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Research Article

Comparative studies of whole mount staining of bones and cartilage of selected bony fishes by Alizarin red S and Alcian blue

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ABSTRACT

Comparative anatomical studies of bones and cartilages are rare in ichthyology as there is lack of reliable protocol for preparing the specimens that demonstrate all the bones and cartilages. The shapes and sizes of skeletal elements with their appropriate location were determined by whole mount skeletal staining. This technique is important for detecting changes in skeletal patterning. In the present study, five different fishes such as *Amblypharyngodon mola, Macrognathus aureus, Puntius sarana, Sciades parkeri* and *Ambassis vachellii* were treated with Alcian blue and Alizarin red S for the staining of cartilage and bone, respectively. Cartilages were stained blue because Alcian blue is a cationic dye which binds the sulphated GAGs and glycoproteins as cartilage contains higher concentration of GAGs than any other tissues. Bones were stained red because Alizarin red S is an anionic dye which binds with the calcium present in bones. Formalin was used as a fixative as it is a common fixative for museum specimen. This paper describes a procedure for rendering cartilage blue, bone red and soft tissue translucent or transparent in whole vertebrate specimen. This cleaning and staining procedure is thus readily applicable to comparative studies in anatomy, embryology, and systematic zoology.

INTRODUCTION

In almost every experiment of skeletal phenotypes, the whole-mount skeletal preparation is the first step of analysis. Whole-mount skeletal staining determines the exact shapes and sizes of skeletal elements in their appropriate locations. Since the original procedure for whole-mount bone staining was first reported by O. Schultze (1897) which has undergone various modifications. Clearing and staining of whole small vertebrates for demonstration of bone **(**Taylor, 1967) or bone and cartilage **(**G. Dingerkus & L. D. Uhler, 1977**)** are methods used widely in comparative vertebrate osteology. Such cleared and stained preparations of whole fish specimen provide osteological data, source of a large portion of characters used in studies of fish phylogeny (G. D. Johnson & W. D. Anderson, 1993). A common method is a double-staining procedure in which differential staining of cartilage with Alcian blue is combined with bone staining with Alizarin red S (E.V. Simons & J. R. Van Horn, 1971). As a cationic dye, Alcian blue binds strongly to sulphated GAGS and glycoproteins, while Alizarin red S, an anionic dye binds to cationic metals such as calcium (R. W. Horobin, 2010). For fish, hydrogen peroxide (H_2O_2) can be used to bleach the soft tissue, although small air bubbles that result from the reaction with endogenous enzymes should be removed manually (E. H. Park & D.S. Kim, 1984). The technique is thus the main and important method for detecting changes in skeletal patterning.

The present study was carried out to study the skeletal framework of different fishes by a non-destructive bone staining protocol that retains small or incompletely calcified immature bones in their intact position and to assess the skeletal maturation

MATERIALS AND METHODS

Four different edible, bony fishes such as *A. mola, M. aureus, Puntius sarana, Sciades parkeri* and *Ambassis vachellii* were collected from local market of Ranchi, Jharkhand. Fishes were killed and the scales and gut were removed. Then specimens were fixed in 10% formalin for 5-7 days depending on the size of the specimens. Specimens were washed thoroughly to wash the fixative. Then specimens were placed in Alcian blue solution for one to two days for cartilage staining and were transferred to two

changes of 95% ethanol, 3 to 4 hours in each change, then through successive solution of 75%, 50% and 30% ethanol for 3 to 4 hours each. The specimens were placed in Trypsin solution for several days to weeks depending on the solution and the amount of fat it contains. The specimens were placed in a lightbox which accelerated the clearing process. The specimens were transferred to 0.5% aqueous potassium hydroxide about 3 hours for small size fish and 6 to 7 hours for large size fish to wash away the enzyme solution and build a KOH environment to help the penetration of Alizarin red S into bone. Then the specimens were transferred to Alizarin red S solution for about 24 hours until the bones were distinctly red or reddish purple and then were transferred to 0.5% KOH for 2 to 3 hrs for decolourization and bleaching. Finally, the specimens were transferred through solutions of 30%, 50% and 70% ethanol, leaving the specimens in each solution for about 3 hrs to prevent fading and diffusion of the stain. For proper differentiation, specimens were transferred to 0.5% KOH overnight. The specimens were transferred to 30% glycerine and 0.5% KOH until it sinks, finally stored in 100% glycerine with thymol

