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Whole-mount Bone Staining of Selected Cyprinidae Fishes of Punatsangchu, Bhutan

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Abstract

Whole-mount bone staining was used to study skeletal system and reproductive toxicology for a long time. Alizarin red S and Alcian blue were used for a decade to stain specimens, and it is still widely used today. The study was conducted to standardize the rapid whole-mount bone staining protocol for larger specimens of selected cyprinidae and to compare meristic and morphometric data before and after staining, which further generates comparative information on the osteological characteristics of the fishes found in Punatsangchu River. The specimens were collected using purposive sampling and stored in 10% formalin. The protocol of the whole-mount bone staining method was modified by changing the concentration of reagent and the timing required based on the size of the specimens. Formalin, Triton X-100, and potassium hydroxide were used as fixative solutions. The enhancement solution consisted of ethylene glycol, Triton X-100, and potassium hydroxide. Alizarin red S and ethylene glycol were used as the bone staining solution and Alcian blue as the cartilage staining solution. Tween 20 and potassium hydroxide were used as clearing solutions. Details of the meristic counts were compared following both pre- and post-staining procedures. In all four species, the meristic counts revealed significant differences (p= .03, N=20). However, comparative morphometric measurements revealed no significant difference between and within species (p=1.00). The strong stout spines on the dorsal and anal fins remained the same, indicating that bone staining was useful in species identification.

Keywords: Ichthyological taxonomy, meristic count, morphometric measurement, whole-mount bone staining.

Introduction

Whole-mount bone staining was first used and reported by (Schultze, 1897). It was used mainly to study the skeletal system and reproductive toxicology testing. After the first report of the original processes of whole-mount bone staining, there have been numerous modifications done to the procedures (Sakata et al., 2018). Among the various methods being modified, removing soft tissues with a solution containing potassium hydroxide (KOH) and staining bone with alizarin red S had been used long time as a standard method for staining bone in small vertebrates (Dawson, 1926).

Clearing and staining of complete vertebrate bone and cartilage is the current method widely used in vertebrate osteology (Song & Parenti, 1995). Ashiwa and Hosoya

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(1998); Norris (2001); Sanger and Mccune (2002); Shantakumar and Vishwanath (2006) as cited in Sakata et al. (2018) helped in considering and knowing the main taxonomic characteristics for the identification and classification of fish at higher levels through bone staining. Studying skeletal system and meristic counts through bone staining appears favorable in identifying fishes (Shabnam, Sharma & Dhanze 2016).

The traditional bone staining methods reported by Cumley, Crow, and Griflfen, (1939) took several weeks and months to complete the whole procedure. However, the rapid and non-destructive protocol for whole-mount bone staining of small fish and *Xenopus*, reported by Sakata et al. (2018), modified the protocol, which completed whole procedures quickly. This method provided clear, transparent, and non-destructive specimens, which was also time-saving.

The present study aimed to develop a standard protocol for whole-mount bone staining of selected Cyprinidae fishes. It also aimed to compare osteological characteristics and help confirm species through comparative meristic counts. This study is first of its kind from Bhutan and will serve as an important baseline information reference for future studies.

Materials and Methods

Study Area

Punatsangchu, one of the river basins in Bhutan, originates in northern part of the country and drains into the Bramaputra River in the Indian state of Assam (Bhutan Himalaya, 2011). Phochu and Mochu are the two major tributaries joining to become Punatsangchu below Punakha Dzong. Another major tributary, Dangchu joins Punatsangchu below Wangdue Phodrang Dzong. Further to the south, the west flowing Harachu joins the river at Taksha. As Punatsangchu makes its progress to the south, other major tributaries namely Burichu, Changcheychu, Kalikhola and Dagachu join at various points. Punatsangchu covers almost five districts from its origin in the extreme north of Gasa to the southern foothills of Dagana before entering the Indian plain (Figure 1).

