In situ Visualization and Characterization of Intermuscular Bone Development in Milkfish (*Chanos chanos*)

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Introduction

Milkfish (*Chanos chanos*) is one of the most important finfish species in Philippine aquaculture. However, its processing and consumer acceptability are hindered by the presence of "spicule-like" intermuscular bones (IBs), a feature that has received limited research attention. The milkfish aquaculture industry needs to address these limitations using informed strategies with a solid biological basis. However, there is a significant knowledge gap in the development of milkfish IBs. Thus, this study set out to provide a detailed characterization of milkfish IBs.

Materials & Methods

Hatchery-bred milkfish at larval (9.0–11.0, 14.0–15.0, and 18.0–20.0 mm standard length [SL]; n = 30 per group), juvenile (~110 mm SL; n = 4), and sub-adult (~250 mm SL; n = 3) stages were collected for tissue clearing and differential bone staining. This technique was used to visualize in situ both the cartilaginous and skeletal elements of the specimen, including the IBs. After staining, the samples were preserved and documented. We also conducted a preliminary molecular investigation and identified likely homologs of IB-related genes to gain insight into the molecular basis of IB formation in milkfish.

Results & Discussion

The morphological characterization of milkfish IBs is still relatively understudied, despite the species' economic importance. The findings of this study show that at size 9.0-11.0 mm SL, the axial skeleton is cartilaginous, and no IBs are formed yet. Vertebral ossification begins at 14.0-15.0 mm SL, preceding IB formation. By 18.0-20.0 mm SL, the vertebrae are fully ossified, and IBs first appear posteriorly, extending anteriorly (Fig. 1).

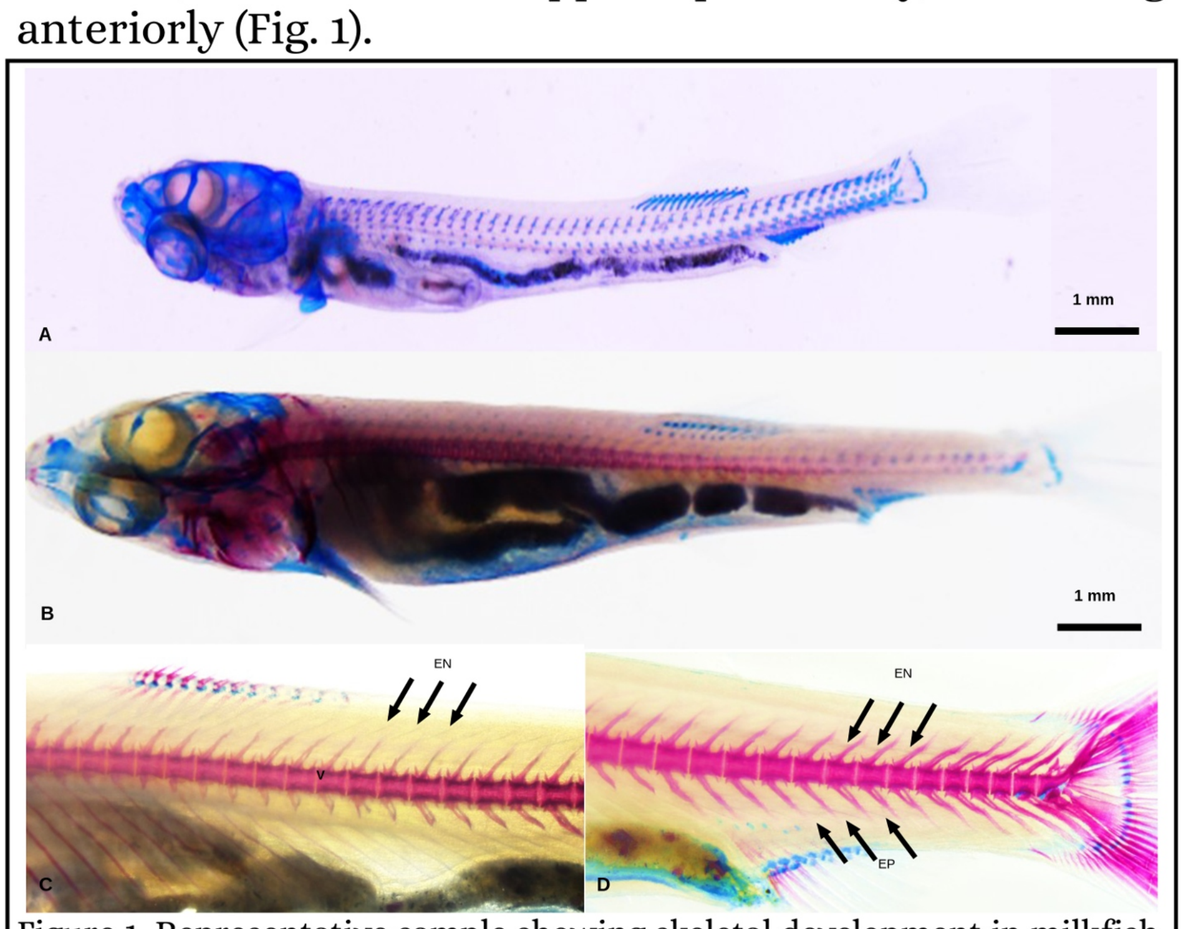


Figure 1. Representative sample showing skeletal development in milkfish. (A) Fry measuring 9–11mm SL has a cartilaginous axial skeleton. (B) At size 14.0-15.0 mm SL, vertebrae start to ossify as indicated by the red staining. Samples are viewed at 6.7x magnification. At size 18.0-20.0 mm SL, IBs are notable at 10x magnification (C, D), showing ossification from posterior region towards the anterior region. EN = Epineurals, EP = Epipleurals, V = Vertebra

Ossification continues until the juvenile stage. Sub-adult specimens have completely developed IBs. Milkfish possesses six IB morphological types: 1) two-end-multi-forked, 2) one-end-multi-forked/one-end-unequal-bi-forked, 3) one-end-unequal-bi-forked (⊦ -type), 4) one-end-multi-forked, 5) non-forked (I-type), and 6) Y-type.

