appear in native waters but with rare occurrence due to post-mating isolation.

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Authors' contributions: Chiao-Chuan Han designed the study, and collected specimens for the study. Tsair-Bor Yen performed the microphotography, chromosome analyses, and data analyses. Nian-Cih Chen assisted the DNA extraction, PCR, chromosome preparation, and feeding fish. Mei-Chen Tseng performed DNA related analyses, analyzed all specimens, and wrote the manuscript. Mei-Chen Tseng, Chiao-Chuan Han, and Tsair-Bor Yen participated in revising the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials: The key datasets, 18S rDNA sequences, were deposited in the publicly available NCBI gene bank (Accession nos. MF 598161 and MF 598162). Other data are available in the publication.

Consent for publication: Not applicable

Ethics approval consent to participate: All procedures in the research conducted in the manuscript have followed the ethic standards of the responsible committee on laboratory animal experiment by IACUC of National Pingtung University of Science and Technology (Approved no. NPUST-IACUC-102-013, Issued date: August 2013, Expired date: July 2017).

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