RESULTS AND DISCUSSION

Study of bones

The result obtained from the clearing process was clear vision of bones. This process made the specimens completely clear without skinning. Details of the skeletal system were observed in specimens with high transparency. All the bones in all the specimens were clearly visible (Fig.1. A, B, C, D, E). The bones were lower jaw, premaxillary, cranium, opercular bones, pelvic bones, ribs, abdominal vertebra, caudal vertebra, hypural, fins. There were variations in the bone structure of all the five species of fishes. Some have modifications in their bone structure and due to the deposition of calcium the bones are stained light and dark red colour.

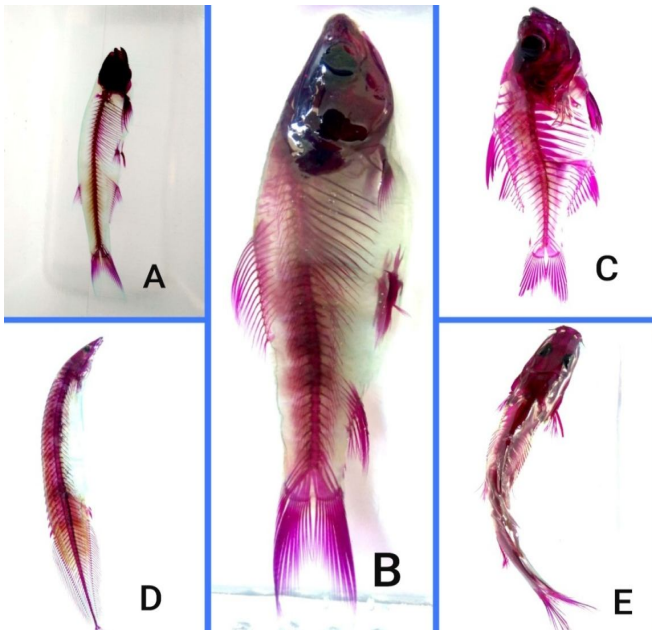
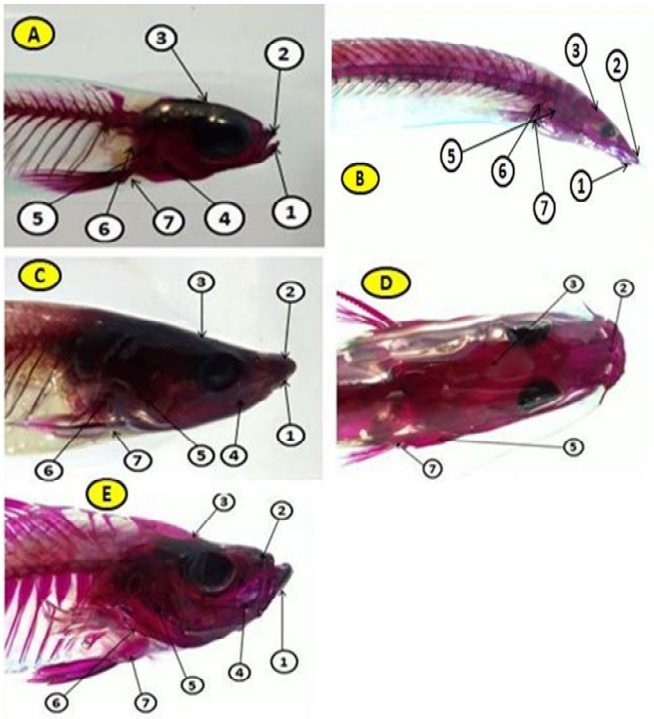


Fig. 1. Showing the Bone staining of A. *A. mola*, B. *P. sarana*, C. *A. vachellii*, D. *M. aureus*, E. *S. parkeri.*

