Scale surface topography of a vulnerable cyprinid fish, Schizothorax plagiostomus from Kashmir Himalayas using Scanning Electron Microscopy (SEM)

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INTRODUCTION

The Kashmir valley is located in the Himalayan Mountain system along the eastern area, between the Pir-Panjal and Zanskar ranges. It has a unique position in harboring rich and diverse types of aquatic habitats, occupying 6% of its total land area (Zutshi and Gopal, 2000). There are lentic and lotic waterbodies in the valley well known around the world. Over the years, extensive research has been conducted on the water bodies of the Kashmir valley, particularly focusing on the rich ichthyofauna inhabiting its lakes and streams. Fishes belonging to the families Cyprinidae, Cobitidae, Siluridae, Poecilidae, Sisoridae and Salmonidae are found in the valley (Bhat *et al.,* 2010). The major ichthyofauna of Kashmir is represented mainly by the Central Asiatic fauna in which genus *Schizothorax* is predominant (Sunder *et al.,* 1979). The genus *Schizothorax* is the most diversified Schizothoracine, possessing more than 100 species and subspecies and is represented in Kashmir waters by *S. niger* , *S. curvifrons* ,*S. esocinus* , *S. labiatus and S. plagiostomus* (Wani*et al* ., 2018). *Schizothorax plagiostomus* (locally known as Khont) stands out with its elongated body, projecting snout, and lower jaw with a sharp edge. It has distinctive features like expanded lower lip fold. This fish prefers fast flowing streams in Kashmir (Kullander*et al* ., 1999). This is an important food fish of Kashmir Himalayas. The fish has been declared vulnerable by International Union for Conservation of Nature (IUCN) on 25th April 2022. The vulnerable state of this indigenous fish species reflects the broader challenges confronting Kashmir's water bodies, constantly subjected to ecological changes due to various anthropogenic factors (habitat degradation, overfishing and water pollution). Inadequate attention and insufficient measures exacerbate the risks faced by this fish species; therefore, necessary conservation measures should be taken to conserve this vulnerable species. This highlights the importance of my work where use of SEM can enhance taxonomic precision and contribute valuable insights into the morphological features of various species facing threats. This innovative approach not only aids in accurate identification but also forms a foundation for framing effective conservation strategies. By providing a precise tool for species identification, my work extends its benefits to other vulnerable species globally. Application of scanning electron microscopy (SEM) in fish taxonomy is relatively recent development (Delmater & Courtenary, 1974; Kaur & Dua, 2004; Johal *et al* ., 2006; Liu *et al* ., 2008). The scanning electron microscope (SEM) is one of the most versatile instruments available for the examination and analysis of microstructure morphology and chemical composition characterizations (Zhou *et al* ., 2007). It is a type of electron microscope that uses a focused beam of high-energy electrons to scan the surface of a sample to create detailed images of its topography, composition, and other properties. Scanning electron microscopy (SEM) has significantly advanced morphological studies, particularly in the investigation of fish scale morphology (Echreshavi *et al.,* 2021; Esmaeili *et al.,* 2019; Sadeghi *et al.,* 2021). This instrument has greatly enhanced our understanding of the macro- and microstructural morphology of scales, revealing numerous new features that are now effectively utilized in ichthyological research. Since its invention, SEM has made substantial contributions to the field. Fish scales are dermal elements embedded in the dermis and epidermis of most fish species, appearing as thin or thick plates (Wainwright, 2019). These scales come in various forms and shapes, and historical research has established a framework for categorizing them, which is fundamental for describing their morphology (Wainwright, 2019). The use of scales in fish classification dates back approximately 188 years, when Swiss Zoologist Louis Agassiz (1833-1843) first employed them as a taxonomic tool. He classified fish into four groups based on their scales: Placodermi, Ganoidei, Ctenoidei, and Cycloidei. Since then, numerous ichthyologists have described fish scales and utilized their morphology for taxonomy (Goodrich, 1907, 1909; Lagler, 1947; Lanzing & Higginbotham, 1974; Lippitsch, 1990; Williamson, 1851). Fish scales exhibit various structural features such as type, shape, size, circles, radii, lepidonts, granule configurations and lateral line structures, which have been used in fish identification and classification at species and higher taxonomic levels (Batts, 1964; Hughes, 1981; Kaur and Dua, 2004). Tzeng *et al* . (1994) emphasized the utility of fish scales in comprehending the life history of a fish, including age composition and growth rate. Various researchers, such as Kobayashi (1953), Dulce-Amor *et al* . (2010), Esmaeli *et al* . (2012), and Zubia *et al* . (2015), have utilized scales to study phylogenetic relationships, systematic classification, and sexual dimorphism. Fish scales have proven to be effective bioindicators of water pollution (Kaur & Dua, 2012). Various pollutants have been identified in the environment as a consequence of urbanization, industrialization, and technological advancements (Kaur & Dua, 2012). Aquatic organisms are biologically sensitive and can respond to changes in water quality, with fish scales being among the first structures to be affected by environmental changes, including pollution. Surface morphology and microstructures of scales have been used to identify various fish groups, including cichlids (Lippitsch, 1990), tripterygiids (Jawad, 2005), cyprinids (Jawad, 2005), synodontids (Jawad & Al-Jufaili, 2007), cyprinodontids/aphaniids (Esmaeili *et al.,* 2019; Esmaeili & Gholami, 2007; Ferrito *et al.,* 2003; Ferrito*et al.,* 2009; Teimori *et al.,* 2017), mugilids (Esmaeili*et al.,* 2014), and clupeids (Dizaj *et al.,* 2020). Schizothoracids have been subjected to various morphological, biological, and molecular studies (Lin *et al.,* 2010; Nie *et al.,* 2014; Zhou *et al.,* 2015; Zhang *et al.,* 2017; Hussain*et al.,* 2018; Farooq *et al.,* 2019; Gu *et al.,* 2019). However, information on detailed scale morphology and microstructures using SEM of genus *Schizothorax* have not been provided in literature yet.

This study represents the first attempt to utilize Scanning Electron Microscopy (SEM) to describe the ultrastructural features of the scales of the vulnerable cyprinid fish *S. plagiostomus* , collected from the Jhelum River in Kashmir. Our objectives are (i) to describe and emphasize the microstructural morphology of the scales, and (ii) to identify potential topographical and structural characteristics of the scales that can be used as taxonomic tool.

Materials and Methods

Sampling site and studied taxa

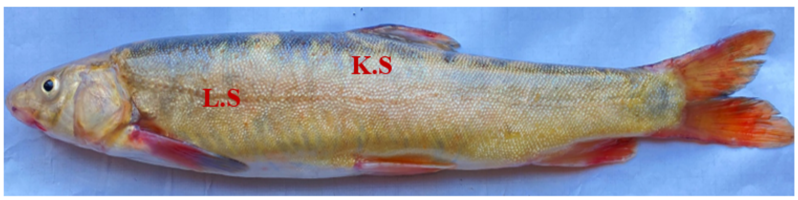
To study the morphological characteristics and ultrastructural design of scales in S. plagiostomus, a total of 12 fish specimens of same size were collected from River Jhelum (32@58′- 35@38′N, 73@23′-75@35′E) using cast net in December 2023. Samples were transported to Fisheries Resource Management (FRM) laboratory of Faculty of Fisheries, SKUAST-K in insulated boxes containing ice packs.

