

## Comparative analysis of intermuscular bones in fish of different ploidies

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We documented the number, morphology, and distribution of intermuscular bones in five fishes of different ploidy: *Carassius auratus* (Abbr.WCC,  $2n=100$ ), *Carassius auratus* variety PengZe (Abbr.PZCC,  $3n=150$ ), improved triploid crucian carp (Abbr.ITCC,  $3n=150$ ), improved red crucian carp (*Carassius auratus* red var., Abbr.IRCC, ♀,  $2n=100$ ), and improved allotetraploids (Abbr.G×AT, ♂,  $4n=200$ ). The number of intermuscular bones in WCC, PZCC, and G×AT ranged from 78 to 83 ( $\bar{x}=81$ ), 80 to 86 ( $\bar{x}=84$ ), and 77 to 84 ( $\bar{x}=82$ ), respectively. The numbers in ITCC and IRCC were significantly lower, ranging from 77 to 82 ( $\bar{x}=79$ ) and 58 to 77 ( $\bar{x}=71$ ), respectively. The average number of intermuscular bones in each sarcomere, ranked in order from highest to lowest, was 0.721 (WCC), 0.673 (PZCC), 0.653 (G×AT), 0.633 (ITCC), and 0.608 (IRCC). There was no difference between ITCC and G×AT or between G×AT and PZCC. However, the average number of intermuscular bones in the sarcomeres of ITCC, WCC, and PZCC differed significantly, as did that of IRCC and the four other kinds of fish. The intermuscular bone of these five fishes was divided into seven shape categories, non-forked (I), one-end-unequal-bi-fork (└), one-end-equal-bi-fork (Y), one-end-multi-fork, two-end-bi-fork, two-end-multi-fork, and tree-branch types. Generally, the morphological complexity was higher in the anterior intermuscular bones than in the posterior body. The number of intermuscular bones was similar but not equal between the left and right sides of the body. ITCC had significantly fewer intermuscular bones than either WCC or PZCC, making it of greater commercial value. Additionally, IRCC and ITCC had fewer intermuscular bones than WCC. Our observations are significant in both fish bone developmental biology and genetic breeding.

**intermuscular bone, epineural bone, epipleural bone, morphology, genetic breeding**

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Intermuscular bones (IB) are unique to teleost fish, and are more common in the lower teleosts (non-spined fin fish) [1]. They are small, hard-boned spicules located in the muscle tissue on both sides of the vertebrae, and consisting of membranous ossifications of connective tissue in the muscular septum [1]. Depending on the site of attachment, in-

termuscular bones are divided into three categories from dorsal to ventral: epineurals (EN), which attach to the neural arches; epipleurals (EP), which attach to the hemal arches or ribs; and epicentrals, which attach to the central vertebrae [1]. This small bone has gradually been lost during fish evolution, and in the spiny finfish Perciformes it has almost completely disappeared [2].

The earliest members of Teleostei possessed a relatively simple form of intermuscular bone. During the subsequent

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evolution of this line, the morphology of this structure has changed significantly. In particular, intermuscular bone morphology has become extremely complex with the evolution of the Cyprinids [3]. Patterson et al. [2] and Johnson et al. [4] have made detailed observations and analyses of the intermuscular bone of 125 teleostean fish species, including China's major freshwater Cyprinids. The study of small intermuscular bone morphology in fish has primarily involved monographs of fish anatomy [5–7]. As early as the 1960s, Chinese researchers began to recognize the importance of studying small intermuscular bones in fish. For example, Bing [8] first documented the morphology of IB in small juvenile carp (*Cyprinus carpio*). More recently, Dong et al. [9] published their preliminary observations on the IB of silver carp, bighead carp, bream, and allogynogenetic silver crucian carp and farmed fish of the common carp family. Similarly, other researchers have documented IB morphology and/or counts in silver carp grass carp and catfish [10–12]. Despite the attention, there are very few observational studies on the number, morphology, and distribution of intermuscular bones in fish of different ploidy levels. To address this gap, we document the number, morphology, and distribution of intermuscular bones in five fishes of different ploidies, *Carassius auratus* (WCC,  $2n=100$ ), *Carassius auratus* var. PengZe (PZCC,  $3n=150$ ), triploid crucian carp ( $3n=150$ , ITCC (Grant No. GS-02-001-2008) produced by mating improved  $4n$ AT males with females of improved IRCC), improved red crucian carp (*Carassius auratus* red var., IRCC, ♀,  $2n=100$ ), and improved allotetraploids ( $G \times AT$ , ♂,  $4n=200$ ). Our results provide a morphological basis for describing both fish bone developmental biology and improving genetic breeding.

## 1 Materials and methods

### 1.1 Samples

WCC were collected from Dongting Lake, PZCC were purchased from the Erliban fish market at Hunan Normal University, and  $G \times AT$ , IRCC, and ITCC were obtained from the Engineering Center of Polyploid Fish Breeding of the Education Ministry located at Hunan Normal University. Fifteen individuals of each type were used for the experiments.

### 1.2 Measurement, counting, and X-ray map creation

Total length, body length, and head length were measured with vernier calipers and each fish was also weighed. An X-ray diagram of each individual was obtained from the Hunan Provincial Institute of Traditional Chinese Medicine, Department of Radiology.