Comparative study of skulls

At the lower jaw of skull region, premaxillary, cranium opercular bones, pectoral arches and pelvic bones were found. In *A. mola*, the lower jaw and premaxillary were upward in position because they are surface feeder. Cranium and opercular bones were deeply stained, it showed that it contains high amount of calcium. Though the opercular bones were deeply stained the maxillary and pectoral arch were not clearly visible but the pelvic bones were clearly identified (Fig. 2A). Pelvic fins arose from the pelvic bones. In *M. aureus*, the lower jaw was small, and the upper jaw was pointed (Fig. 2B). Cranium and opercular bones were clearly visible and lightly stained than *A. mola.* In *P. sarana*, the bones were dark stained due to presence of large amount of calcium. Ventrally the lower jaw was present. The upper jaw covered the lower jaw. The cranium and the opercular bones were clearly visible and larger in size. The pelvic bines were clearly identified (Fig. 2C). *Scisdes parkeri*have ventrally flattened head. A pair of barbels arose from the anterior part of the mouth. Nostril pores were clearly visible. On the either side of the head a pair of eyes was present. The skull was lightly stained than *A. mola* and *P. sarana.* From the base of pelvic bone, a pair of spines like structures arose. The upper jaw covered the lower jaw (Fig. 2D). In *A. vachellii*, the skull bones were slightly stained which means it contains lower amount of calcium. The lower jaw was upward in position that means they are surface feeder. The opercular bones were flattened and the pelvic bone was present comparatively in upper level than other four fishes. Pectoral arches and the opercular bones were clearly visible (Fig. 2E).

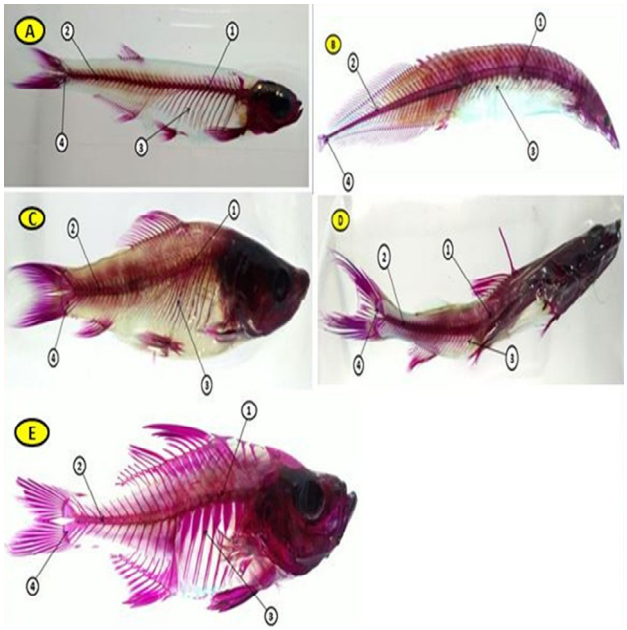


1. Lower jaw, 2. Premaxillary, 3. Cranium, 4. Maxillary, 5. Opercular bone, 6. Pectoral arch, 7. Pelvic bones.

Fig.2. Comparative structure of Skull bones.
(A) *A. mola*, (B) *M. aureus*, (C) *P. sarana*, (D) *S. parkeri*, (E) *A. vachellii.*

Comparative study of vertebral column

In this study we observed that the vertebral column originated from the head region and extended up to the caudal fin with many branching. It was identified as abdominal vertebra, caudal vertebra extended up to caudal fin with hypural. Hypural were the flattened structure. Ribs were developed from the vertebral column. The ribs at the abdominal region of *M. aureus* were shorter in size as comparison to the other four species. In *A. vachellii*, the ribs at the abdominal region were flattened in their structure (Fig. 3. A, B, C, D, E). The length of vertebral columns were about 3.9c.m, 4.5c.m, 4.6cm of three specimen of *A. mola*, 11.2c.m, 7c.m, 10c.m, 7c.m of *M. aureus, P. sarana, S. parkeri* and *A. vachellii* respectively.