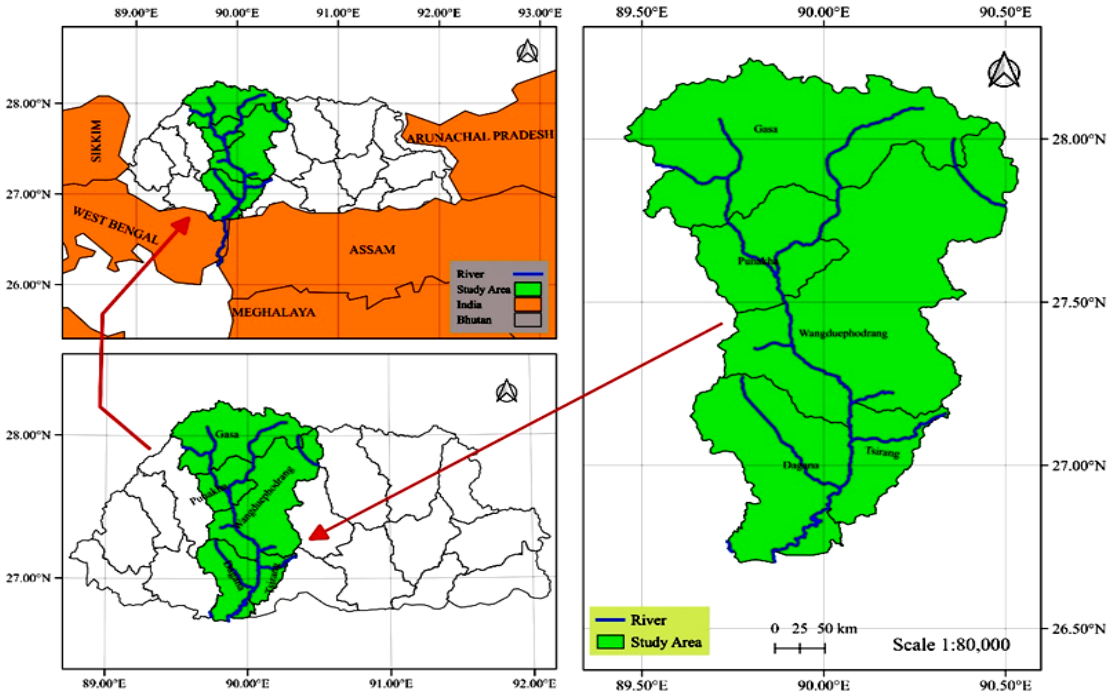

Figure 1: Study area of Punatshangchu basin along with its major tributaries

Specimen preservation

The specimens were collected from Punatsangchu river basin following purposive sample plots. The specimens were euthanized and fixed in 10% formalin and then preserved in 70 % ethanol in the field during data collection.

Morphometric measurements and meristic counts

Morphometric and meristic data were generated using digital calipers by measuring all the fish samples collected during the fieldwork. Various landmarks (Bookstein, 1991; Armbruster, 2012 and Lim et al., 2016) such as measurement of the distance between the dorsal-fin base and anal-fin base were done mainly for morphometric data.