### 1.3 Anatomy and morphology of intermuscular bones

After measurement of the fresh experimental fish, each individual was parceled in gauze and boiled. The gauze was removed when the fish had cooled and the skin carefully peeled off using tweezers. The morphological distribution of intermuscular bones was documented based on the muscle divisions along the spine and dorsoventrally in the sections before and after the abdominal cavity. Each fish was divided into four sections: the trunk including the axis muscle, the trunk under the axis muscle, tail hindquarters shaft muscle, tail hindquarters shaft under muscle. Beginning from the left trunk on axis on the left side of the fish, followed by the left trunk under axis, the left tail shaft, and the left tail hindquarters shaft, each sarcomere was dissected. Following this, the IBs were removed and arranged in order. The same process was carried out on the right side of the body. The IBs were photographed in order with a 550D Canon digital camera.

## 2 Results

### 2.1 Comparison of IB numbers in different fish

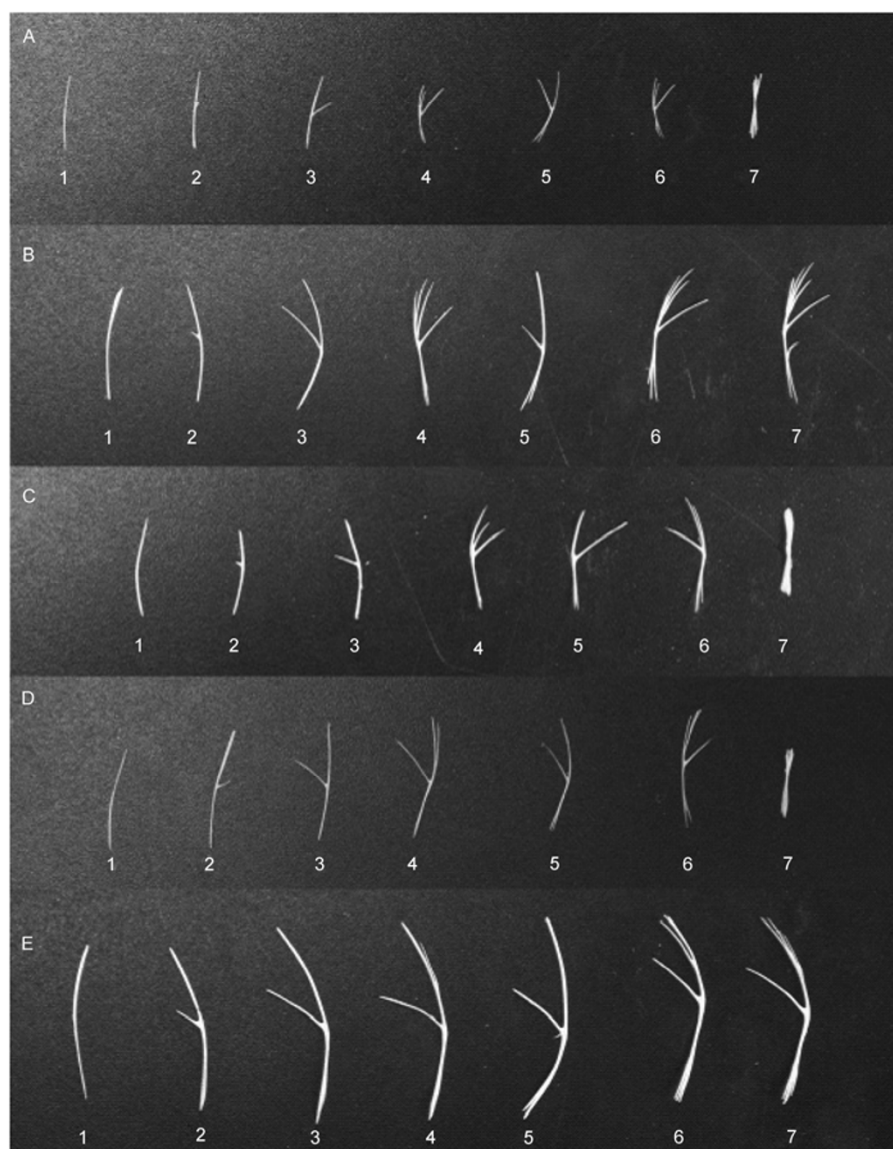
The number of IB in WCC, PZCC, and  $G \times AT$  fell within the range 78–83 ( $\bar{x}=81$ ), 80–86 ( $\bar{x}=84$ ), and 77–84 ( $\bar{x}=82$ ), respectively. The count was slightly lower in ITCC and IRCC, ranging from 77 to 82 ( $\bar{x}=79$ ) and from 58 to 77 ( $\bar{x}=71$ ), respectively (Table 1).

The IB count did not differ significantly between PZCC and  $G \times AT$ ; however, there was a significant difference between IRCC and  $G \times AT$ , PZCC, and ITCC, and between ITCC and the other three fish ( $P < 0.05$ , for all comparisons) (Table 2 presents a similar subset of the group mean, using sample size=15).

The IB count in each sarcomere ranked in order from highest to lowest was 0.721 (WCC), 0.673 (PZCC), 0.653 ( $G \times AT$ ), 0.633 (ITCC), and 0.608 (IRCC) (Table 3, a similar subset group mean, using sample size=15). The count of IB in  $G \times AT$  was not significantly different from that in ITCC or PZCC. However, the average number of IBs in the sarcomeres of ITCC, WCC, and PZCC differed significantly ( $P < 0.05$ ), as did that of IRCC and the other four kinds of fish ( $P < 0.05$ ).

### 2.2 Comparison of IB morphology

The five groups of fish possessed seven forms of IB, including non-forked (I), one-end-unequal-bi-fork ( $\vdash$ ), one-end-equal-bi-fork (Y), one-end-multi-fork, two-end-bi-fork, two-end-multi-fork, and tree-branch forms (Figure 1). The numbers of the seven different types of IB found in the left sides of each group are shown in Table 4. PZCC, ITCC,  $G \times AT$ , IRCC, and WCC weighed 312, 483, 196, 228, and 54 g, respectively (Table 1).



