Karyotype and evolution analysis of vulerable fish *Onychostoma lini* from China

Yinggui Dai
College of Animal Sciences
Guizhou University
Guiyang 550025, China
Email: daiygui@163.com

***Abstract*—The karyotype and chromosomal characteristics of the vulnerable species *Onychostoma lini* (Wu 1939) from China were studied by examining metaphase chromosome spreads obtained from kidney. According to the 200 metaphase spreads from 10 specimen of *Onychostoma lini*, captured from the Duliu river (located in the Pearl River system), China, the chromosome formula in the species might be described as 2n=50=12M+8SM+4ST+26T and FN=70. The mean values of chromosome lengths in *Onychostoma lini* ranged from 7.975 to 14.270 μm, and the haploid chromosome length of the species was 289.111±27.767 μm. This study provides first knowledge on karyotypes in *Onychostoma lini* which may facilitate aquaculture, conservation practices of the species. Also, the evolutionary level of *Onychostoma lini* is preliminarily analyzed based on the karyotype of the species in this paper.**

Key words*—*Vulnerable species*, *Onychostoma lini*, *karyotype*, *chromosome*, *evolution

I. INTRODUCTION

Karyotype studies have provided basic information on the number, size and morphology of chromosomes [1][2] and increased the knowledge of evolutionary mechanisms and genetic question in the species investigated [3][4]. Since the 1960s, karyological studies in teleost fishes have made valued contributions in the fields related to genetics, taxonomy, systematics and environmental toxicology [5]. The study of fish chromosomes has become an active area of research in recent decades [6] .

The family Cyprinidae in the Order Cypriniforme is one of the richest and most important family of fish, and its members are distributed throughout the world [7]. So far, a total of about 1500 species have been recorded in the family worldwide [8].

The Genus *Onychostoma*, the members of which are characterized by a straight mouth on the ventral side of rostrum and 5 branched anal fin rays, belongs to the Subfamily Barbinae in the Family Cyprinidae. The genus is mainly distributed south to the Yangtze River in China [9] and only a few species in *Onychostoma* occur in the rivers in Cambodia, Laos and Vietnam in the Southeast Asia [10] [11] [12]. The *Onychostoma* in China comprises 14 species (subspecies) (Table I), which are all commercially exploited in China. The species in the genus can only live in fast-flowing rivers with saturated dissolved-oxygen, no species in the genus has been cultured in aquaculture farms so far. Up to now, the natural populations of members in the genus have become depleted because of the river pollution, dams on the rivers and over-fishing. As a result, the species *Onychostoma rara* in the genus is inscribed as the endangered species, and both *O. alticorpus* and *O. lini* are listed as the vulerable species in China at present [13].

TABLE I. SPECIES IN THE GENUS *Onychostoma* [9]

Species	Distribution
Onychostoma alticorpus	Taiwan
O. angustistomata	Upper Yangtze River
O. barbata	Pearl River, Wujiang River, Yuanjiang River
O. barbatula	Lower Yangtze River, Pearl River, Minjiang River, Lingjiang River, Taiwan
O. brevis	Upper Yangtze River
O. daduensis	Upper Yangtze River
O. gerlachi	Lancang River, Red River, Pearl River, Hainan Island
O. lepturus	Guangxi, Guangdong, Fujian, Hainan Island
O. lini	Yuanjiang River, Lower Pearl River, Dingjiang River, Jiulong River
O. macrolepis	Yangtze River, Huaihe River, Weihe River, Haihe River
O. ovalis ovalis	Red River
O. ovalis rhomboids	Pearl River, Wujiang River,
O. rara	Yuanjiang River, Xijiang River
O. sima	Upper and middle reaches of Yangtze River, Pearl River

A total of 6 species, including *Onychostoma simus*, *O. gerlachi*, *O. macrolepis*, *O. rara*, *O. alticorpus* and *O. barbatula* have been investigated karyotologically in the genus *Onychostoma* so far [14] [15] [16][17][18]. *Onychostoma lini* lives in the Yuanjiang River (located in the Yangtze River system), the lower Pearl River, and the Dingjiang River and the Jiulong River in Fujian, China [9]. Although morphological and taxonomic characteristics and population genetics of the species have been studied [9] [19], little information is known about the karyotype and evolution of *Onychostoma lini* till now.