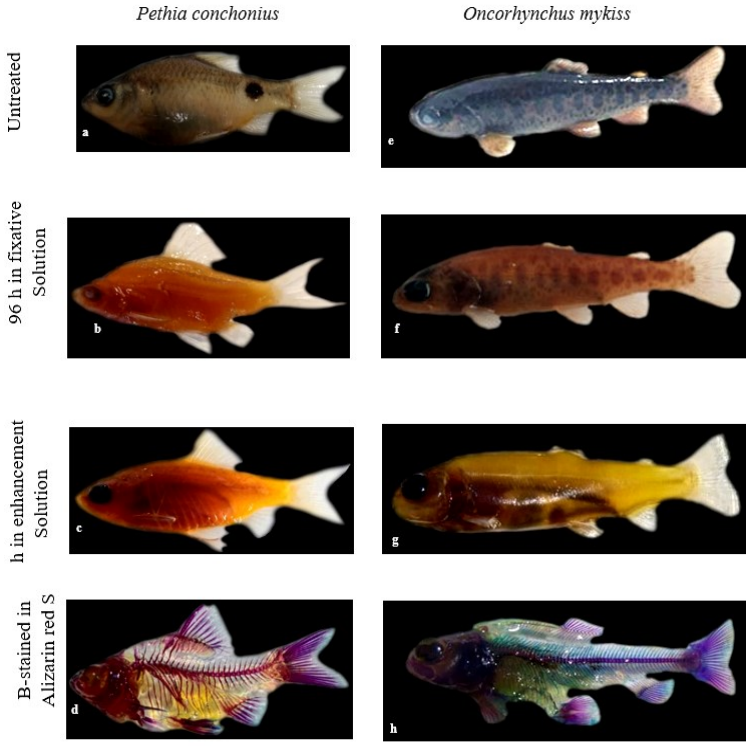

Figure 2: Efficiency of the reagent in decolorization and clearing specimens at 42^0C for 96 hours both in fixative as well as in enhancement solution and bone staining (a-d is *Pethia conchonius*) (e-h is *Oncorhynchus mykiss*)

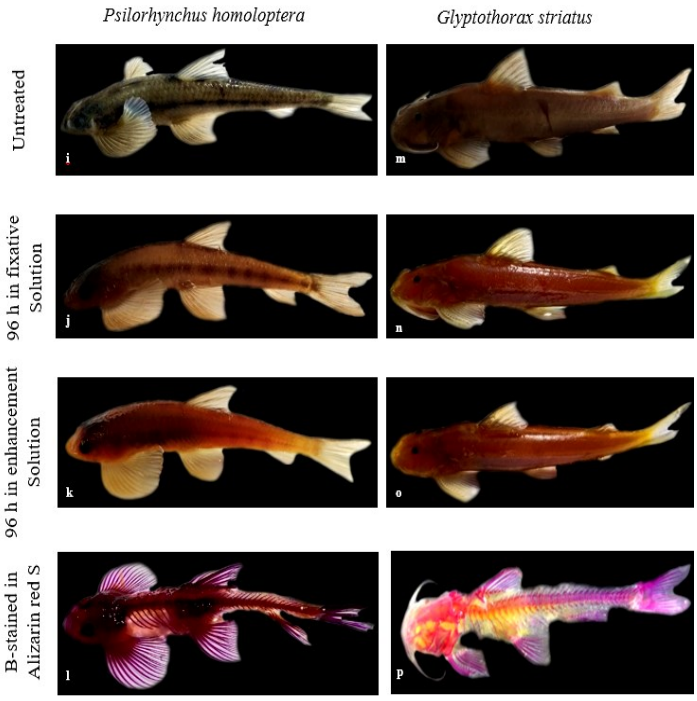

Figure 3: Efficiency of the reagent in decolorization and clearing specimens at 42^0C for 96 hours both in fixative as well as in enhancement solution and bone staining (i-l is *Psilorhynchus homoloptera*) (m-p is *Glyptothorax striatus*).

Fixation and clearing

The fixative solution containing 10% Formalin, 10% Triton X-100 and 2% potassium hydroxide (KOH) was mixed with 100 ml of distilled water. While incubating the specimen for 96 hours at 42^0 C, the solution became transparent through bleaching which was enough to move to the next bone staining procedure. (For skinning and evisceration, it can be done after incubating the specimen for 48 hours in a fixative solution). After incubating for 96 hours, it was possible to see the bones in the dorsal and ventral regions without staining. However, the rate of bleaching depends on the size of the specimen. The above procedures were applicable for the specimens ranging from (25.04 mm to 116.46 mm). To increase the transparency rate, removing internal organs was necessary, as it took longer to dissolve

pigments and tissues.

To improve and increase transparency efficiency, the specimens were immersed in the enhancement solution containing 40% ethylene glycol, 10% Triton X-100, and 2% potassium hydroxide (KOH). The specimens were incubated for 96 hours at 42^0C, which helped enhancing transparency and making color lighter, especially on the body surface. However, the eyes and skull region remained unchanged, and it needed 48 to 72 hours to make them completely transparent. Immersing into the enhancement solution provided greater efficiency in making the specimen transparent. But if kept for more than 96 hours in enhancement solution, the specimens spoiled and failed to retain its original shape and often broke and dissolved the bones.