**Figure 1** Illustration of the various types of intermuscular bone observed in fish of different ploidy levels. A, WCC. B, PZCC. C, IRCC. D, G×AT. E, ITCC. 1, I type (not-fork type); 2, † type (one-end-unequal-bi-fork type); 3, Y type (one-end-equal-bi-fork type); 4, one-end-multi-fork type; 5, two-end-bi-fork type; 6, two-end-multi-fork type; 7, tree-branch type.

**Table 1** Length, weight, and IB count in fish of different ploidy levels

Fish	Weight (g)		Standard length (cm)		Amount of IB	
	Range	Average	Range	Average	Range	Average
WCC	42.0–58.0	48.0±5.8	11.8–12.9	12.2±0.4	78.0–83.0	81.2±1.7
PZCC	250.0–477.0	327.0±61.1	20.1–24.5	21.8±1.4	80.0–86.0	83.8±1.4
IRCC	123.0–314.0	217.0±65.3	14.5–19.5	17.0±1.8	58.0–77.0	70.5±6.4
G×AT	114.0–290.0	196.0±57.3	16.2–21.5	18.7±1.5	77.0–84.0	82.3±2.5
ITCC	210.0–593.0	390.0±90.6	19.0–27.0	23.1±2.4	77.0–82.0	79.1±2.0

WCC had five kinds of epineural IBs (the I, Y, two-end-bi-fork, two-end-multi-fork, and tree-branch types), four kinds of epipleural IBs (I, †, Y), and two-end-multi-fork types. PZCC has seven kinds of epineural IBs, but only four kinds of epipleural bone (I, †, Y, and tree-branch types).

Only 20% of IRCC possessed the tree-branch type, and the remaining 80% possessed the other six forms of epineural bone, with five forms of the epipleural bone (I, †, Y, one-end-multi-fork, and two-end-multi-fork forms). The remaining two groups of fish had all seven epineural IB

**Table 2** Two-way analysis of variance of IB count in fish of different ploidies

	Fish	N	A subset of the alpha=0.05		
			1	2	3
The one-way ANOVA	IRCC	15	70.500		
	ITCC	15		79.083	
	G×AT	15			82.250
	PZCC	15			83.833
	Significance		1.000	1.000	0.294

**Table 3** Two-way analysis of variance on IB count in the sarcomeres of fish of different ploidies

	Fish	N	A subset of the alpha=0.05			
			1	2	3	4
The one-way ANOVA	IRCC	15	0.6078			
	ITCC	15		0.6327		
	G×AT	15		0.6528	0.6528	
	PZCC	15			0.6727	
	WCC	15				0.7208
	Significance		1.000	0.092	0.095	1.000

**Table 4** Numbers of various types of intermuscular bone (IB) in the left side of fish of different ploidies<sup>a)</sup>

Fish	Location	Amounts of various types of IB							Amount of IB
		I type	┆ type	Y type	One-end-multi-for k type	Two-end-bi-fork type	Two-end-multi-for k type	Tree-branch type	
WCC	LTOA	3	0	9	0	8	2	0	22
	LTHS	4	0	1	0	0	0	1	6
	EN	7	0	10	0	8	2	1	28
	LTUA	3	0	4	0	0	0	0	7
	LTHU	4	1	0	0	0	1	0	6
	EP	7	1	4	0	0	1	0	13
PZCC	LTOA	4	3	5	3	6	1	0	22
	LTHS	4	0	1	0	0	0	1	6
	EN	8	3	6	3	6	1	1	28
	LTUA	5	0	2	0	0	0	0	7
	LTHU	5	0	0	0	0	0	1	6
	EP	10	0	2	0	0	0	1	13
IRCC	LTOA	1	1	13	4	1	1	0	21
	LTHS	1	2	1	0	0	0	0	4
	EN	2	3	14	4	1	1	0	25
	LTUA	1	1	3	1	0	0	0	6
	LTHU	2	0	0	1	0	1	0	4
	EP	3	1	3	2	0	1	0	10
G×AT	LTOA	4	2	5	3	2	7	1	24
	LTHS	2	1	0	0	0	1	0	4
	EN	6	3	5	3	2	8	1	28
	LTUA	4	0	4	1	0	0	0	9
	LTHU	4	1	0	0	0	0	0	5
	EP	8	1	4	1	0	0	0	14
ITCC	LTOA	2	2	5	0	4	8	3	24
	LTHS	3	0	2	1	0	0	0	6
	EN	5	2	7	1	4	8	3	30
	LTUA	0	2	4	0	0	0	0	6
	LTHU	2	0	1	0	0	2	0	5
	EP	2	2	5	0	0	2	0	11