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This study is the first attempt to investigate the karyotypes of *Onychostoma lini* and provide detailed information on the number, size and morphology of chromosomes of the species. Also, the evolutionary level of *Onychostoma lini* will be preliminarily discussed based on the karyotype of the species in this research.

II. Material and Methods

A. Material

Ten individuals of *Onychostoma lini* were collected from the Duliu River (located in the Pearl River system in the South China) in March, 2009 for karyotype analysis. The specimen of *Onychostoma lini* were 174-265mm in standard length and 110.6-324.9g in weight. The fish were kept in 400L aquarium at 18°C before experiment.

B. Methods

To stimulate and increase cell mitotic divisions, the fish of *Onychostoma lini* received a celiac injection of phytohaemagglutinin from the base of pectoral fin at a final dose of 10 μg/g body weight in 0.2mL distilled water solution after they were maintained in the aquarium for 24 hours. Twenty hours after the phytohaemagglutinin injection, the specimen of *Onychostoma lini* were injected into the abdominal cavity with colchicine at the base of pectoral fin at a final dose of 5 μg/g body weight. Then the fish were killed and their anterior kidneys collected 4 hours after the colchicine injection.

The tissue from the anterior kidney of each individual in *Onychostoma lini* was sliced into small pieces and minced completely in a culture dish. The visible biomembrane was discarded from the tissue. Then, the tissue was immersed in a cold 0.075M KCl for hypotonic treatment for 45min in a 10-mL centrifuge tube. The hypotonized tissue was centrifuged at 1000 r/min for 10min. The supernatant was discarded and the cold fresh Carnoy's fixative (3:1 v/v methanol and glacial acetic acid) was added and mixed completely with the tissue in the centrifuge tube. After 30 min, the mixture was centrifuged at 1000 r/min for 10min. The supernatant was discarded and the fresh fixative was added and mixed completely with the tissue in the centrifuge tube, and then the mixture was centrifuged at 1000 r/min for 10min. Another two replacements of fixative were repeated before spreading.

After fixation, the mixture was dropped onto the glass slides which were kept at 4°C in a refrigerator for 20 min in advance, from a height of about 50 cm and air-dried at room temperature. The slides were stained with 3% Giemsa buffered solution (pH 7.2) for 30 min, then gently washed with distilled water and air-dried. Mitotic metaphases were observed using an Olympus CH20 microscope with an oil immersion lens at 10×100 magnification. The chromosomes at the metaphase stage of somatic cells were photographed with a digital image capture system (DM200, Beijing Groupca Technology Co., Ltd, Beijing, China). Karyotyping was conducted according to 35 best mitotic metaphases images in *Onychostoma lini*. The numbers of chromosomes in the somatic cells were counted, and chromosome morphometric data were determined using ADOBE PHOTOSHOP 6.0 photographic software. The average length of the short and long arms and the chromosome, and the centromeric index (CI, the ratio of the short arm length to the length of the chromosome), arm ratio (the ratio of the long arm length to the short arm length of the chromosome) and relative length (the percentage of absolute length of each chromosome pair in the sum of absolute lengths of total chromosome pairs in a somatic cell) were then calculated for each chromosome pair.