Variation in IB complexity has been associated with evolutionary history, as exhibited by close relatives Cyprinidae and Chanidae (milkfish) (Patterson and Johnson 1995). Further differences in IB complexity between anterior and posterior regions, as well as the ossification process, may reflect the biomechanical demands of subcarangiform swimming (Danos and Ward 2012; Yao et al., 2015).

Additionally, preliminary molecular investigation shows that milkfish *runx2b* and *bmp6* share conserved functional domains with their zebrafish counterparts (Fig. 2), which regulate osteoblast differentiation and IB ossification (Li et al., 2009; Nie et al., 2022), suggesting that these genes may serve similar roles in milkfish. This provides the first molecular insight into IB formation in milkfish.

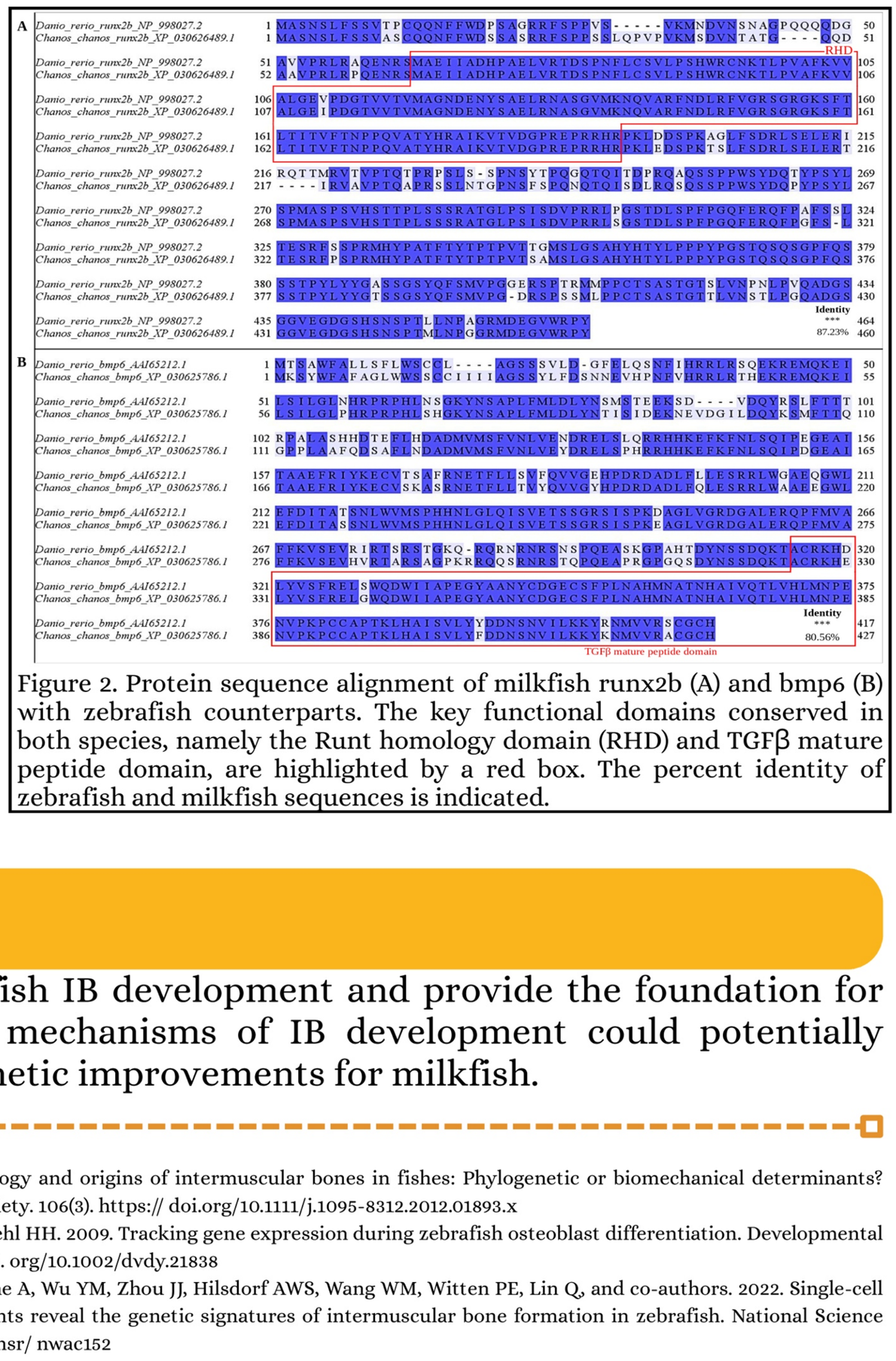


Figure 2. Protein sequence alignment of milkfish runx2b (A) and bmp6 (B) with zebrafish counterparts. The key functional domains conserved in both species, namely the Runt homology domain (RHD) and TGFβ mature peptide domain, are highlighted by a red box. The percent identity of zebrafish and milkfish sequences is indicated.

Conclusion

Overall, these findings advance understanding of milkfish IB development and provide the foundation for future molecular studies. Elucidating the underlying mechanisms of IB development could potentially inform interventions such as breeding strategies and genetic improvements for milkfish.

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