Scale preparation for Scanning Electron Microscopy (SEM) imaging

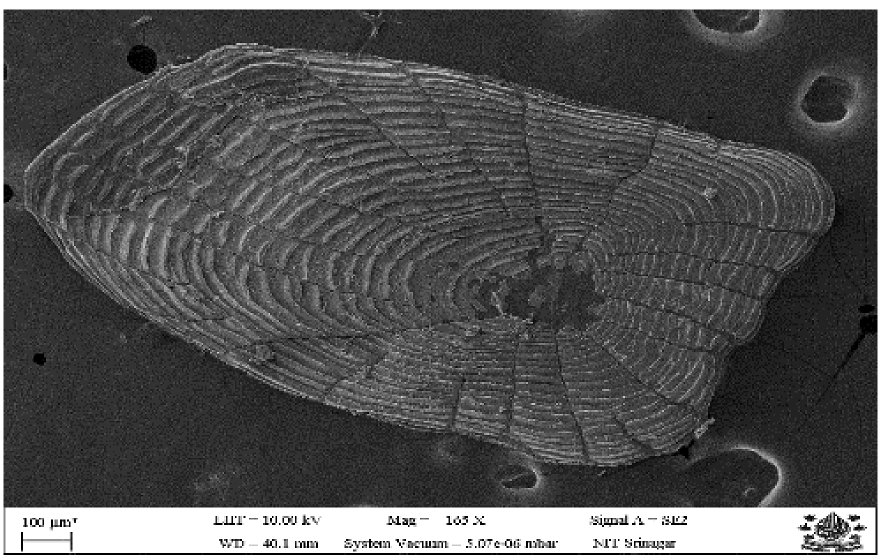
Fish total length and weight were measured using digital vernier caliper and weighing balance before scale removal. Key scales and lateral line scales (Figure 2) were used in the present study following Raffealla*et al.,* 2020 and Dey *et al.,* 2014. Key scales are the scales taken from the region below the dorsal fin and above the lateral line. Scales taken from the middle of lateral line are referred to as lateral line scales. In this study, a total of 240 scales were examined which comprised 20 scales from each specimen. Utmost care was taken so that no damage to the scales would occur while removing them from the selected regions with fine forceps. For the preparation of scales, Teimori *et al.* (2017), Esmaeili *et al.* (2012) and Lippitsch (1990) were followed. The scales were rinsed in distilled water to remove impurities followed by immersion in 5% potassium hydroxide (KOH) solution for a duration of 40 minutes to remove soft tissues from surface. It was followed by dehydration process using ethanol solutions of varying concentrations (30%, 50%, 70%, and 90%) each for a duration of 20 minutes and dried on filter paper. To prevent curling of scale margins, the scales were delicately positioned between two microslides for a period of 2-3 days. For SEM imaging, the prepared scales were carried to the Central Research Facility Centre, National Institute of Technology (NIT)-Srinagar where scales were attached to metal stubs with double adhesive tape, ensuring that the dorsal surface was oriented upward, while the ventral surface was securely affixed to the tape (Dey *et al.,* 2014). Metal stubs were then gold coated to a thickness of 100 Å in gold coating unit. The samples coated with gold were introduced into the SEM's holding chamber for in-depth analysis. The scales were observed in the SEM using the secondary electron emission mode, employing an accelerating voltage of 20 kV. Tilt control was set at 0° to precisely position the samples in a horizontal plane. The working distance was maintained at 8 mm.

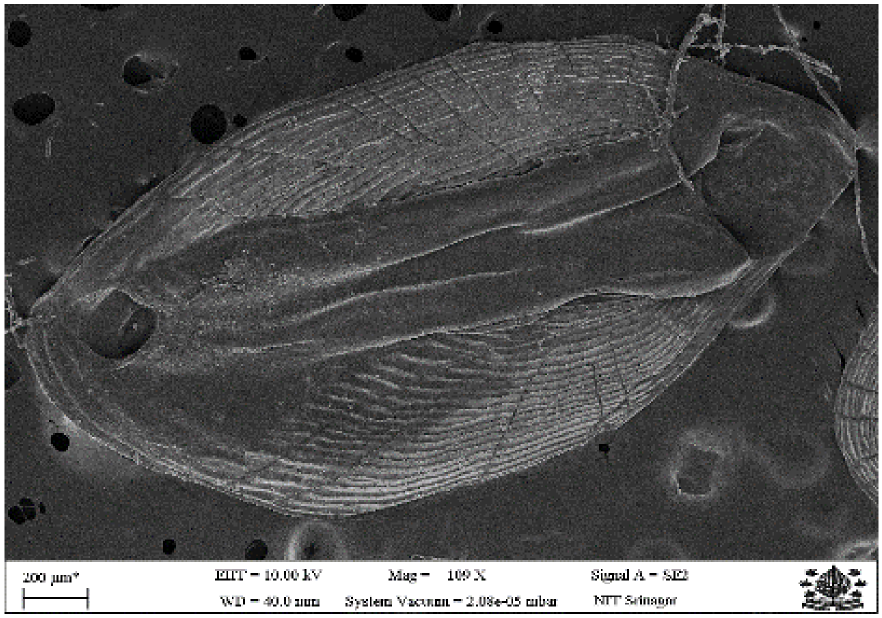
Scale measurements

Various parameters of scale components including length and width of scale, inter-radial distance of circuli, width of circuli, inter-circular space, width of radii, and more, were measured. These measurements were extracted from the SEM micrographs, utilizing reference bars present in the images (Dey *et al.,* 2014). Length of scale (LS) was calculated as anterior-posterior length or the maximum longitudinal diameter of the scale (Figure 3a), width of scale (WS) as maximum transverse diameter of the scale (Figure 3b), anterior focus length (AFL) was measured from anterior margin of scale to focus (Figure 3c), location of focus or Focus index(Fi) was calculated as the ratio of anterior focus length (AFL) to length of scale (LS) (Dey *et al.,* 2014; Teimori *et al.,* 2017; Jufaili *et al.,* 2021).**If the value of Fi <0.20, focus is said to be anterior in position; if Fi = 0.21-0.40, focus is said to be antero-central in position; if Fi = 0.41-0.60, focus is said to be central in position; if Fi = 0.61-0.81, focus is said to be postero-central in position and if Fi> 0.81, focus is said to be posterior in position** (Brager, 2016; Sabbah *et al.,* 2020; Echreshavi *et al.,* 2021). Morphological descriptive analysis was carried out using R studio version 4.2.2 (2023).



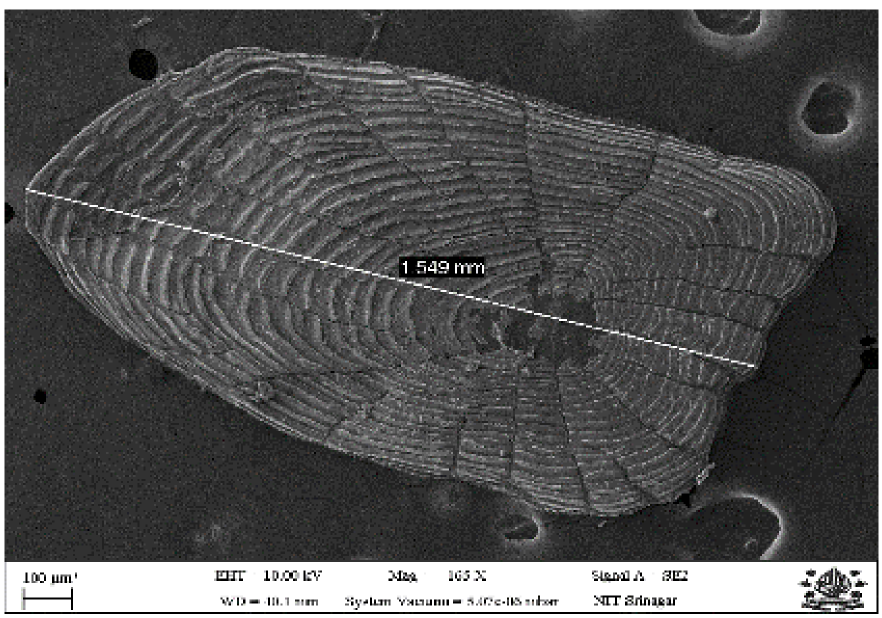
(a)