The chromosomes in each spread were paired and counted using the criteria of maximum resemblance based on the total length and the CI [2]. The chromosome pairs were classified into metacentric (M), submetacentric (SM), subtelocentric (ST) and telocentric (T) groups and counted respectively following the criteria used by Levan, Fredga and Sandberg (1964) [20]. In this resarch, metacentric and submetacentric chromosomes were considered to have two arms, and telocentric and subtelocentric chromosomes to have only one. The fundamental number (FN) of chromosome arms was then calculated by summing up the arm numbers of all types of chromosomes in a somatic cell in *Onychostoma lini*. Finally, the karyotype of the species was constructed by placing the chromosome pairs into the mentioned groups on the basis of centromic position and arranging the homologous pairs in decreasing length order within each group. The ideogram was arranged using EXCEL 2007 (Microsoft) to provide the common feature of the chromosomes for the species.

III. Results

Two hundred mitotic metaphases (more than 10 per fish) from 10 individuals of *Onychostoma lini* were available for the kayotype analysis of this species. The count of chromosomes varied from 46 to 54 per metaphase, with a mode of 50 representing 77.00% of the metaphases (Fig. 1). The heteromorphic sex chromosomes and micro-chromosomes were not identified in the metaphase spreads.

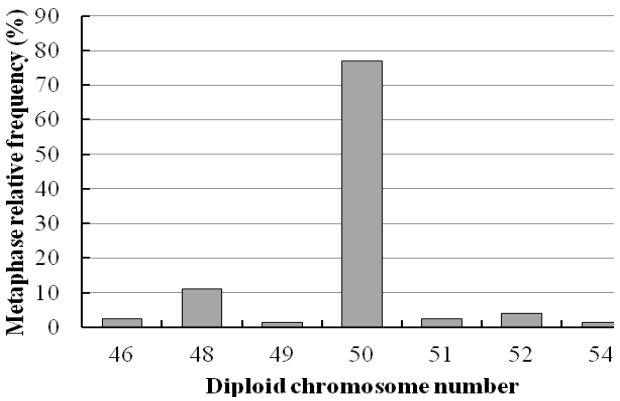

Fig. 1. Frequency distribution of chromosome numbers recorded in 200 diploid metaphases of *Onychostoma lini*.

The average length of the chromosomes ranged from 7.975 to 14.270 μm according to the measurements of 35 best mitotic metaphases (Table II). There were 6 pairs of metacentric chromosomes (M) in the species. Their lengths of the long arm, short arm and chromosome were 7.262±1.238--

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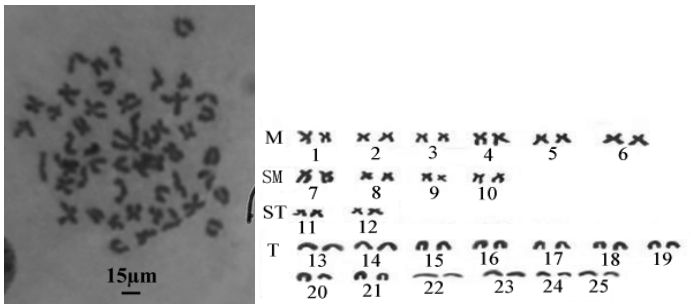
8.606±1.682, 5.061±0.846--6.192±1.038 and 12.521±1.675--14.043±2.744μm, respectively. The ranges of the relative length, the CI and arm ratio of these chromosome pairs were respectively 4.341±0.507--4.846±0.754%, 0.382±0.016--0.460±0.021 and 1.176±0.102--1.624±0.105. There were 4 pairs of submetacentric chromosomes (SM). The long arm, short arm and total lengths of them were 9.202±1.625--9.819±2.020, 4.142±0.975--4.930±1.039 and 13.385±2.442--14.270±2.901μm respectively. Their relative length, the CI and arm ratio were 4.635±0.745--4.923±0.814 %, 0.296±0.020--0.345±0.021 and 1.906±0.186--2.394±0.218, respectively.