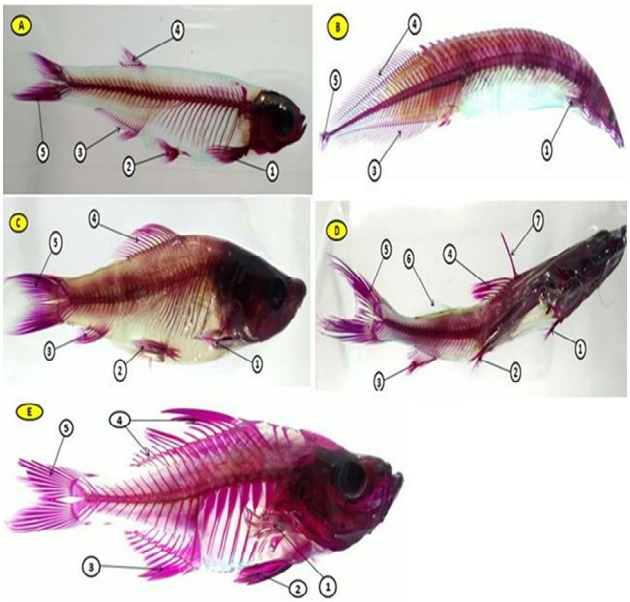


1. Abdominal vertebra, 2. Caudal vertebra, 3. Ribs, 4. Hypural

Fig. 3. Comparative Structure of Vertebral column.
(A) *A. mola,*(B) *M. aureus,* (C) *P. sarana,* (D) *S. parkeri,* (E) *A. vachellii.*

Comparative study of fins

Though the fin rays and spines contain calcium, they were stained by Alizarin red S. All the fishes had a few fins at different places which were clearly visible (Fig. 4. A, B, C, D, E). One pair of pectoral fins, one pair of ventral or pelvic fin and one anal fin were present in *A. mola.* At the dorsal region, one dorsal fin was also present at the posterior side (Fig. 4A). The bifurcated tail or caudal fin was present which was jointed with hypural. But in *Macrognathus aureu,* the structure and position of the fins were different from other four species (Fig. 4B). One pair of pectoral fins was present, but no pelvic fin was present. The anal fin and dorsal fins were elongated up to the caudal region. The caudal fin was exceedingly small but was not bifurcated in comparison to other specimens. At dorsal region (before the dorsal fin) 3-4 no. of spines were observed. *P. sarana* had a pair of pectoral fins, one pair of pelvic fins and one anal fin. One dorsal fin was present. The bifurcated anal fin was connected to hypural (Fig. 4C). In*S. parkeri,* the structure of fins was different from the others. At the pectoral fin, a pair of ciriated spines was present. As other species had the pelvic fin and the anal fin, these fins were also present here. Aciriated spine was present at the beginning of dorsal fin which was absent in other species (Fig. 4D). The second dorsal fin was adipose fin, which was also absent in other specimens. In *A. vachellii,* two dorsal fins were present where the other specimen had single dorsal fin. There was some space present between the two dorsal fins which separate them from each other. Fin rays were present only in pectoral fins but in other fins, flattened spines were observed. The caudal fin was also different in structure in comparison with other four species (Fig. 4E)



1. Pectoral fin, 2. Pelvic or ventral fin, 3. Anal fin, 4. Dorsal fin, 5. Caudal fin, 6. Adipose fin, 7. Ciriated spine

Fig. 4. Comparative of different fins.
(A) *A. mola,* (B) *M. aureus,* (C) *P. sarana,* (D) *S. parkeri,* (E) *A. vachellii*