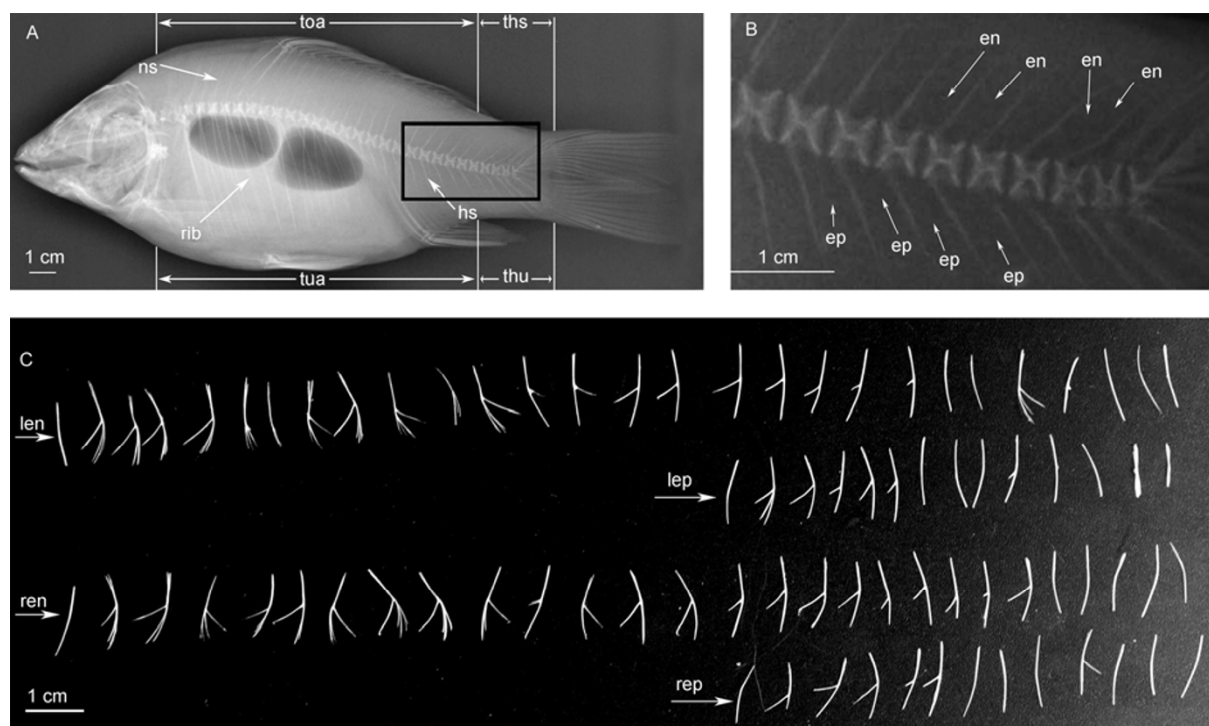
a) LTOA, left trunk along axis; LTUA, left trunk under axis; LTHS, left tail hindquarters shaft; LTHU, left tail hindquarters under shaft; EN, epineural bone; EP, epipleural bone.

types. G×AT and ITCC only had four of the epipleural bone types: the I, 1, Y, and two-end-multi-fork types. In summary, the variation in IB morphology was lower in WCC than in PZCC, ITCC, and G×AT. The epipleural IBs were more complex than the epineural IBs.

### 2.3 IB distribution

IBs are long, thin linear bones about 1/3 to 1/2 the length of the ribs. They exhibit complex morphology (described

above) and are arranged in an orderly fashion in each sub-section layer of the large lateral muscle tissue from head to tail [3]. The IB distribution varied among the five types of fish (Figures 2C–6C). IB distribution differed between the left and right sides, but not significantly. The specific locations of EN and EP were identified using detailed skeletal X-rays (Figures 2A and B to 6A and B). The IB counts on both sides of the body in our experimental fish were not exactly equal, but were quite similar. IB numbers varied (Table 5) among sections of the same fish.

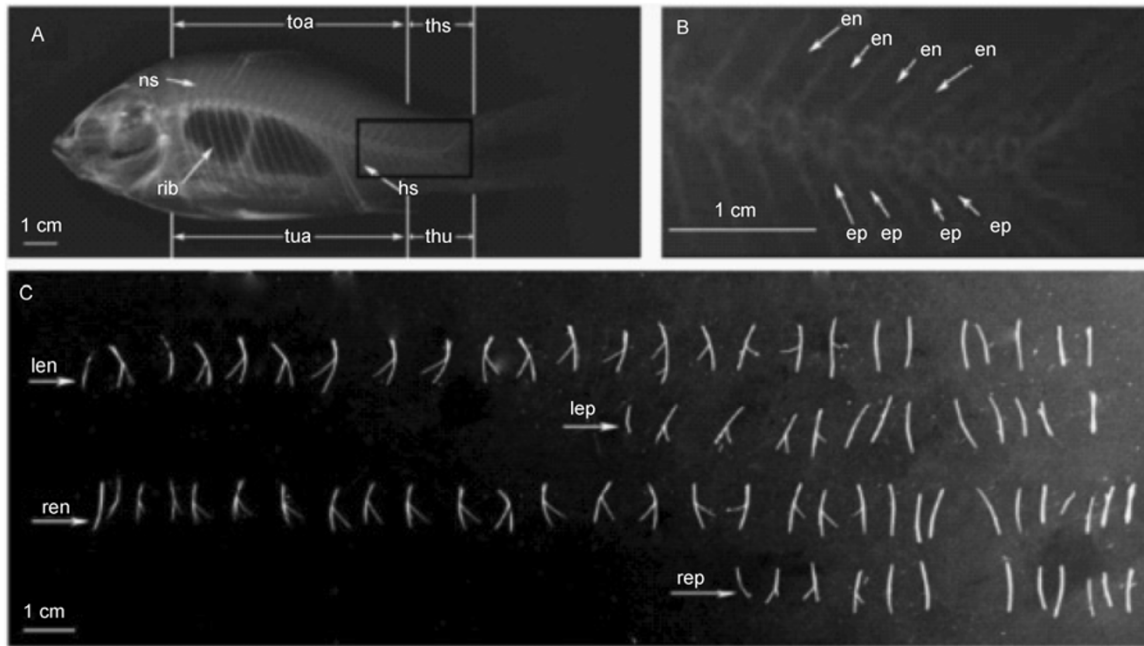


**Figure 2** PZCC intermuscular bone morphology. A, Whole-mount X-ray diagram of skeleton of PZCC. Toa, trunk on axis; tua, trunk under axis; ths, tail hindquarters shaft; ths, tail hindquarters shaft under; rib; ns, neural spine; hs, hemal spine. B, Enlarged view of the panel highlighted in A. en, epineural bone; ep, epipleural bone. C, The anterior region intermuscular bones. Len, left epineural bones; lep, left epipleural bones; ren, right epineural bones; rep, right epipleural bones.