Only 2 pairs of subtelocentric chromosomes (ST) were measured in the metaphase spreads. Their long arm, short arm and total chromosome lengths were respectively 8.859±1.420--9.250±1.380, 2.469±0.375--2.592±0.338 and 11.451±1.714--11.719±1.675μm. The relative length, the CI and arm ratio of these chromosome pairs were 3.973±0.566--4.064±0.531%, 0.211±0.018--0.227±0.015 and 3.419±0.305--3.771±0.405 respectively. However, as many as 13 pairs of telocentric chromosomes (T) were identified in the species. The lengths of the long arm, short arm and chromosome were 7.975±0.734--12.569±1.502, 0 and 7.975±0.734--12.569±1.502μm respectively. The ranges of the relative length, the CI and arm ratio of these chromosome pairs were respectively 2.765±0.176--4.347±0.302%, 0 and ∞. The haploid chromosome length of the species was 289.111±27.767 μm.

Therefore, the karyotype formula of *Onychostoma lini* could be summarized as 2n=50=12M+8SM+4ST+26T, and the arm number was FN=70. An example of the metaphase spreads and the karyotype of the species were shown in Fig. 2, and its ideogram was demonstrated in Fig. 3.



(a) (b)

Fig. 2. (a) Metaphase chromosomes of *Onychostoma lini*. (b) Karyotype of *Onychostoma lini* (2n=50).

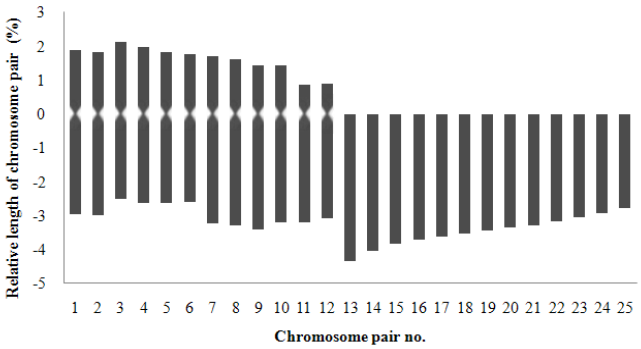


Fig. 3. Ideogram of *Onychostoma lini* (n=25). The plus values represent the relative lengths of short arms of chromosome pairs, and the minus values those of long arms of chromosome pairs.

IV. DISCUSSION

The chromosomes of teleost fish differ from those of other vertebrates due to the small size and the high and variable number of chromosomes in somatic cells of the teleost fish. This is the main cause of technical difficulties in the karyogical study of teleost fish [5]. Usually, the mitotic metaphase cell in blood and kidney tissue *in vivo* or *in vitro* of teleost fish can present clear chromosome spreads. The chromosome slides for optical microscopy in this study were prepared from the anterior part of kidney *in vivo* of *Onychostoma lini*. With this technique, the preparation of the slides was inexpensive and the result could be obtained quickly. However, the success of this technique could be ultimately determined by three key procedures in this research: Injection dose and exposure time of phytohaemagglutinin, those of colchicine and exposure time of hypotonic treatment. During the chromosome preparation of the fishes, the phytohaemagglutinin, which is a stimulator of cell division, could increase and synchronize the mitosis of kidney cells of fish. However, the colchicine, as a mitotic inhibitor, might block quickly-proliferating cell populations at the metaphase stage. In this research, the optimum phytohaemagglutinin treatment was determined to be an injection of a dose of 10 μg/g body weight of fish for 20 hours in *Onychostoma lini*. After the phytohaemagglutinin treatment, the kidney tissue in the species should receive 4 hours exposure to colchicines via an injection of the inhibitor into ventral cavity at a dose of 5μg/g body weight of fish. Additionally, the exposure time of hypotonic treatment was important to form complete and clear metaphase spreads. In this study, the best chromosomal spreads were obtained from the kidney cells via hypotonic treatment in the cold 0.075 M KCl for 45 min in the species.

