Journal of Applied and Natural Science
17(2), 767 - 774 (2025)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Reporting a new ciliate, *Hemiurosomoida linea* n.sp. from a freshwater pond of Rajghat, Delhi, India

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Article Info
https://doi.org/10.31018/jans.v17i2.6547
Received: January 17, 2025
Revised: May 26, 2025
Accepted: June 01, 2025

How to Cite
Naqvi, I. *et al.* (2025). Reporting a new ciliate, *Hemiurosomoida linea* n.sp. from a freshwater pond of Rajghat, Delhi, India. *Journal of Applied and Natural Science*, 17(2), 767 - 774. https://doi.org/10.31018/jans.v17i2.6547

Abstract
Hemiurosomoida was established as a new genus by Singh and Kamra in 2015 based on its morphology, morphogenesis and molecular analysis. Based on their morphology, the members of this genus were earlier erroneously placed in the genus *Oxytricha*, then shifted to another genus *Urosomoida*, before establishing the new genus *Hemiurosomoida*. However, a detailed molecular analysis based on small subunit ribosomal RNA (SSU rRNA) showed that the members of this genus are rather placed far apart from the other two genera in the phylogenetic tree. Therefore, they are now placed in a new genus *Hemiurosomoida*. To date, four species of this genus have been reported, *viz. H. longa*, *H. warreni*, *H. tibetensis* and *H. koreana*. In the present investigation, a new freshwater species, *Hemiurosomoida linea* n. sp. was reported from a freshwater pond located in Rajghat, Delhi, India. Diagnostic features of *H. linea* include- a flexible body with an average size of 81x18 µm (protargol stained cells); narrowly elongated cells with a rounded anterior end and a tapering posterior end; the presence of 2 macronuclear nodules and 2 micronuclei; the presence of only 17 Frontal-Ventral-Transverse (F_{1-8}, V_{1-5}, T_{1-4}) cirri, the transverse cirri (T_{1-4}) being arranged in a linear row; four dorsal rows of bristles (DK_{1-3} and DM_1); 2 caudal cirri; an average of 19 adoral membranelles; 17 right marginal and 17 left marginal cirri; no fragmentation of DP_3; a flat and narrow buccal cavity, and undulating membranes (UMs) in typical *Oxytricha* pattern. Thus, this study has unravelled and confirmed that *H. linea* was a new species and helped expand the knowledge base of ciliate biodiversity in India.

Keywords: Ciliates, *Hemiurosomoida*, Morphogenesis, Morphology, Urosomoididae

INTRODUCTION

Ciliates inhabit a wide range of environments, such as soil, fresh and salt waters, etc., and are one of the most species-rich groups of Protozoa (Small and Lynn, 1985; Bai *et al*., 2020; Lian *et al*., 2020; Wu *et al*., 2020; Zhao *et al*., 2020). Among the members of the phylum Ciliophora, the Hypotrichid ciliates have the most complex morphology and ontogenesis (Stein, 1859; Doflein, 1901). Hence, it is being studied the most in various fields of biology. Recent studies have reported numerous hypotrich taxa, demonstrating that this group is relatively more diverse than previously assumed (Berger, 1999; Foissner, 2016; Song and Shao, 2017; Hu *et al*., 2019; Chen *et al*., 2020; Dong *et al*., 2020; Lu *et al*., 2020; Paiva, 2020; Park *et al*., 2020; Shao *et al*., 2020; Wang *et al*., 2020; Xu *et al*., 2020; Zhang *et al*., 2020; Li *et al*., 2021; Wang *et al*., 2021).
The hypotrich ciliates include *Oxytricha, Paraeuplotes, Scuticociliate, Uroleptusa, Urosomoida*, and *Hemiuro-*