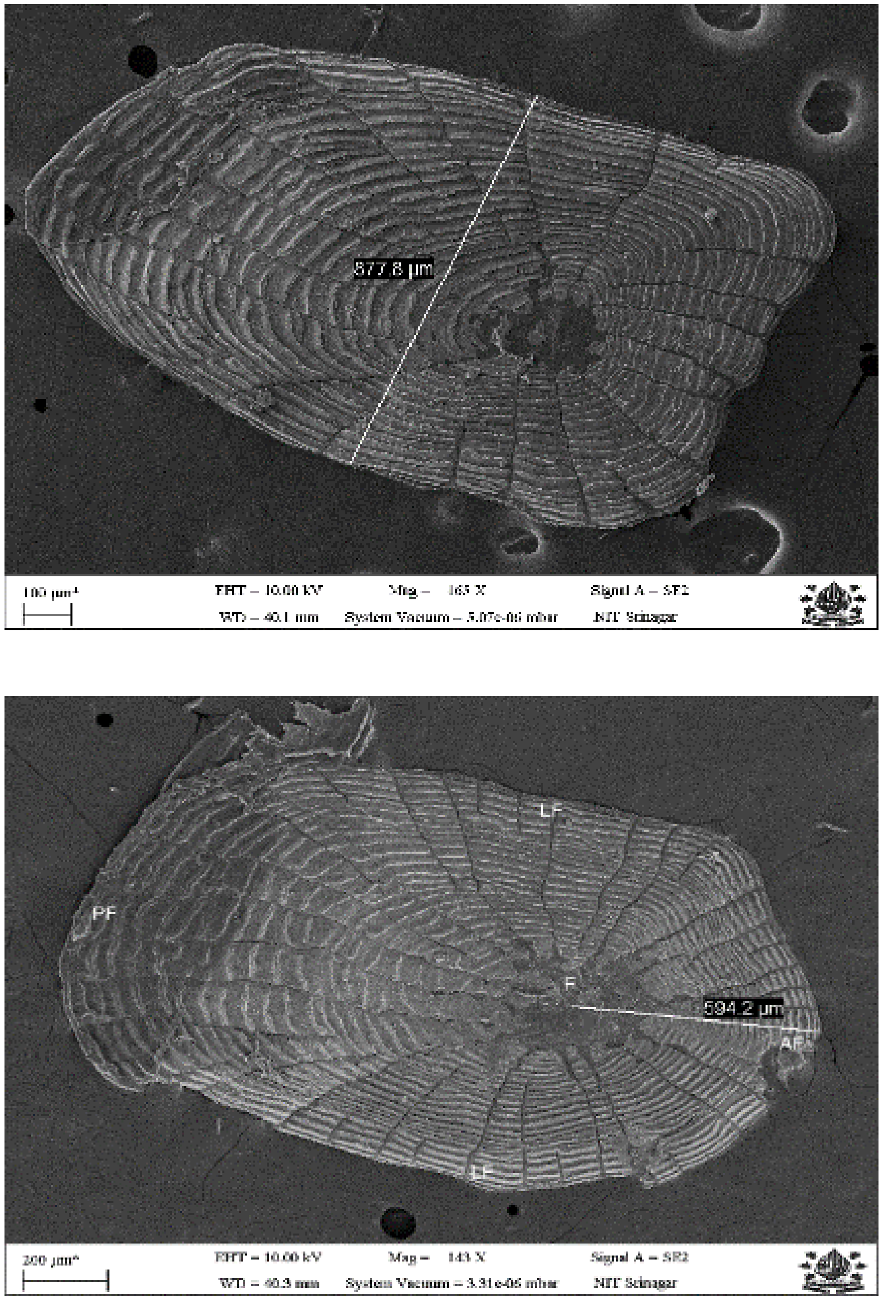




(b) (c)

FIGURE 2: (a) Image of *Schizothorax plagiostomus* showing 2 selected body regions (KS=key scales, LS=lateral line scales) where scales were removed from left side of fish. (b) Image of key scale. (c) Image of lateral line scale.





(a) (b) (c)

FIGURE 3: Linear measurements used for the estimation of focal index (Fi), (a) length of scale (LS). (b) Width of scale (WS). (c) Anterior focus length (AFL).

Results

Scale morphology and microstructural design

The general morphology of scales from the two selected regions is shown in figure 4. The scales of the studied cyprinid fish were cycloid, lacking ctenii on the posterior part. Each scale had a focus, dividing it into anterior, posterior, and lateral fields. The anterior field was embedded in the skin and overlapped by the next scale's posterior side. The ventral part was shiny and smooth, while the dorsal part was rough and convex. From the focus, circuli (growth lines) emerge, densely packed in the anterior part and more widely spaced in the posterior part. The scales feature three types of radii: primary radii extending from the focus to the margin, secondary radii not reaching the margin, and tertiary radii extending from midway to the margin. Lateral line scales have a canal along the anterior-posterior axis with distinct openings.

(a)

(b)

FIGURE 4: Morphological characteristics of the fish scale used in this study.

(a) Key scale (b) Lateral line scale.

Scale type

Cycloid type of scale was shown by *Schizothorax plagiostomus* due to absence of any ctenii on its posterior region (Figure 5a).

Scale shape

Polygonal and cordate shaped scales were found in the studied*Schizothorax* species. The rostral margin or front edges of the scales were often smooth, rounded and occasionally wavy (Figure 5b-c).

Focus

Α ςλεαρ φοςυς, ρουνδεδ ιν σηαπε, ωας πρεσεντ (Φιγυρε 5δ-φ). Τηε αρεα ενςλοσεδ βψ τηε φοςυς ωας σμοοτη. Τηε μεαν αλυε οφ σιζε οφ φοςυς ωας 122.90 μμ (Ταβλε 1). Ιτ ςαν βε ςονςλυδεδ τηατ τηε ποσιτιον οφ φοςυς ιν τηις σπεςιες ωας αντερο-ςεντραλ ας τηε μεαν αλυε οφ φοςαλ ινδεξ ωας 0.316 (Ταβλε 1).