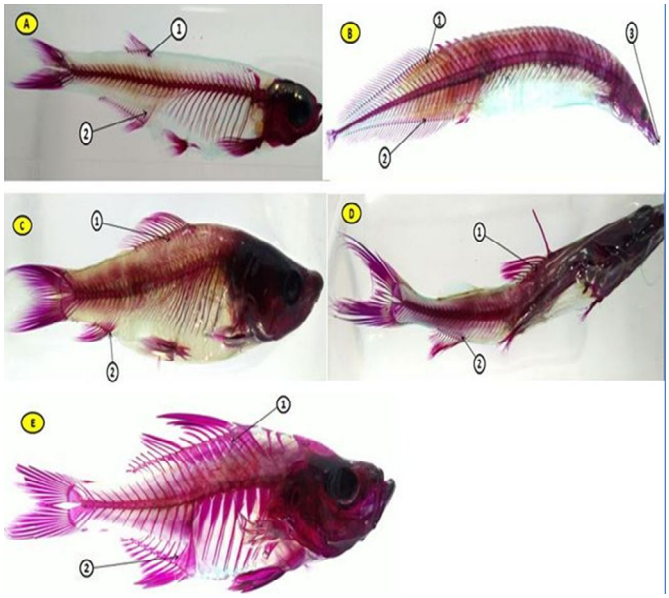
Comparative study of cartilage

In this study, it was observed that every specimen had inter neural and inter haemal radial cartilage (Fig. 5. A, B, C, D & E).But they have difference in their structure. In *A. vachellii,*the inter neural and inter haemal bones were flattened in structure in comparison with other fishes (Fig. 5E). In *M. aureus,* at the anterior part of mouth the presence of cartilage was noticed (Fig. 5B).

After staining with Alcian blue the cartilage were appear bluish in colour without transparency. It is clearly noticed that there are very a smaller number of cartilages are present in all the specimens like interneural, interhaemal, radial cartilage (Fig. 5). In *M. aureus*the pointed end of mouth was noticed as the presence of cartilage which was stained as blue (Fig. 5B).

Researchers in ichthyology generally prepare fishes for osteological studies by using a common whole-mount procedure known as clearing and staining (Dingerkus and Uhler, 1977; Potthoff, 1984). Whole mount skeletal staining permits evaluation of the shapes and sizes of skeletal elements in their appropriate location and it helps in the study of skeletal development, maintenance, and regeneration; demonstrate that clearing and staining causes fish to shrink. In total, relative to their live length, cleared-and-stained specimens shrink approximately 3% to 6%

(Mabee Paula M.et. al., 1998). The technique is thus the major method for detecting changes in skeletal patterning. Alizarin compounds, either in the form of alizarin red S (ARS) or alizarin complexone (ALC), have long been used to stain the mineralized skeleton in fixed specimens from all vertebrate groups (A. Bensimon-Brito et. al., 2016).



1. Inter neural radial cartilage, 2.Inter haemal radial cartilage, 3.Mouth cartilage.

Fig. 5. Comparative structure of Cartilage.
(A) *A. mola,* (B) *M. aureus,* (C) *P. sarana,* (D) *S. parkeri,* (E) *A. vachellii*

In this comparative study, five different fishes were used. This experiment followed the pathway of the fixation, decolourization, and transparentizing of specimens at the same time (Hiromi Sakata et. al., 2018). This paper describes a procedure for rendering cartilage blue, bone red and soft tissue translucent or transparent in whole vertebrate specimen. Alcian blue and Alizarin red S were used to stain cartilage and bone, respectively. Cartilages were stained blue because Alcian blue is a cationic dye which binds the sulphated GAGs and glycoproteins. It was also used for the staining of cartilage of chicken embryo (J. L. Ojeda *et al., 1970*). Bones were stained red because Alizarin red S is an anionic dye which binds calcium. For the perception of the bone structure, Alizarin recolouring strategy was utilized by T. Potthoff (1984) and was utilised with some alternation to the stain the entire fish to watch the bone structure by S. Kameswari et. al. (2019).In our procedure formalin was used as a fixative. This is a significant modification because formalin is the common fixative for museum specimen. This cleaning and staining procedure is thus readily applicable to comparative studies in anatomy, embryology, and systematic zoology.

CONCLUSION

Vertebrate biologists have been studying comparative anatomy using Alizarin red S stained skeleton since the beginning of the 20th century. The present study creates contrast between tissue and high lights the bones and cartilages distribution of five different fishes, facilitating the recognition and description of the structures. Thus, the staining of bones helps in disease detection and deposition of calcium in different species. Our procedure is simple and non-destructive and should therefore be useful for examining the skeletal framework. Another advantage of our procedure is that it resulted in less or no structural damage. This beneficial feature contributed to reducing the time and labour required for preparation of highly cleared bone stained specimen

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