somoida, among several others. *Hemiurosomoida*, however, was established recently as a new genus by Singh and Kamra in 2015 based on its morphology, morphogenesis and molecular analysis studies. Previously, members of this genus were erroneously classified as '*Oxytricha* Bory de Saint-Vincent' (Gelei and Szabados, 1950; Ganner *et al*., 1987) but later transferred to a new genus *Urosomoida*, based on the typical feature of fewer frontal-ventral-transverse cirri, and the lack of dorsal fragmentation (Foissner *et al*., 1991), until its further separation into a new genus. Shao *et al*. (2012) also confirmed this placement but noted that it was just preliminary due to the lack of molecular data on *Urosomoida agilis*. Moreover, the members of the genus *Oxytricha*, *Urosomoida* and *Hemiurosomoida* are indistinguishable in their microscopic live cell morphology; nevertheless, the molecular study based on the 18S rRNA sequence has confirmed that the genus *Hemiurosomoida* should not be clustered with *Urosomoida* or *Oxytricha* species (Singh and Kamra, 2015).

H. longa is the type species of the genus *Hemiurosomoida*. The typical identifying features unique to this genus include the reduced number (less than 18) of fronto-ventral-transverse (FVT) cirri and the lack of dorsal kinety fragmentation. Other features include- a flexible body; adoral zone of membranelles (AZM) formed like a question mark (**?**); undulating membranes in *Oxytricha* pattern; one right and one left marginal row; three dorsal kineties with one caudal cirrus each on kinety 1 and 2; one dorso-marginal row; Streak V and VI of proter originate from streak V and VI of opisthe. The dorsal morphogenesis is in the typical *Urosomoida* pattern (Singh and Kamra, 2015; Chen *et al*., 2021; Kouser *et al*., 2022; Omar *et al*., 2024).

Hemiurosomoida was placed in the family Urosomoididae, which was established by Foissner (2016) based on the fewer frontal-ventral-transverse cirri and the lack of dorsal fragmentation. There are four recognized species of this genus: *H. longa* (Gelei and Szabados, 1950; Singh and Kamra, 2015), *H. warreni* (Chen *et al*., 2021), *H. tibetensis* (Kouser *et al*., 2022) and *H. koreana* (Omar *et al*., 2024).

The present study was aimed at elucidating the richness of Indian freshwater ciliate biodiversity. In that pursuit, a new freshwater ciliate, *Hemiurosomoida linea* n. sp. was discovered in a pond water sample from Rajghat, Delhi, India. The morphology and morphogenesis observations justify the generic designation of this species.

MATERIALS AND METHODS

Collection of Water samples and processing

The water samples were collected from a man-made pond with a depth of about 2 m at Rajghat in Delhi (28º38′26.16″N, 77º14′57.84″E). The water samples, including aquatic plant roots, were collected from the littoral zone using a beaker and were immediately transported to the laboratory and kept in troughs along with the plants. The samples were kept at room temperature and fed with algae *Chlorogonium elongatum ad libitum* (Ammermann *et al*., 1974). The samples were then filtered through a series of Nytex meshes (60 to 120 µm) to eliminate crustaceans, debris, and other unwanted particles. The filtered samples were kept in small petri dishes at room temperature for 15-20 days to allow the excystment of the ciliate species.

The emerged ciliates were then maintained at 23±1ºC in a biological oxygen demand (B.O.D.) incubator in Pringsheim's culture media containing 0.85 mM calcium nitrate [$Ca(NO_3)_2.4H_2O$], 0.35 mM potassium chloride [KCl], 0.08 mM magnesium sulphate [$MgSO_4.7H_2O$], and 0.11 mM sodium hydrogen phosphate [$Na_2HPO_4.2H_2O$] (Chapman-Andersen, 1958; Pringsheim, 1964). The ciliate cultures were routinely supplemented with food, *i.e., Chlorogonium elongatum*.