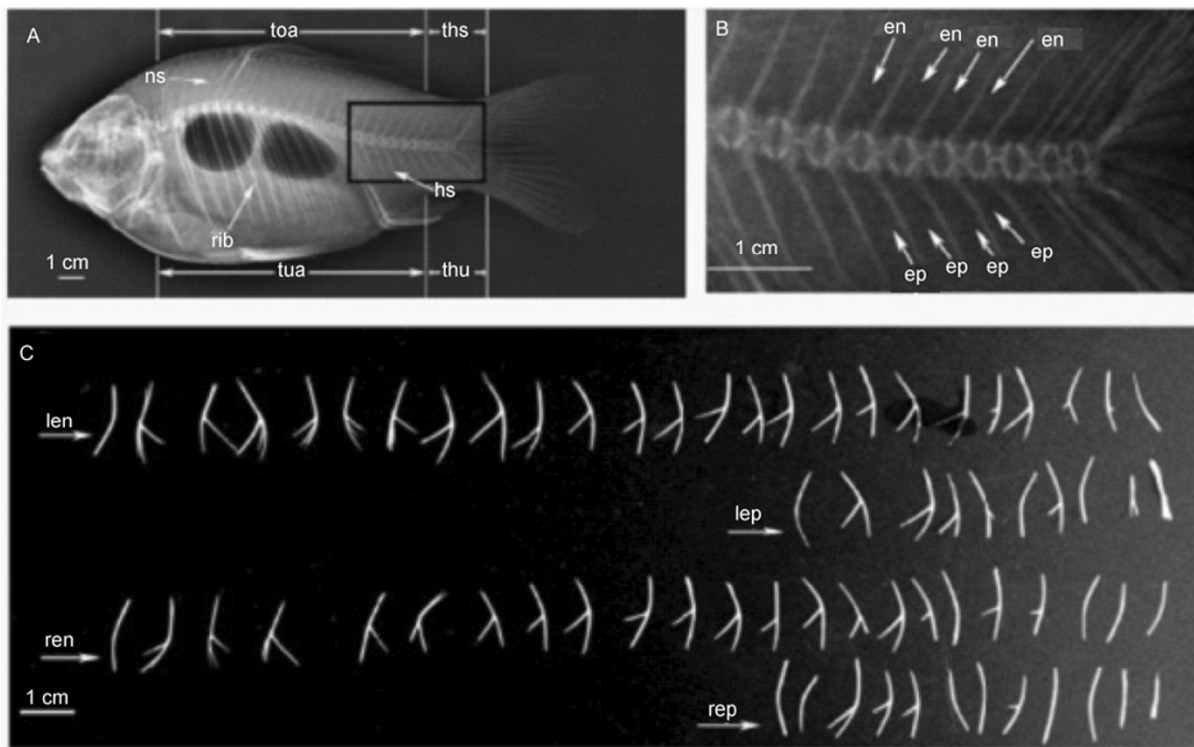
**Table 5** Number of intermuscular bones in fish of different ploidy<sup>a)</sup>

Fish	WCC	PZCC	IRCC	G×AT	ITCC
LTOA	22	22	21	24	24
LTUA	7	7	6	9	6
LTHS	6	6	4	4	6
LTHU	6	6	4	5	5
LS	41	41	35	42	41
RTOA	21	23	19	24	23
RTUA	6	6	7	9	5
RTHS	6	7	5	4	5
RTHU	7	7	5	4	5
RS	40	43	36	41	38
AN	81	84	71	83	79
AN/ES	0.721	0.672	0.608	0.653	0.633