Circulus/ circuli

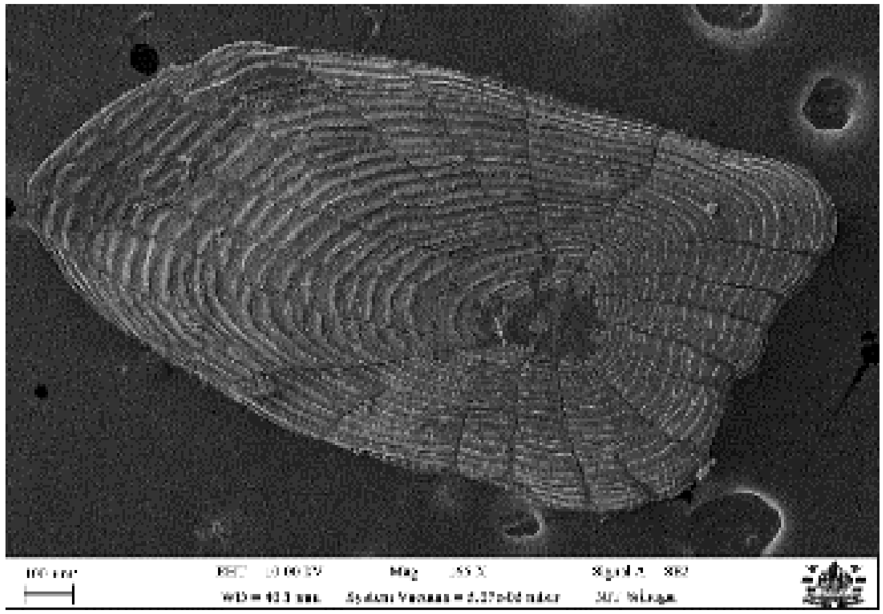
Τηε ςιρςυλι ωερε διστινςτ ανδ δισςοντινυους ανδ αρρανγεδ ιν ςιρςυλαρ παττερνς (Φιγυρε 5γ-ι). Ιν τηε αντεριορ φιελδ, τηε ςιρςυλι ωερε ςλοσελψ σπαςεδ ωιτη μεαν αλυε οφ ιντερςιρςυλαρ σπαςε οφ 14.91 μμ (Ταβλε 1). The mean value of number of circulii were 24 (Table 1) in the anterior field. There was an almost equal number of circuli in the transitional area between the anterior and posterior fields, while their number decreases in the posterior field. The shape of circuli was convex. Smooth circuli were present in this species due to the absence of lepidonts on circuli.

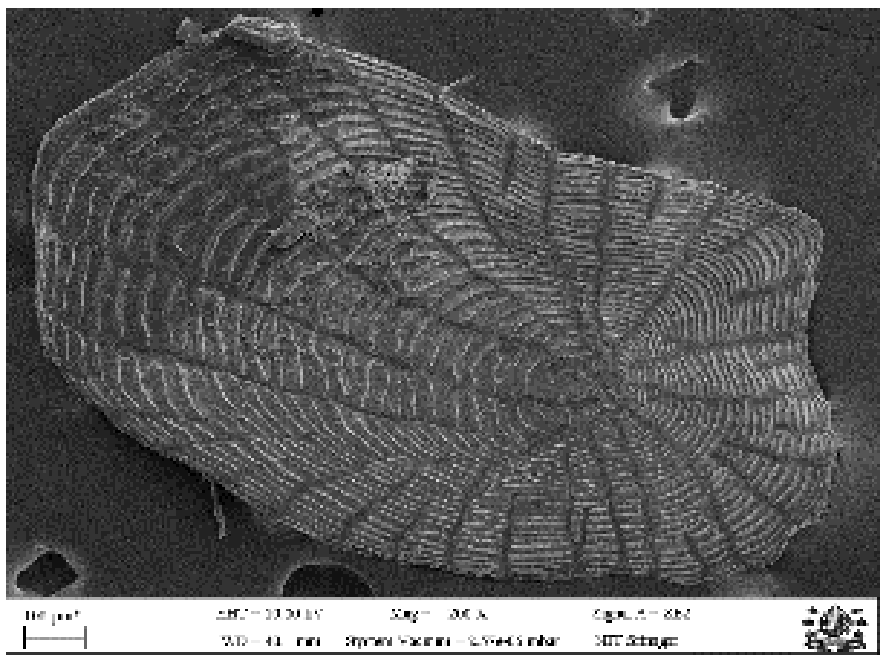
Radius/radii

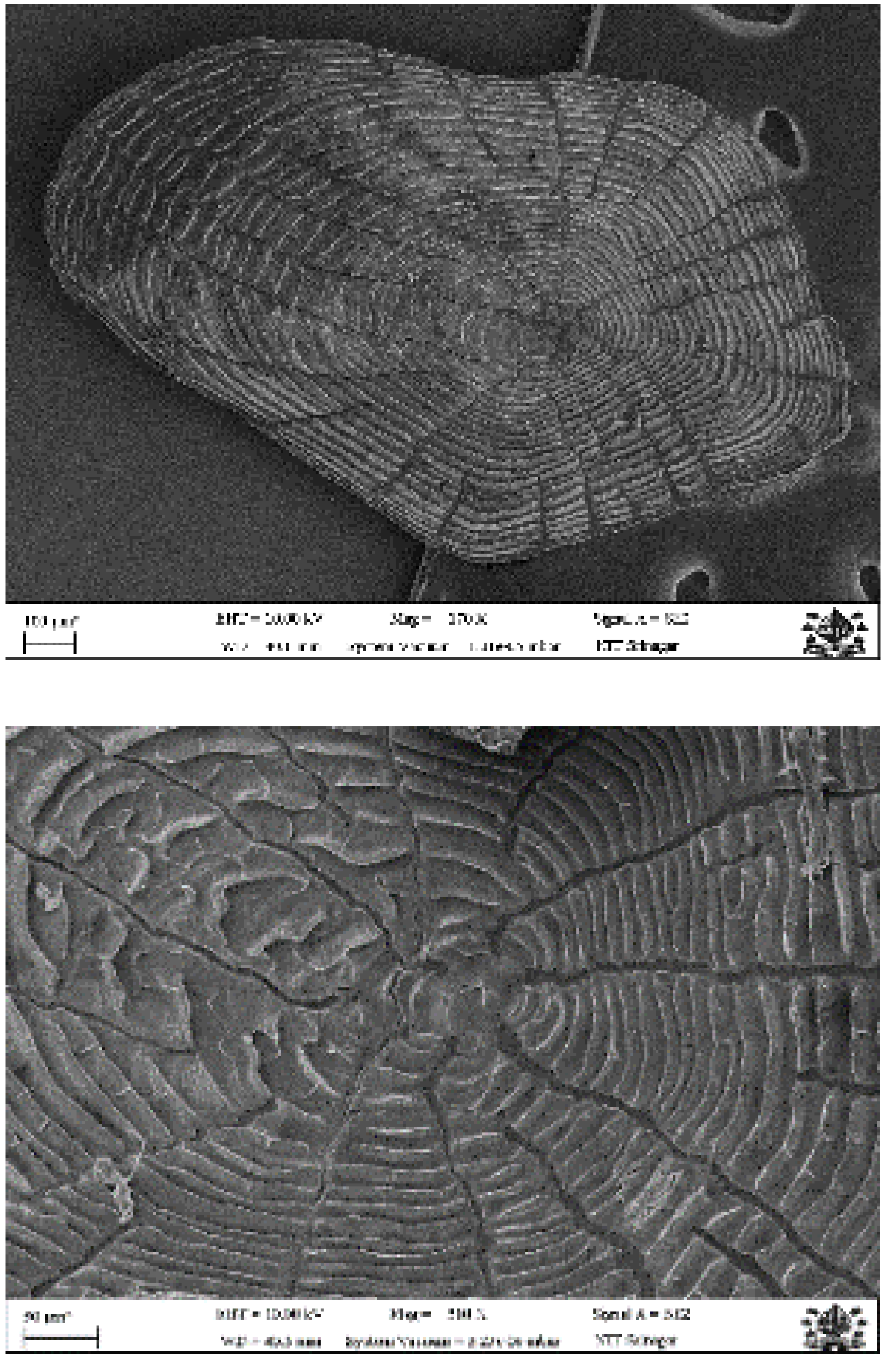
Scales possess radii in nearly all four fields (anterior, posterior, and laterals) and was thus categorized as tetra-sectioned type (Figure 5j-l). The radii extending towards the posterior were longer compared to the others. Generally, the scales exhibited three types of radii: primary, secondary, and tertiary with primary radii being more numerous than secondary and tertiary ones. All radii were oriented parallel to each other, with a well-aligned arrangement in the posterior field .The average number of radii were 11 and the width of radii was 9.70**μμ** in the anterior field (Table 1).