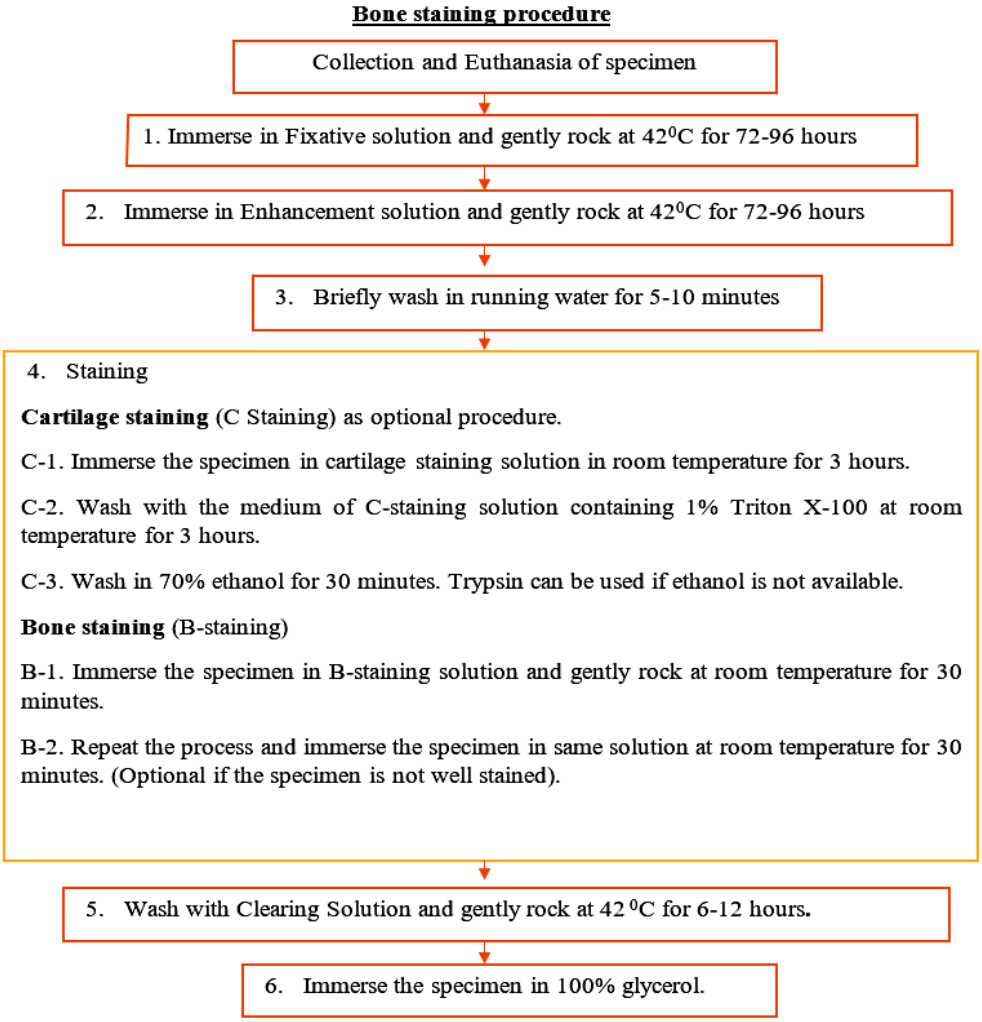

Figure 4: Flow chart of bone staining procedure which allows to obtain fine bone-stained specimen. Cartilage staining was done as a double staining before bone staining. Fixative Solution: 10% formalin, 10% Triton X-100, 2% potassium hydroxide (KOH). Enhancement Solution: 40% ethylene glycol, 10% Triton X-100, 2% potassium hydroxide (KOH). Cartilage Staining Solution: 70% ethanol, 20% acetate and 0.015% Alcian blue. Bone Staining Solution: 0.05% Alizarin red S, 20% ethylene glycol and 1% potassium hydroxide. Clearing Solution: 20% Tween 20, 1% KOH.