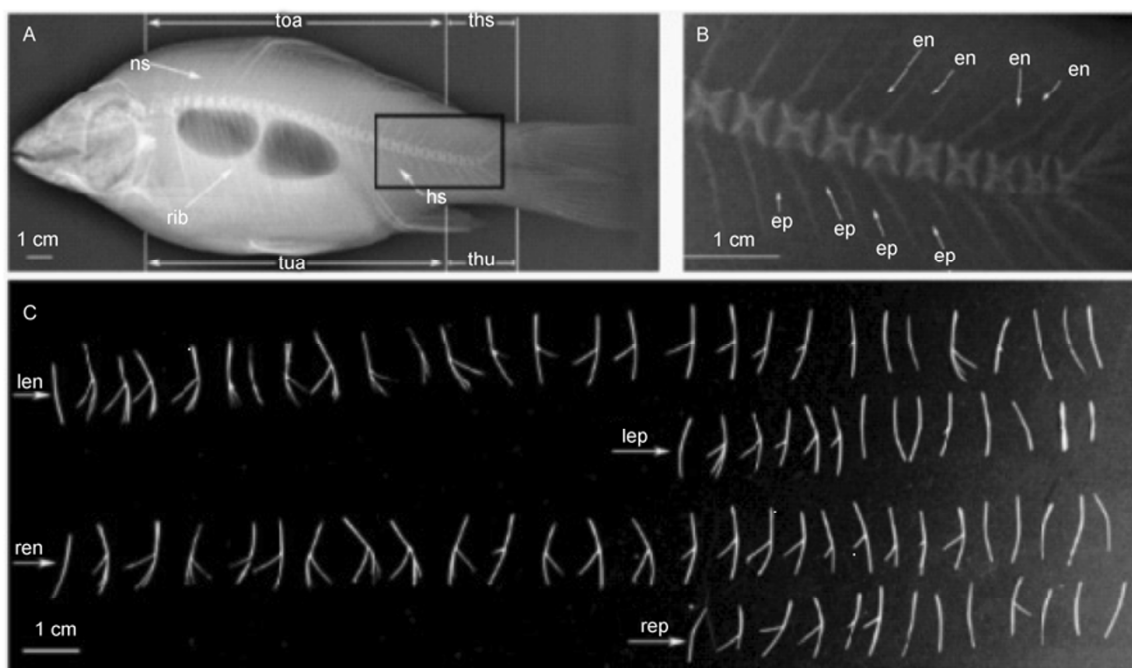
a) LTOA, left trunk along axis; LTUA, left trunk under axis; LTHS, left tail hindquarters shaft; LTHU, left tail hindquarters under shaft; RTOA, right trunk along axis; RTUA, right trunk under axis; RTHS, right tail hindquarters shaft; RTHU, right tail hindquarters under shaft; LS, left subtotal of IB; RS, right subtotal of IB; AN, average number of IB; AN/ES, average number of IB in each sarcomere.



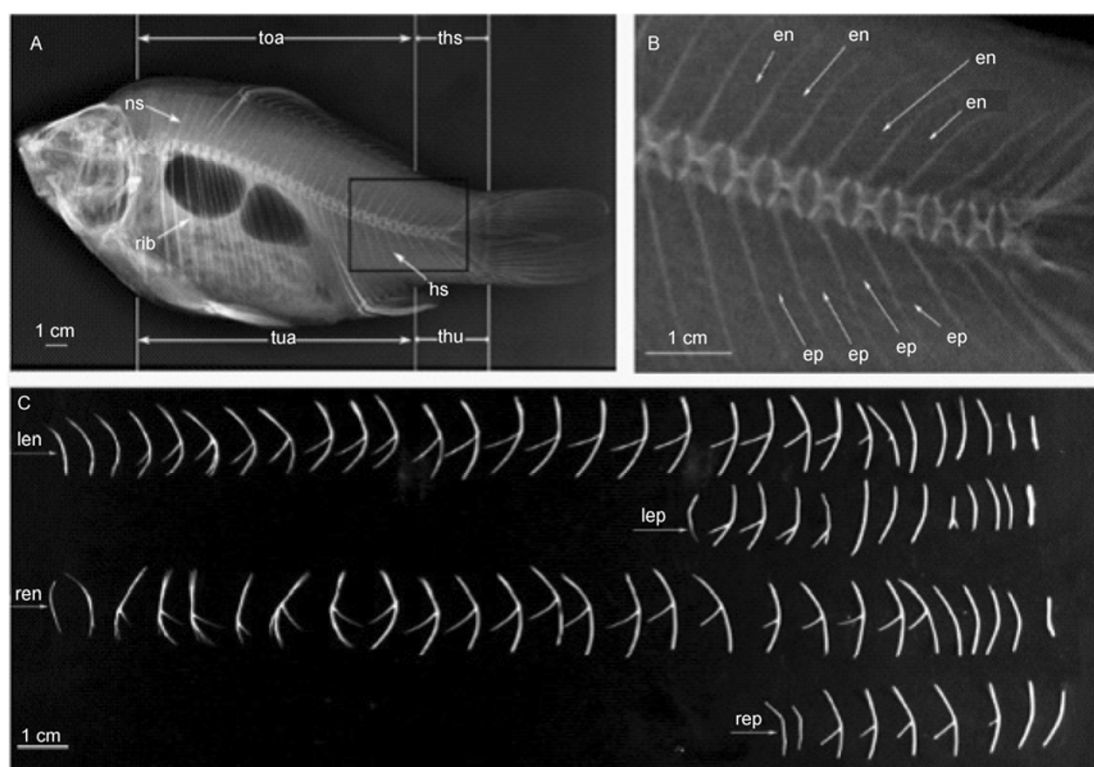
**Figure 3** WCC intermuscular bone morphology. A, Whole-mount X-ray diagram of WCC skeleton. Toa, trunk on axis; tua, trunk under axis; ths, tail hindquarters shaft; thu, tail hindquarters shaft under; rib; ns, neural spine; hs, hemal spine. B, Enlarged view of the panel in A. en, epineural bone; ep, epipleural bone. C, The anterior region intermuscular bone. Len, left epineural bones; lep, left epipleural bones; ren, right epineural bones; rep, right epipleural bones.



**Figure 4** IRCC intermuscular bone morphology. A, Whole-mount X-ray diagram of the IRCC skeleton. Toa, trunk on axis; tua, trunk under axis; ths, tail hindquarters shaft; thu, tail hindquarters shaft under; rib; ns, neural spine; hs, hemal spine. B, Enlarged view of the panel in A. en, epineural bone; ep, epipleural bone. C, The intermuscular bone posed from anterior region. len, left epineural bones; lep, left epipleural bones; ren, right epineural bones; rep, right epipleural bones.