Lateral line scale

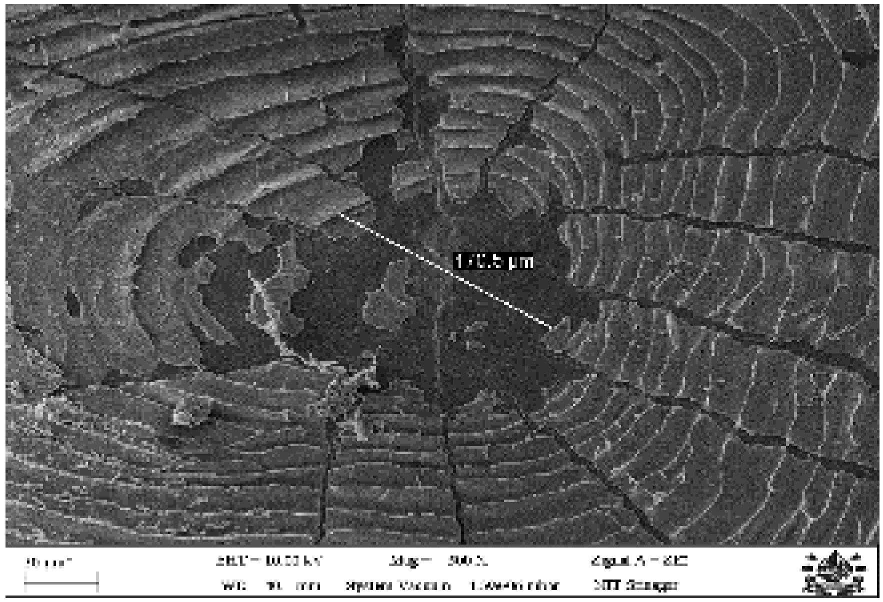
Lateral line scales in Schizothoracids included four parts viz. anterior, posterior and two lateral regions. The focus on these scales was absent and instead they have a channel existing along the anterior-posterior axis of the scales with two openings: anterior opening and posterior opening (Figure 5m-n). **Τηε μεαν αλυε οφ λενγτη οφ λατεραλ λινε ςαναλ ωας 1.52 μμ ανδ σιζε οφ αντεριορ οπενινγ οφ λατεραλ λινε ςαναλ ωας 354.61 μμ (Ταβλε 1).**

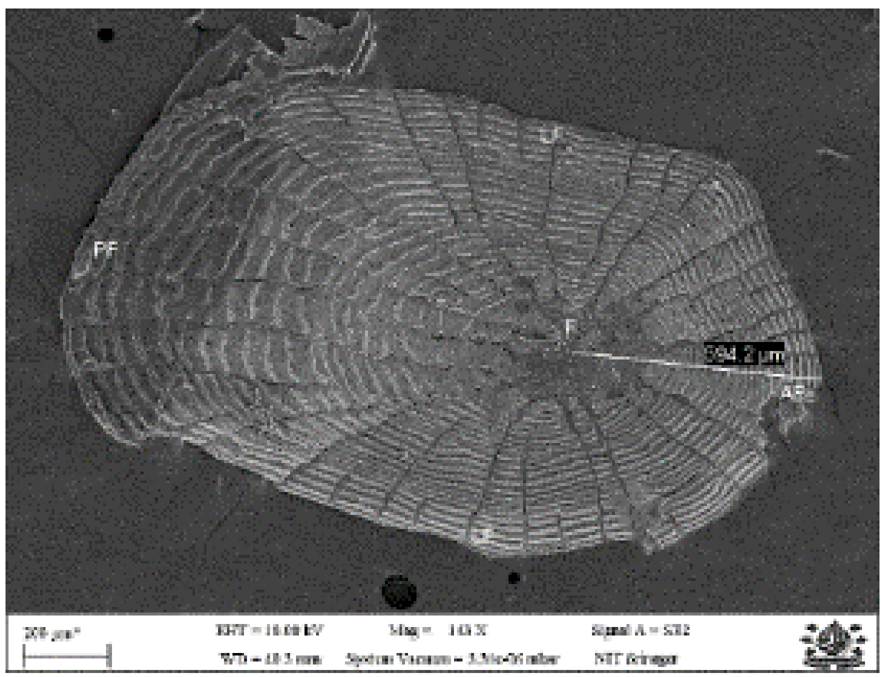






(a) (b) (c) (d)