Cartilage staining (C-Staining)

After completing the fixation and clearing procedure, the specimens were washed with distilled water and immersed into cartilage staining solution containing 70% ethanol, 20% acetate and 0.015 % alcian blue. The incuba-

tion was done for 2 to 3 hours at room temperature depending on the size of the specimens. The specimens that were less than 50 mm was incubated for 2 hours, and those more than 50 mm were incubated for 3 hours. This staining was rather taken as an optional in case of double staining.

Bone staining (B-staining)

After completing C-staining, the specimen was washed with distilled water and immersed into bone staining solution and gently rocked for 30 minutes at a room temperature.

Observations and meristic count

Images of specimens stained with alizarin red S were observed using a fluorescence microscope by attaching a DSLR camera that provided a highly magnified image that provided detailed structures of bones and fin rays. Dino-Lite camera and Vivo 1935 mobile phone were also used to take normal photos in the lab.

Table 1: The comparative results of meristic counts of four different fish species before and after bone staining

Species	Test values	Dorsal		Pectoral		Ventral		Anal		Caudal		
		DS	DB rays	PS	PB rays	VS	VB rays	AS	AB rays	CS	CP	CPr
Garra annandalei	Median	1.5	8.5	1	13	1	7.5	1.5	5	17	2	9.5
	Std. Deviation	0.52	0.52	0	0.48	0	0.52	0.527	0	0	0	5.79
	p value	0.03	0.03	1	0.05	1	0.03	0.03	1	1	1	0.03
Barilius barna	Median	1.5	7	1	10	1	8	2.5	7.5	17	2	11
	Std. Deviation	0.52	0	0	1.05	0	0	0.52	0.52	0	0	5.27
	p value	0.03	1	1	0.03	1	1	0.03	0.03	1	1	0.03
Barilius bendelisis	Median	1.5	7.5	1	10	1	8	2.5	7.5	17	2	12.5
	Std. Deviation	0.52	0.52	0	1.05	0	0	0.52	0.52	0	0	6.85
	p value	0.03	0.03	1	0.03	1	1	0.03	0.03	1	1	0.03
Neolissochilus hexagonolepis	Median	2.5	9.5	1	14.5	1	8	2.5	5	17	2	11.5
	Std. Deviation	0.99	0.52	0	1.06	0	0	0.52	0	0	0	3.68
	p value	0.04	0.03	1	0.06	1	1	0.03	1	1	1	0.03

Note: *N*=20.

DS=Dorsal spine, DB rays= Dorsal branched rays, PS=Pectoral spine, PB rays=Pectoral branched rays, VS=Ventral spine, VB rays=Ventral branched rays, AS=Anal spine, AB rays=Anal branched rays, CS=Caudal spine, CP=Caudal principal rays, CPr=Caudal pro-current rays.

Results and Discussion

Protocol for staining

The whole-mount bone staining protocol was standardized through modification of rapid and non-destructive bone staining procedure (Figure 4). To standardize protocol for whole-mount bone staining, twenty species of Cyprinidae (ranging from 25.08 mm to 116.46 mm) were tested using Alizarin red S and Alcian Blue. Three species of Psilorhynchidae, two species of Siluridae and Salmonidae were also

used mainly to test the efficacy of Alizarin red S and Alcian Blue solution aside Cyprinidae. The efficacy of Alizarin red S and Alcian Blue was significant in all the specimens tested in laboratory. However, in some of the species such as *Garra* with thick deposition of tissues had to be removed using forceps.

Unlike the rapid and non-destructive methods proposed by Sakata et al. (2018), this procedure took longer, considering the specimens' larger size. The protocol took 7-8 days to complete the whole process of bone staining for larger specimens (25.08 mm to 116.54 mm). More time was consumed in making the specimen transparent by incubating the specimen in a fixative and enhancement solution. Once the specimens were made well transparent before staining, the consequent staining and clearing were completed within 24 hours.

Comparative meristic counts before and after bone staining

Comparative evaluation of changes in the meristic count before and after bone staining revealed significant difference between the meristic counts of pre and post staining, (p= .03, N=20). The significant difference indicated that meristic counts before bone staining was not as effective as whole-mount bone staining through Alizarin red S and Alcian blue.