**Figure 5** G×AT intermuscular bone morphology. A, Whole-mount X-ray diagram of the G×AT skeleton. Toa, trunk on axis; tua, trunk under axis; ths, tail hindquarters shaft; thu, tail hindquarters shaft under; rib; ns, neural spine; hs, hemal spine. B, Enlarged view of the panel in A. en, epineural bone; ep, epipleural bone. C, The anterior region intermuscular bone. len, left epineural bones; lep, left epipleural bones; ren, right epineural bones; rep, right epipleural bones.



**Figure 6** ITCC intermuscular bone morphology. A, Whole-mount X-ray diagram of the ITCC skeleton. Toa, trunk on axis; tua, trunk under axis; ths, tail hindquarters shaft; thu, tail hindquarters shaft under; rib; ns, neural spine; hs, hemal spine. B, Enlarged view of the panel in A. en, epineural bone; ep, epipleural bone. C, The intermuscular bone posed from anterior region. len, left epineural bones; lep, left epipleural bones; ren, right epineural bones; rep, right epipleural bones.

### 3 Discussion

China is a significant contributor to the global aquaculture industry, accounting for more than two-thirds of world aquaculture production [14]. Chinese aquaculture production has increased from less than 1 million tons per year 50 years ago to 38.3 million tons per year in 2010, making it the fastest growing of the agricultural and food industries. Aquaculture production accounts for 71.3% of total aquatic products [15], and up to 70% of world production. Some farmed fish require a great deal of processing, for example making fish fillets or balls. The presence and morphology of the IB influences this processing and affects the freshness of the fish, resulting in many IB fish being unattractive to consumers. Therefore, the presence of IB has a negative impact on the economic and food value of a species. Given this, knowledge of IB tissue distribution is important in determining the relative merits of farming a given type of fish [16]. Studies suggest that the main role of IB is to support the large side muscle. Therefore, relatively loose fleshed fish should have more IBs [3]. Another role of IB is conduction of muscle strength. Other studies have shown that in fish with higher muscle protein content, muscle strength conduction is more likely dependent on muscle fiber, and they therefore have fewer IBs [9]. However, this remains speculation, and there is no direct evidence that proves this effect. Higher Teleostei do not have IB, so it is highly likely that fish without IB suffer no adverse effects from their absence. Lv's analysis [10] of IB evolution in the lower Teleostei indicates that they may be a trace organ. Given this, it seems likely that some species could be targeted to select for lower IB counts.

#### 3.1 Comparison of IB numbers in fish of different ploidies

The IB numbers were highest in WCC, PZCC, and G×AT, followed by ITCC and IRCC, which had the lowest number. The average IB count in each sarcomere differed significantly between ITCC and PZCC or WCC ( $P < 0.05$ ). In addition to having a lower IB count, ITCC also exhibits fast growth, large body size, high protein content, fresh meat, infertility, disease resistance, cold tolerance, and hypoxia tolerance, making it commercially attractive.

Our research indicates that IRCC possess a number of improved features, such as a smaller head, higher body, and shorter caudal peduncle. Additionally, they are bisexually fertile, mature at one-year, and have the ability to self-mate. Moreover, use of IRCC as the maternal line to produce improved triploids could increase the yield of meat and thereby benefit commercial growers. IRCC and their progeny also exhibit a number of remarkable reproductive advantages, including higher fertility and hatching rates, higher gamete production, and longer idiophase, which can directly

improve reproduction [17]. Lv et al. [10] reported greater numbers of IBs in the sarcomere of carp and WCC (0.638 and 0.721, respectively) than in IRCC (0.608). Thus, creating IRCC via fish distant hybridization and genetic improvement methods suggests it is possible to produce superior varieties with lower IB counts.

The average IB count was significantly higher in WCC sarcomeres than in the other four types of fish (Table 3), all of which have been genetically improved through artificial methods. Using distant hybridization technology combined with gynogenesis, G×AT and IRCC were obtained through artificial breeding over several generations [18]. ITCC were then obtained from a large scale crossing between G×AT and IRCC [13]. Thus, enhanced varieties of fish that have lower IB counts can be produced by artificial methods.

The distant hybridization of red crucian carp (♀) and carp (♂) combined with gynogenesis produced genetically stable allotetraploid crucian carp [18]. Further manipulation using distant hybridization and genetic improvement methods can produce varieties with fewer intermuscular bones. Distant hybridizations are defined as crosses between distantly related species or genera, and allow the transfer of the genome of one species to another, resulting in phenotypic and genotypic changes in the progeny [18]. Distant hybridization results in genome-level alterations, including diploid, triploid, tetraploid hybrids, and diploid gynogenetic organisms with sub genome-level alterations, such as the formation of the micro-chromosomes. These outcomes depend on the genetic relatedness and similarity in chromosome numbers of the parents. The combination of the parents' beneficial traits results in phenotypic heterosis in the offspring, including increased growth rate, survival, and disease resistance. For the purposes of researching teleost evolution and improving breeding protocols, distant hybridization is a proven method for producing fish of different ploidies [18]. The summary of genetic rule and pathways in crucian carp distant hybridization for getting polyploid fish will provide important theoretical guidance for more excellent polyploid fish group using hybrid breeding in the cyprinid economic fish or the other one [19]. Gui found that allogynogenetic crucian carp lines only produced gynogenetic offspring through heterologous sperm stimulation, and more importantly, demonstrated that the dual reproductive modes of gynogenesis and sexual reproduction coexist in the polyploid gibel carp. The breeding of allogynogenetic silver crucian carp, gynogenetic reproduction to activate eggs, and embryonic development methods with the heterologous sperm of other fish species have been widely used in aquaculture [20].