The study of karyotype is important in aquaculture in connection with the use of chromosome manipulation techniques [21] [22] which may facilitate the culture of endangered fishes and then reduce the fishing pressure on their natural stocks [23]. This research provides first information on the chromosomes and karyotype of *Onychostoma lini* which will be useful for the selective breeding and stocks maintaining of this valuable and vulnerable species, and may ultimately improve aquaculture, conservation and restocking plans of *O. lini.*

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A few studies have used fish standard karyotypes to examine taxonomic or systematic problems [24]. To date, the cytogenetic investigations in the Cyprinidae have been reported for 410 species belonging to 152 genera, and the diploid chromosome number in cyprinid fishes ranged from 34 to 446 [25]. Arai (1982) [26] considered that karyotype of 2n=50 chromosomes was the primitive one in Cyprinidae on the basis of karological analysis of 141 species of cyprinid. According to Ojima (1985) [27], 2n = 50 was not only the most typical number but also an ancestral number of chromosomes in the karyotypes of the cyprinid fishes. Yu et al. (1987) [28] thought that the cyprinid fishes probably differentiated into two large branches in the process of phyletic evolution: the Leuciscin lineage and the Barbinae one. Compared with Leuciscin lineage, the Barbinae one was probably close to the cyprinid ancestors [29], and the diploid chromosome numbers in most of the genera and species in the subfamily Barbinae were 50 [28]. In this study, the species *Onychostoma lini*, belonging to Barbinae and Cyprinidae, had 2n=50 chromosomes, which indicated that the species had a primitive number of diploid chromosomes and probably was close to ancestral species in Cyprinidae.

Up to now, totally seven species in *Onychostoma* have been investigated karyologically (Table III) and all had 2n =50

TABLE III. KARYOTYPES OF SPECIES IN THE GENUS *Onychostoma*

Species	2n	Chromosome formula	FN	Resource
Onychostoma alticorpus	50	6m+26sm+18 st,t ♀ 6m+27sm+17 st,t ♂	82 ♀ 83 ♂	Chen (2010)[16]
O. barbatula	50	10m+24sm+16 st,t ♀ 10m+23sm+17 st,t ♂	84 ♀ 83 ♂	Chen (2010)[16]
O. gerlachi	50	12m+12sm+14st+12t	74	Gui et al.(1986)[14]
O. macrolepis	50	16m+14sm+8t	80	Pang et al.(2012)[18]
O. rara	50	12m+16sm+10st+12t	78	Dai et al.(2012)[17]
O. sima	50	10m+16sm+16st+8t	76	Li et al.(1986)[15]
O. lini	50	12m+8sm+4st+26t	70	

chromosomes, which hinted that the type of 2n=50 chromosomes probably represented the typical pattern of diploid chromosome number in the genus *Onychostoma*. However, the seven species studied in *Onychostoma* revealed the difference of karyotype in FN and chromosome formula. Of the seven species, *Onychostoma lini* had the lowest FN but the most subtelocentric and telocentric chromosomes, while *O. alticorpus* and *O. barbatula* possessed more submetacentric and metacentric chromosomes and higher FN than the remaining species. All uniarmed karyotypes were considered to be ancestral in fish species [30], which meant that the fish species with more subtelocentric and telocentric chromosomes and lower FN had a relatively lower evolutionary level. In general, the primitive species has a wider distributional area than the evolutionary one in a genus of fish. Of the seven species, *Onychostoma sima* lives in the upper and middle reaches of the Yangtze River and the Pearl River, *O. gerlachi* is distributed in the Lancang River, the Red River, the Pearl River and the Hailan Island, *O. macrolepis* ranges in the Yangtze River, and the Huaihe River, the Haihe River and the Weihe River (located in the Yellow River system) , *O. rara* occurs only in the Yuanjiang River (located in the Yangtze River system) and the Xijiang River (located in the Pearl River system), *O. alticorpus* is only distributed in Taiwan, *O. barbatula* occurs only in the Southeast China including the lower Yangtze River, the Pearl River, the Minjiang River and the Lingjiang River and Taiwan and *O. lini* ranges in the Yuanjiang River (located in the Yangtze River system), the lower Pearl River, and the Dingjiang River and the Jiulong River in Fujian, China (Table I). The geographical distribution pattern shows that the species *Onychostoma lini* ranges in a wider distributional area but *O. barbatula* and especially *O. alticorpus* occur in a relatively narrower area than the remaining species within the seven species in China. According to the FNs and chromosome formulas as well as the geographical distribution pattern of the seven species, *Onychostoma lini* should represent the comparatively primitive species while *O. alticorpus* and *O. barbatula* might be the diverged and evolutionary one among the seven species in *Onychostoma*.