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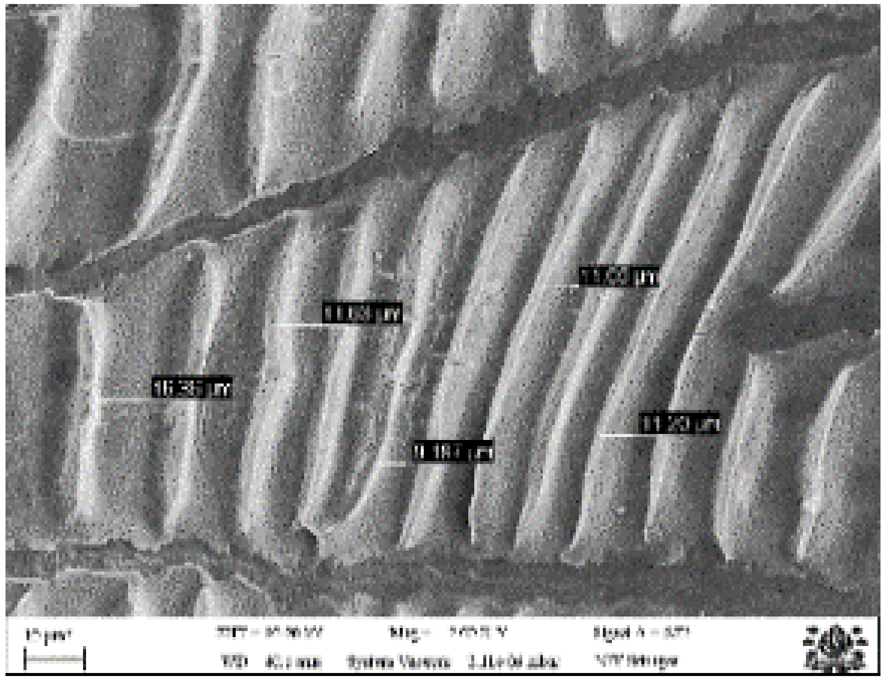
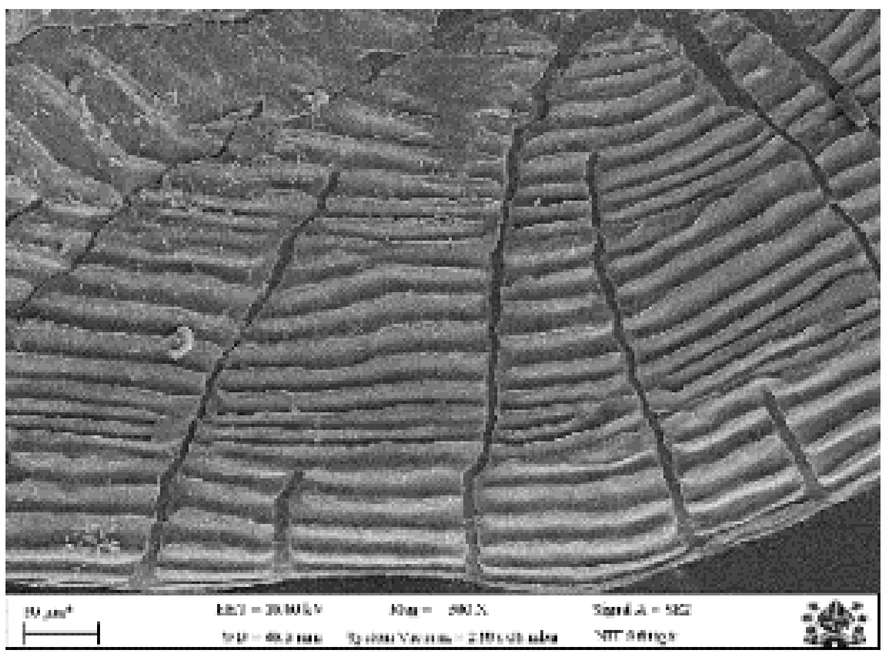
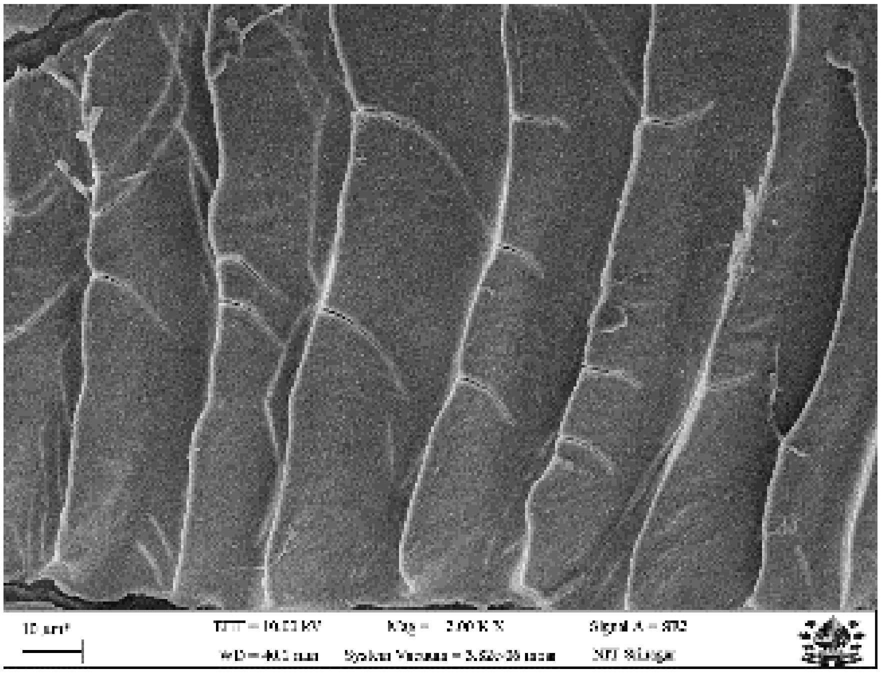
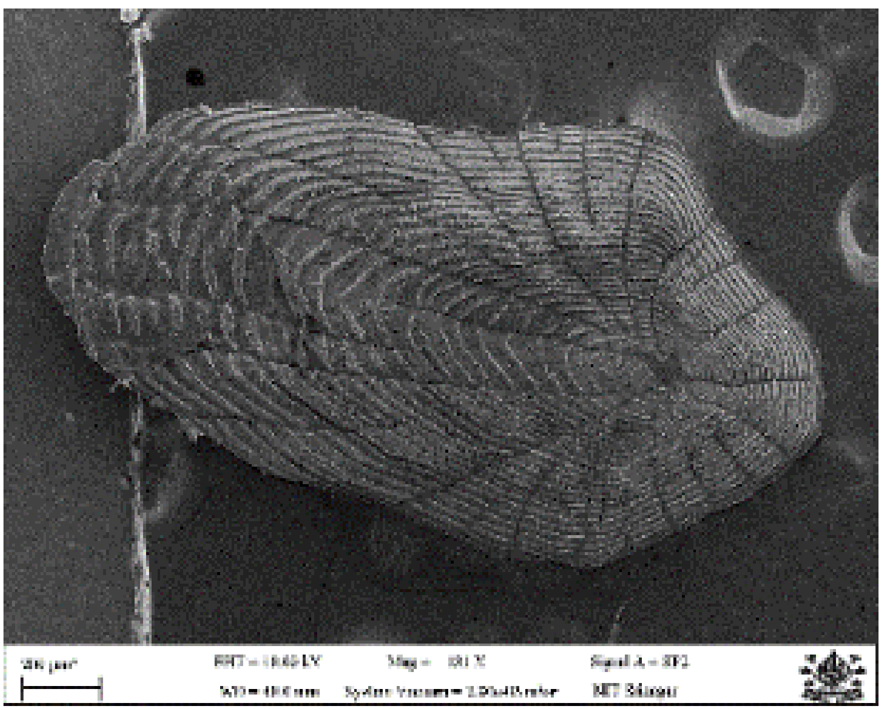
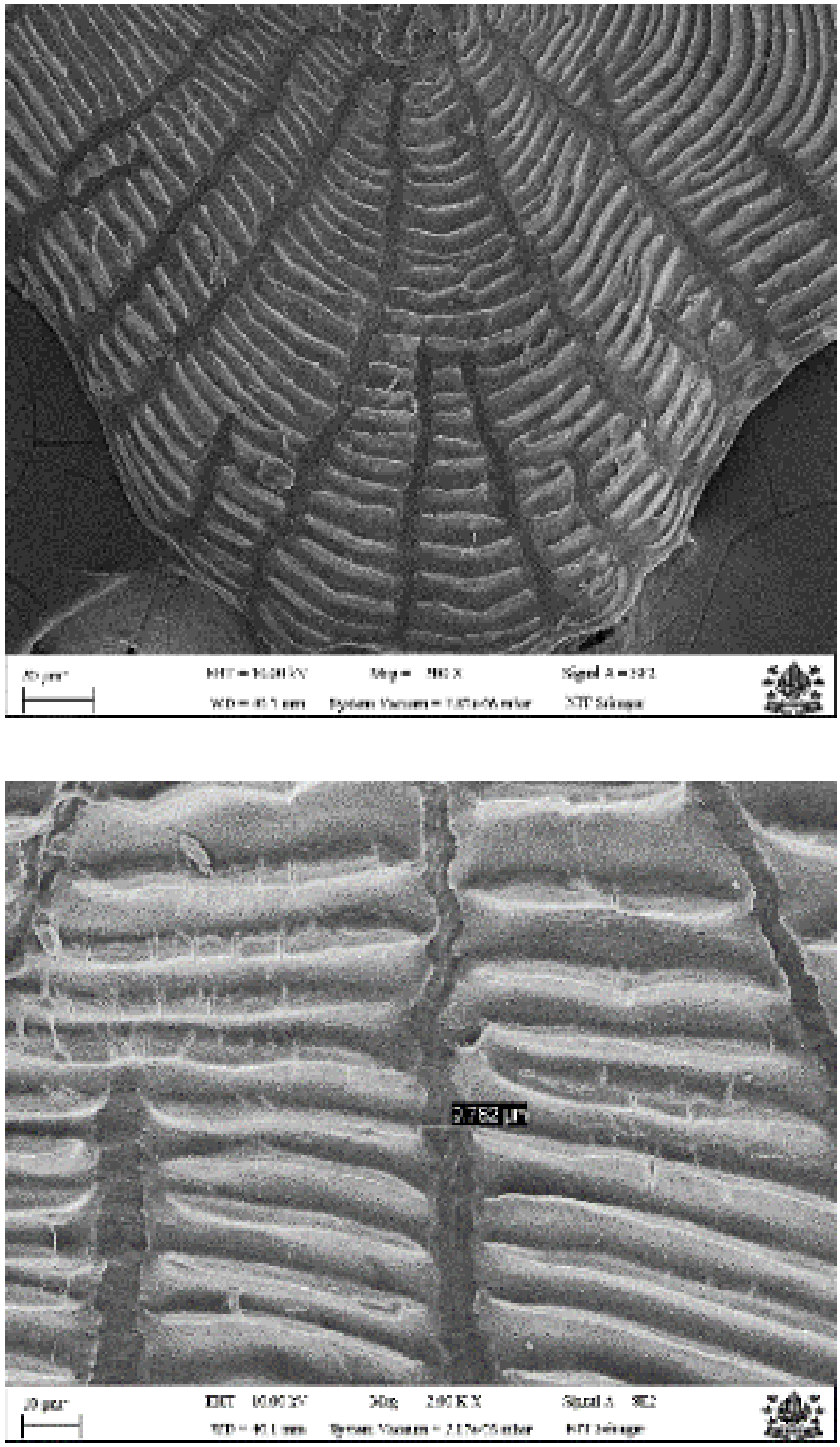