Bone stained with Alizarin red S and Alcian blue did reveal distinctly all meristic rays enabling counting with certainty. Overall, the study revealed that meristic count after whole-mount bone staining through Alizarin red S and Alcian blue was more effective than meristic counts without staining (Table 2).

Comparative meristic analysis of spines and fin rays based on different lengths

In order to understand the developmental characteristics of the strong stout spine and fin rays, five different lengths of *Garra annandalei*, 45.12 mm - 94.05 mm, standard length (Figure 5, A), *Barilius barna*, 37.18 mm – 60.77 mm, standard length (Figure 5, B), *Berilius bendelensis*, 58.40 mm – 101.45 mm, standard length (Figure 5,C) and *Neolossochilus hexagonolepis,* 25.08 mm -94.20 mm, standard length (Figure 5,D) were assessed based on the meristic count after bone staining. The results of the meristic count revealed that spines and fin rays remained the same for all the specimens irrespective of length and size. This confirmed that invisible strong stout spines at the anterior insertion of the fin do not grow longer as the specimen grows bigger which is usually overlooked during meristic counts if not stained.

Comparative morphometric analysis between and within species

The morphometric measurements within the species of the same genus, *Barilius barna* and *Barilius bendelisis,* and within different genera *Barilius barna* and *Garra anandalei,* under the same family were compared with bone staining data to determine their significance in identifying species in ichthyological taxonomy. Non-parametric tests were conducted to assess variation among the same genera. The results revealed no significant differences between different genera and within the same genus in all the morphometric measurements (p>.05, N=20). This finding indicated that morphometric measurement is less ascertaining compared to bone staining. Therefore, morphometric data alone may not be sufficient to identify and describe a species.

Structure of vertebrae

To assess the variation of vertebrae structures of the fishes, 14 different species of four different genera with maximum numbers of species were selected to compare the number of vertebrae along the vertebrae column. All four genera had different vertebrae columns. The *Barilius* group had 40 vertebrae; the anterior region comprised 19 vertebrae columns (V1-V19), and the posterior vertebrae comprised 21 vertebrae columns (V20-V40) (Figure 6, A). The *Schizothorax* group had 46 vertebrae columns. The abdominal region was 22 (V1-

V22), and the caudal region comprises 24 (V23-V46) (Figure 6, B). The *Psilorhynchus* group also had 46 vertebrae columns. However, the abdominal region had 20 (V1-V20), and the caudal region had 26 (V21-V46) (Figure 6, C). *Garra* group comprised 31 vertebrae columns. The abdominal region consisted of 14 vertebrae (V1-V14) and the caudal region consists of 17 vertebrae (V15-V31) (Figure 6, D).