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TABLE II. NUMERAL CHARACTERISTICS OF THE KARYOTYPE IN *Onychostoma lini* SHOWING THE MEAN VALUES OF MEASUREMENTS OF THE 35 BEST MITOTIC METAPHASES

Chromosome pair no.	Long arm (μm)	Short arm (μm)	Total length (μm)	Relative length (%)	Centromeric index (CI)	Arm ratio	Classification
1	8.558±1.765	5.486±1.030	14.043±2.744	4.846±0.754	0.392±0.020	1.560±0.126	M
2	8.606±1.682	5.294±0.965	13.899±2.614	4.801±0.770	0.382±0.016	1.624±0.105	M
3	7.262±1.238	6.192±1.038	13.454±2.206	4.644±0.528	0.460±0.021	1.176±0.102	M
4	7.550±1.080	5.739±0.876	13.289±1.831	4.597±0.457	0.432±0.026	1.325±0.141	M
5	7.536±1.120	5.321±0.858	12.857±1.835	4.448±0.476	0.414±0.029	1.428±0.163	M
6	7.461±0.961	5.061±0.846	12.521±1.675	4.341±0.507	0.403±0.028	1.492±0.164	M
7	9.339±1.925	4.930±1.039	14.270±2.901	4.923±0.814	0.345±0.021	1.906±0.186	SM
8	9.477±1.693	4.663±0.862	14.139±2.444	4.908±0.831	0.330±0.025	2.048±0.235	SM
9	9.819±2.020	4.142±0.975	13.961±2.926	4.832±0.924	0.296±0.020	2.394±0.218	SM
10	9.202±1.625	4.183±0.891	13.385±2.442	4.635±0.745	0.311±0.022	2.225±0.216	SM
11	9.250±1.380	2.469±0.375	11.719±1.675	4.064±0.531	0.211±0.018	3.771±0.405	ST
12	8.859±1.420	2.592±0.338	11.451±1.714	3.973±0.566	0.227±0.015	3.419±0.305	ST
13	12.569±1.502	0	12.569±1.502	4.347±0.302	0	∞	T
14	11.657±1.483	0	11.657±1.483	4.028±0.283	0	∞	T
15	11.088±1.323	0	11.088±1.323	3.832±0.226	0	∞	T
16	10.731±1.212	0	10.731±1.212	3.710±0.197	0	∞	T
17	10.430±1.192	0	10.430±1.192	3.604±0.163	0	∞	T
18	10.169±1.123	0	10.169±1.123	3.515±0.144	0	∞	T
19	9.922±1.127	0	9.922±1.127	3.429±0.154	0	∞	T
20	9.703±1.128	0	9.703±1.128	3.353±0.157	0	∞	T

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21	9.470±1.058	0	9.470±1.058	3.274±0.155	0	∞	T
22	9.161±1.105	0	9.161±1.105	3.165±0.162	0	∞	T
23	8.832±1.015	0	8.832±1.015	3.053±0.154	0	∞	T
24	8.414±0.881	0	8.414±0.881	2.913±0.176	0	∞	T
25	7.975±0.734	0	7.975±0.734	2.765±0.176	0	∞	T

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