Distant crossing can break through barriers between species, integrate their beneficial characteristics, expand the genetic variation, and produce new variants or species, which may result in enhanced offspring viability, growth potential, stress resistance, yield, and quality [21]. By a combination of distant crossing and gynogenesis, improved



diploids and tetraploids were obtained after selection from multiple generations. Crossing these different ploidy fishes produced the improved triploid crucian carp. The improved triploid crucian carp did not have the deficiencies of the common hybrids so represents a promising new aquaculture species [13]. Additionally, both G×AT and IRCC exhibited enhanced fertility, stress resistance, and had fewer IBs. These advantages are important for commercial level production of ITCC. Furthermore, IB numbers were lower in ITCC than in WCC and PZCC, resulting in the former having higher value as a food fish. This makes it a popular choice with farmers and consumers throughout China.

Among IRCC, ITCC, and G×AT, we observed an increase in the IB count with increasing ploidy, suggesting a positive correlation between the two.

### 3.2 IB morphology in different ploidy fish

Meng and Su [7] divided catfish IB morphology into four types: I type (non-fork and small bifurcation type), 卜 type, Y type, and tree-branch type (chunky, both ends branch a lot). Dong et al. [9] classified it into two types: I type and Y type, the other types are differentiated on the basis of these two shapes. According to their morphological complexity, Lv grouped them into seven types to ease analysis of the evolution of the various IB types. Of the 15 IRCC in our study, three possessed the tree-branch type IBs, accounting for 20% of the total. The morphology observed in WCC was less complex than in PZCC, ITCC, or G×AT. IB exhibits the same arrangement from the tail to the front body ossification as in the zebrafish [22]. Bing observed IB morphology in young carp [8] and noted that every ossification was present in the lateral muscle compartment. Furthermore, the IB was membranous and bony, lacking in cartilage at that stage of morphological development. It was also differentiated from the precursor connective tissue, then exhibited ossification from the posterior to anterior [23]. Bing also found that carp, crucian carp, and four domestic fish (black carp, grass carp, silver carp, and bighead carp) have something in common based on the observation of side muscle compartment spicules in young carp. That is, for more juvenile fish, because of the less developed diaphragmatic muscle and the presence of several phrenic nerves on the first and last shaft muscle, IB had either not formed at all or only part of the fiber had begun to harden. With continued growth, ossification is evident in the diaphragms on both the upper and lower shaft muscles, with each diaphragm containing IBs (except for the area below the trunk shaft) [8]. This suggests that the IB count varies between the larval and adult stages. Generally, young fish have fewer IB than adult fish, though the exact mechanism for this difference requires further study.

In a comparative analysis of IB in the lower teleost fish, Lv et al. [10] concluded that the evolution of IB in cyprinids followed two pathways: (i) I type—卜 type—Y type—

one-end-multi-fork type. The multi-fork type IB appeared for a period in shad, whereas the two-end-bi-fork type did not, suggesting that this type of evolution does take place. (ii) I type—卜 type—Y type—two-end-bi-fork type—two-end-multi-fork type—tree-branch type. Our experimental results agree with this view. Based on observations on IB anatomy in fish of the same type but different sizes, we found that ENs were absent from the area behind the back in small individuals, and subsequently appeared from front to back in larger fish. Our observations agree with those of Patterson et al. [2] and Johnson et al. [4] who compared ENs in fish of the same type but different sizes. This demonstrates that the complex EN patterns, i.e., tree-branch type and two-end-multi-fork type, which appear at the anterior of the cyprinid posterior, require a longer evolutionary period, providing a morphological basis for fish bone development biology. WCC has significantly fewer tree-branch and two-end-multi-fork type IBs than PZCC, IRCC, ITCC, and G×AT. We speculate that there are two reasons for this. First, it is likely that the smaller body size of WCC means its degree of ossification is not yet complete, and it only contains a portion of the morphogenetic IB types. Second, the other four types of fish have been evolving for longer and are older than WCC. This indicates that fish distant hybridization technology and genetic improvement methods may yield further improvements in IRCC, ITCC, and G×AT.

### 3.3 IB distribution in different body parts

The number of intermuscular bones was similar, but not equal, between the left and right sides of the body. The IB count also varied among different regions within the same individual. We observed seven IB forms and the morphological complexity was greater in the anterior than in the posterior of the body.

### 3.4 Significance, prospects, and outlook of IB research

One of the goals in pursuing improved fish morphology is producing new aquaculture species with improved economically important traits. Our observations suggest that genetic improvement methods can have a significant effect on the formation of fish IB. As a result, our study offers insight into fish skeleton developmental biology and genetic breeding.

Although this study signifies a breakthrough in fish IB research, most efforts continue to focus at the morphological level. To date, there are no in-depth studies on the formation, evolution, molecular and genetic mechanisms of IBs, and in particular, the specific role of IBs remains unknown. However, the higher Teleostei do not have IB, so it is quite likely that the absence of IBs does not greatly impact the life of the fish [16].

With complete genome sequencing of some of the main

aquaculture species, ongoing development of genome technologies, somatic cell nuclear transfer, and stem cell technologies, as well as identification and function analysis of candidate genes relative to economically important traits (i.e., reproduction, sex, growth, disease resistance, cold tolerance, and hypoxia tolerance), genetic improvement research in aquaculture species has entered a new era for molecular breeding [15]. In view of the successful application of gene silencing, gene knockout, and green fluorescent protein in fish (such as zebrafish) [24–27], it is possible to suppress IB formation by these methods combined with gene inhibitor technology. This will establish new breeding varieties with high yield and quality from the molecular level, and make a greater contribution to sustainable fisheries and biotechnological innovation.

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