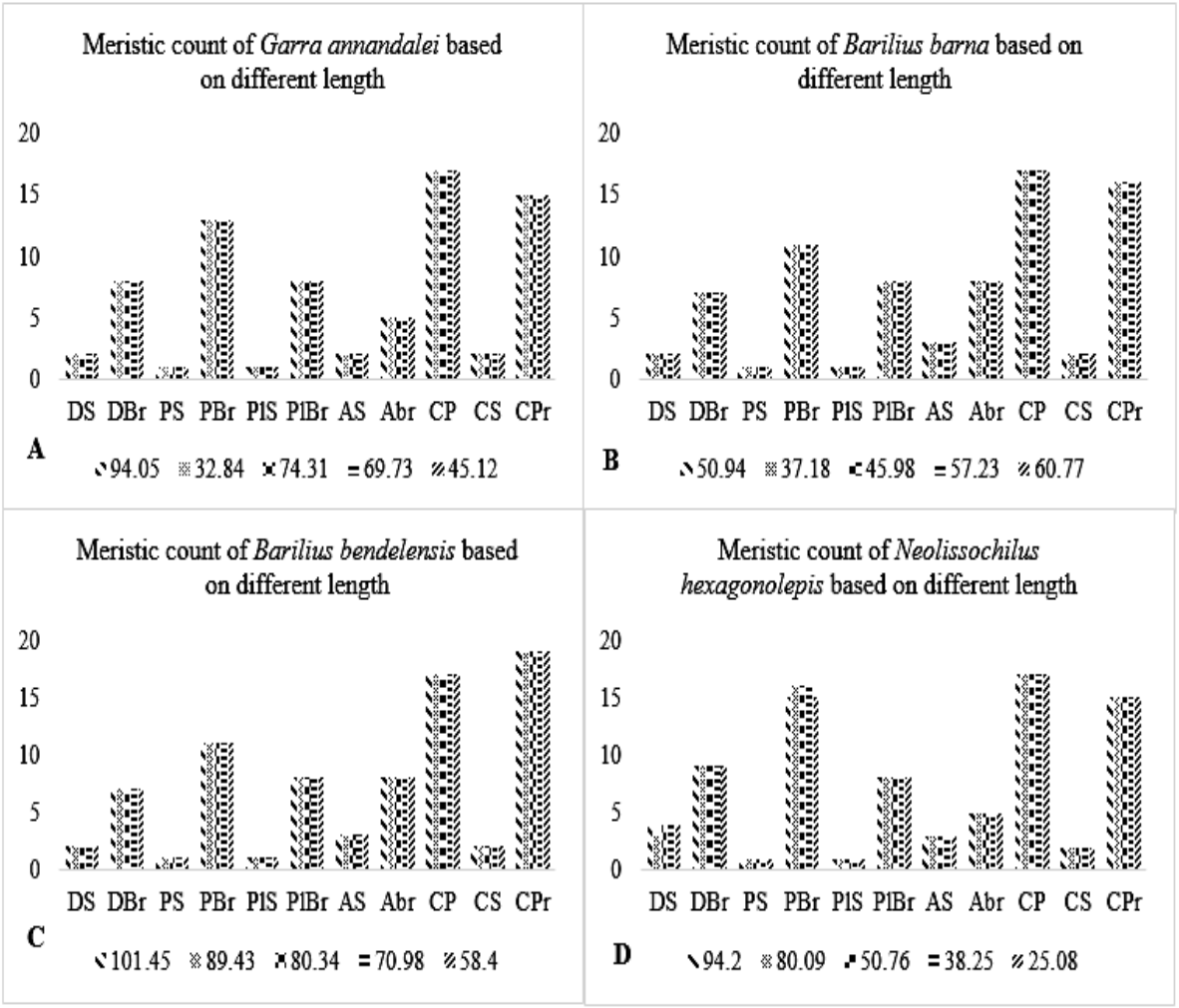

Figure 5: Five different specimens of *Garra annandalei* showing same numbers of fin rays after bone staining (A). Five different specimens of *Barilius barna* showing same numbers of fin rays after bone staining (B). Five different specimens of *Berilius bendelensis* showing same numbers of fin rays after bone staining (C). Five different specimens of *Neolossochilus hexagonolepis* showing same numbers of fin rays after bone staining (D).

Difference in total number of vertebrae column was accounted for and revealed directly by counting and analyzing the total number of vertebrae in the anterior and posterior regions of the vertebrae column in all species. As per Ward and Brainerd (2007), vertebrates display variations in regionalization of their vertebral column. The difference in morphology of vertebrae columns of different regions can be revealed from biometric studies (Desse et al., 1989). Although detailed biometric studies were not done, the variations in the vertebral column for four species were determined based on general regionalization of the vertebral column following Ward and Brainerd (2007).

Table 2: Comparative meristic counts of four different fish species before and after bone staining