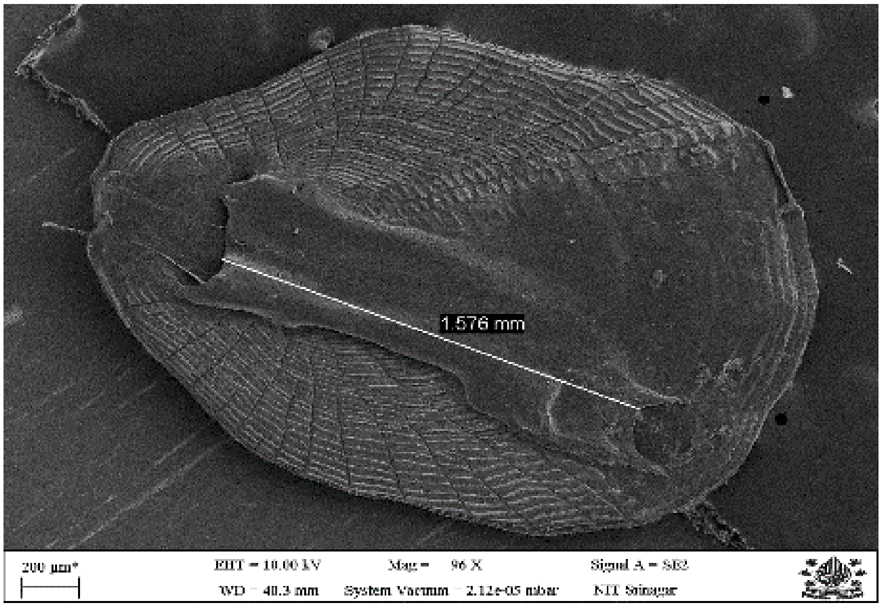
(e) (f) (g) (h)

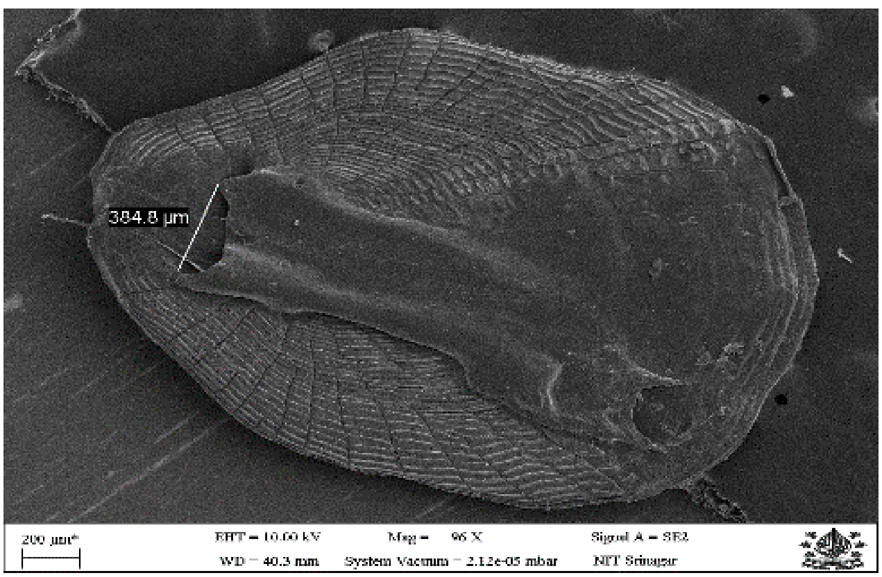

10 μm
Mag = 2.00 K X
Signal A = SE2


Signal A = SE2



(i) (j) (k) (l)





(m) (n)

FIGURE 5: (a) Type of scale. (b) Shape of scale. (c) Rostral margin of scale. (d-f) Shape, size and location of focus. (g-h) Intercircular distance, anterior circuli and smooth circuli (without lepidonts). (j-l) Tetra-sectioned scale, scale showing three types of radii (primary, secondary and tertiary radii), width of radii in anterior field. (m,n) Scale showing length and size of anterior opening of lateral line canal.

Table 1: Descriptive statistics of *Schizothorax plagiostomus*

Variable	**Mean**	**Standard Deviation**	**Coefficient Of variation**	**Minimum**	**Medi**
Length of scale (mm)	1.59	0.13	8.32	1.31	1.59
Width of scale (mm)	0.92	0.12	13.44	0.69	0.92

Size of focus (μm)	122.90	56.30	45.85	73.30	96.70
Location of focus	0.315	0.04	12.70	0.25	0.31
Number of circuli	24.00	2.93	12.07	18.00	25.00
Intercircular space (μm)	14.91	3.17	21.24	11.65	13.89
Number of radii	11.00	1.04	9.50	8.00	11.00
Width of radii (μm)	9.70	1.61	16.61	6.03	9.62
Length of lateral line canal (mm)	1.52	0.13	8.89	1.29	1.59
Size of anterior opening of canal (μm)	354.61	24.89	7.02	331.71	345.5

.

Table 2: Macro and microstructural details of key scales and lateral line scales for *Schizothorax plagiostomus.*

Character	**Key scale (KS)**	**Lateral line scale (LS)**
Type of scale	Cycloid	Cycloid
Shape of scale	Polygonal	Circular, cordate.
Anterior margin	Round and wavy	Wavy
Lateral field	Convex	Convex
Focus shape, size and position	Round, small and antero-central	Absent
Circuli in anterior field	Closely arranged	Closely arranged
Circuli in posterior field	Widely spaced and discontinuous	Widely spaced and discontinuous
Posterior margin.	Round and smooth	Round and smooth
Lepidonts	Absent	Absent
Chromatophores	Absent	Absent

Discussion

Recently, the advent of Scanning Electron Microscopy (SEM) has unveiled numerous new features of teleost scales, enriching ichthyological research (De Lamater and Courtenay, 1973; Hughes, 1981; Jawad, 2005; Teimori, 2016). These advancements have significantly expanded our understanding of fish scales, enhancing their utility in fish taxonomy and phylogeny. Despite this progress, many fish groups including the species studied here, remain insufficiently characterized in terms of their scale features (Esmaeili and Gholami, 2011; Ferrito *et al.,* 2009). In light of this, the present lepidological study aims to identify key micro-characteristics of scales that facilitate species identification and resolve taxonomic ambiguities among endemic*Schizothorax* species.