Sl	SID	DSB	DSA	DBrB	DBrA	PSB	PSA	PBrB	PBrA	PlSB	PlSA	PlBrB	PlBrA	ASB	ASA	ABrB	ABrA	CPB	CPA	CSB	CSA	CPrB	CPrA
1	1	1	2	9	8	1	1	12	13	1	1	7	8	1	2	5	5	17	17	2	2	4	15
2	1	1	2	9	8	1	1	13	13	1	1	7	8	1	2	5	5	17	17	2	2	4	15
3	1	1	2	9	8	1	1	12	13	1	1	7	8	1	2	5	5	17	17	2	2	4	15
4	1	1	2	9	8	1	1	12	13	1	1	7	8	1	2	5	5	17	17	2	2	4	15
5	1	1	2	9	8	1	1	13	13	1	1	7	8	1	2	5	5	17	17	2	2	4	15
6	2	1	2	7	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	16
7	2	1	2	7	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	16
8	2	1	2	7	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	16
9	2	1	2	7	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	16
10	2	1	2	7	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	16
11	3	1	2	8	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	19
12	3	1	2	8	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	19
13	3	1	2	8	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	19
14	3	1	2	8	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	19
15	3	1	2	8	7	1	1	9	11	1	1	8	8	2	3	7	8	17	17	2	2	6	19
16	4	2	4	10	9	1	1	13	15	1	1	8	8	2	3	5	5	17	17	2	2	8	15
17	4	2	3	10	9	1	1	14	16	1	1	8	8	2	3	5	5	17	17	2	2	8	15
18	4	2	4	10	9	1	1	14	16	1	1	8	8	2	3	5	5	17	17	2	2	8	15
19	4	2	4	10	9	1	1	14	16	1	1	8	8	2	3	5	5	17	17	2	2	8	15
20	4	2	4	10	9	1	1	14	15	1	1	8	8	2	3	5	5	17	17	2	2	8	15

Note: SID- 1= *Garra annandalei, 2= Barilius barna, 3= Barilius bendelisis, 4= Neolissochilus Hexagonolepis*

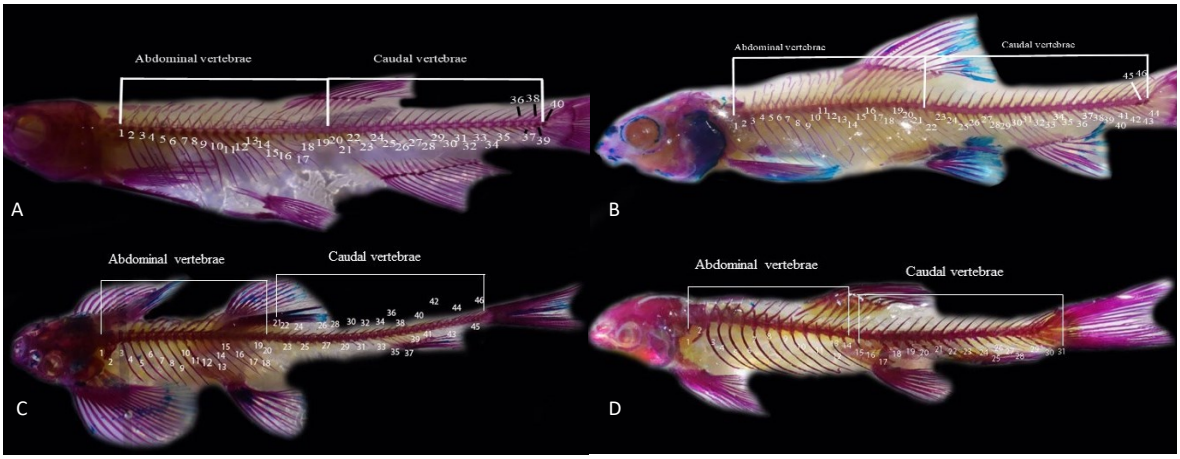

Figure 6: Vertebrae column of (A). *Barilius vagra,* (B). *Schizothorax richardsonii,* (C). *Psilorhynchus homaloptera,* (D). *Garra lissorhynchus*

Conclusion

The whole-mount bone staining method using Alizarin red S and Alcian blue were more useful than both meristic counts and morphometric measurements for species confirmation. The staining time differed based on the size of the specimen. The difference in the meristic counts before and after bone staining concluded that traditional meristic count alone was insufficient for species confirmation. The comparative meristic counts of different lengths of the specimens after bone staining found that the spine rays remained the same for all specimens irrespective of length and size. The bone staining method is also useful in studying variations in the vertebrae column of fishes. The distinct and well-stained bones are more useful for differentiating and studying all parts of bones in three-dimensional angles. This modified protocol of bone staining methods could also be applied in studying osteological characteristics of other small fishes, reptiles, amphibians, birds, and mammals. Our study shows that the correct identification of fish species requires a combination of meristic, morphometric, and osteological studies for species confirmation.

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