General morphology and Scale surface microstructures

Key scales

Scales are found in nearly all significant fish groups and exhibit a wide range of differences in their shape, structure, and development (Sire et al., 2009). Fish scales generally fall into four primary categories: placoid (found in cartilaginous fishes), ganoid (present in sturgeons and gars), cosmoid (seen in lungfishes of the Ceratodidae family and some fossil species), and elasmoid, which include cycloid and ctenoid scales and are predominantly found in teleost fishes (Esmaeili*et al.,* 2019; Wainwright, 2019). The diversity of scales across different fish groups makes them highly valuable for various ichthyological studies, including systematics (Poulet *et al.,*2004; Jawad, 2005; Gholami *et al.,* 2013), ontogeny (Sire, 1986), and phylogeny (Robert, 1993). Cycloid scales, in particular, are found in various fish groups, including those in the order Cypriniformes. Being a cypriniform fish, the general type of scale in*Schizothorax plagiostomus* was cycloid. Detailed studies of cycloid scales have been conducted on several other Cypriniformes, such as *Capoeta damascina* (Valenciennes, 1842), *Catla catla*(Hamilton-Buchanan, 1822), *Hypophthalmichthys molitrix*(Valenciennes, 1844), *Rutilus frisii* (Nordmann, 1840), and *Tor putitora* (Hamilton-Buchanan, 1822) (Esmaeili *et al.,*2007, 2012; Esmaeili and Gholami, 2011). Additionally, the presence of such scales has been documented in cyprinodontoid fishes, including *Cyprinodon variegatus* (Cyprinodontidae) and *Lamprichthys tanganicanus* (Poecilidae) (Rosen and Bailey, 1963).

In the current study, two type of scale shapes i.e., polygonal and circular/cordate were observed. However, a study on the scale surface topography of *Garra sharq* from the Arabian Peninsula revealed that the cycloid scales of this cyprinid exhibited various shapes, including circular (true circular, cordate, and discoidal), polygonal (hexagonal), and oval (true oval) across different regions and three size groups of the fish, with the circular type being the most prevalent. Other research on fish scales has examined both inter- and intra-species variation in overall scale shape (Echreshavi *et al.,* 2021; Gholami *et al.,* 2013; Sadeghi *et al.,* 2021; Teimori *et al.,* 2017). For instance, mullid fish scales have been reported to include intermediate (calyx), polygonal (hexagonal), and oval shapes. Al Jufaili *et al.* (2021) found various shapes in the scales of *A. jayakari*, such as polygonal (hexagonal and pentagonal), circular/discoid, oval/elliptical, and quadrilateral/square, across different size groups and body parts. It is hypothesized that the shape plasticity of scales may help reduce friction drag in fishes while swimming.

One of the detailed features of scales examined by SEM is the focus. The focus is formed during the initial stages of scale development and ontogeny. While typically located centrally on the scale, its position can vary, appearing in the anterior, posterior, antero-central, or postero-central parts (Echreshavi *et al.,* 2021; Sadeghi *et al.,* 2021). In *Schizothorax plagiostomus*, the focus was generally distinct, round and antero-centrally positioned. Additionally, the sculpture of the focus area was smooth. Variation in focus shape has been observed across different fish species, including the mullids, where five types were identified: rectangular (*Upenus doriae*), circular (*U. tragula*), round (*U. sundaicus*), wide round (*U. vittatus*), and semi-round (*Mulloidichthys vanicolensis*) (Echreshavi *et al.,* 2021). Some species, like those in the genus *Sardinella*, lack an obvious and distinct focus, whereas other clupeid fishes have a clear focus, leading to systematic classification challenges. There appears to be a correlation between the scale morphology of *Sardinella* and its molecular identity (Dizaj *et al.,* 2020; Wang *et al.,* 2022), underscoring the importance of scale features in fish identification.

A distinctive feature of cycloid scales is the presence of concentric lines (circuli) and radial grooves (radii) in the anterior field of the scale (Schultze, 2016). In *Schizothorax plagiostomus*, the circuli in the posterior field were discontinuous, while those in the lateral field were continuous. In the anterior field, the circuli were closely spaced, with an average intercircular space of 14.91 µm and an average of 24 circuli. The circuli were convex in shape and smooth due to the absence of lepidonts. Previous studies on cycloid scales have suggested that these scales help reduce friction between the fish body and its aquatic environment (Muthuramalingam *et al.,* 2020; Wainwright, 2019). Consequently, for *Schizothorax* species inhabiting fast-flowing coldwater hillstreams and rivers, this feature likely provides an evolutionary advantage for surviving in such extreme environments.

In the scales of *Schizothorax plagiostomus*, three types of radii—primary, secondary, and tertiary were observed, with primary radii being the most numerous. The average number of radii was 11, and their width in the anterior field was 9.70 µm. According to Johal*et al.* (2006), the variation in the number of radii is associated with the nutritive conditions of the fish; a higher number of radii corresponds to better nutritional status and indicates scale flexibility. The number of radii also depends on the scale's location on the fish's body, showing no significant relationship with the overall scale size (Esmaeili and Gholami, 2011). Additionally, the presence of primary, secondary, and tertiary radii is considered a growth phenomenon (Alkaladi *et al.,* 2013) and is less influenced by the fish's genetic characteristics (Lippitsch, 1990). Radii may appear in various fields: only anterior, as in pickerels (Esox); only posterior, as in shiners (Notropis); both anterior and posterior, as in suckers (Catostomidae) and *R. frisii* (Leuciscidae); or in all four fields, as in barbs (Barbus) (Esmaeili *et al.,* 2012; Esmaeili and Gholami, 2011). However, scales of studied *Schizothorax plagiostomus* displayed a distinct tetra-sectioned form due to the presence of radii in all four fields (anterior, posterior, and laterals). This tetra-sectioned form has also been reported for the scales of *G. rossica* (Esmaeili *et al.,*

2012) and *G. sharq* (Echreshavi *et al.,* 2022). This feature may be considered a distinguishing characteristic for the genus *Garra*, as such an architectural design is not observed in other cyprinid fishes, such as*Rutilus frisii*, *Capoeta damascina*, and *Tor putitora*(Esmaeili *et al.,* 2007; Esmaeili and Gholami, 2011; Johal*et al.,* 1999).

Lateral line scales

Several detailed studies have investigated the morphology and topology of lateral line scales, highlighting their potential use in fish classification (Kaur and Dua, 2004). Research by Mekkawy *et al.*(1999) and Matondo *et al.* (2010) identified the channel openings in the lateral line scales as a distinct and interesting feature. These scales have been utilized to demonstrate their potential in fish taxonomy and classification. Key features such as the canal's position, alignment (straight or oblique), and perforations (anterior, posterior, or lateral) are crucial for fish classification (Delamater and Courtenay, 1973; Tandon and Johal, 1983). In *Schizothorax plagiostomus*, lateral line scales consist of four parts: anterior, posterior, and two lateral regions. Unlike other scales, these lack focus and instead feature a channel running along the anterior-posterior axis, with two openings—an anterior opening, which is wider, and a posterior opening. The lateral line canal resembles a long tube with irregular boundaries. SEM examination of the lateral line scales in*Schizothorax plagiostomus* revealed a straight, central canal originating from the upper margin of the posterior region and extending to the anterior region. The average length of the lateral line canal was 1.52 mm, and the anterior opening measured 354.61 µm.

Conclusion

This study offers a detailed analysis of the scale morphology and microstructures of *Schizothorax plagiostomus* using Scanning Electron Microscopy (SEM). Key findings include polygonal shaped scales with tetra-sectioned configuration, presence of smooth circuli (i.e., without lepidonts), small sized round focus and lateral line scales which lack focus. This study underscores the importance of scale morphology in ichthyological research and reaffirms the utility of SEM in uncovering microstructural scale features critical for species identification and resolving taxonomic ambiguities.

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Conflict of interest

The authors declare that there is no conflict of interest.

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