Laboratory culturing of ciliates

To establish monoclonal cultures, various species of ciliates present in the water samples were identified, isolated, and cultured separately in Pringsheim's media. The ciliates were observed routinely under a stereoscopic microscope (Magnus MS 24), and for final observations (at 100 - 1,000X magnification) and to identify the ciliates, a phase contrast microscope (Nikon Eclipse TS100) was employed.

Protargol staining

Routine protargol (silver albumose or silver proteinate) staining technique was employed to investigate the ciliary bases on the surface of the cells, as well as the infraciliature and nuclear apparatus of the cells from the monoclonal cultures. The staining protocol used in the present study was that of Wilbert (1975) with minor modifications (Kamra and Sapra (1990). Briefly, the cells were fixed for 10 minutes in standard Bouin's fixative. After washing, the cells were coated with Mayer's glycerinated albumin. Then, they were bleached in a 0.6% Sodium Hypochlorite (NaOCl) solution. Subsequently, the cells were impregnated with freshly prepared 2% protargol stain. Following staining, the cells were allowed to develop colour by immersing them in a 'slow developer' solution containing 1.4 g of Boric Acid, 0.3 g of Hydroquinone, 2 g of Sodium Sulphate, and 15 ml of Acetone dissolved in a final volume of 100 ml of double distilled water. The silver-stained cells were then fixed in 5% Sodium Thiosulphate ($Na_2S_2O_3.5H_2O$) solution, dehydrated in alcohol grades, cleared in xylene, and D.P.X. mounted.

Morphometry and morphogenesis

Protargol staining was used to examine the ciliary

structures (Wilbert, 1975; Kamra and Sapra, 1990). The protargol-impregnated specimens were observed under oil immersion (1000X), counted, and morphometric measurements of 20 cells were recorded. The cirri numbering system used was that of Wallengren (1901), Borror (1972), Martin (1982), and Hemberger (1985).

Description: The identified species of the present investigation was described using the nomenclature proposed by Küppers *et al*. (2011), Foissner and Stoeck (2011), and Berger (2008).

Statistical analysis

The data were statistically analyzed using Microsoft Excel software following the morphometric measurements. Parameters calculated included mean, maximum, minimum, standard deviation (SD), and coefficient of variation (CV). The coefficient of variation (CV) was calculated by the formula CV = SD/Mean X 100.

RESULTS

Distribution and Ecology

The new species, *H. linea* n. sp., was discovered from a freshwater pond in Rajghat, Delhi, India.

Taxonomic status

Phylum: Ciliophora
Class: Spirotrichea
Order: Hypotrichida
Family: Urosomoididae
Genus: *Hemiurosomoida*
Species: *linea*

Diagnosis

The protargol stained cells measured approximately 81 x 18 µm. The body of live cells was flexible and exhibited a narrowly elongated shape, with a rounded anterior end and a tapering posterior end. There were two macronuclear nodules and two micronuclei; 17 Frontal-Ventral-Transverse ($F_{1-8}V_{1-5}T_{1-4}$) cirri, with the charact eristic transverse arrangement of cirri in a linear row; four dorsal rows of bristles (dorsal kineties-DM and dorsomarginals-DM) (DK_{1-3} and DM_1), two caudal cirri, and an average of 19 adoral membranelles. Furthermore, there were 17 right marginal cirri (RMC) and 17 left marginal cirri (LMC), with no signs of fragmentation in DP_3. The buccal cavity was flat and narrow, featuring undulating membranes (UMs) that were arranged in the typical *Oxytricha* pattern.

Description (Fig. 1)

These bottom-dwelling cells exhibited rapid creeping movements. Conjugation and encystment occurred frequently. The cells were flexible, dorsoventrally flattened, narrowly elongated, with rounded anterior and tapered posterior ends. The average size of protargol-impregnated non-dividing cells was observed to be around 81x18 µm, (ranging between 75.5-86.4 x 16.7-20.7 mm), with a length-to-width ratio of 4.6:1. Two 15x7 µm (range: 12.40-16.70 x 6.30-8.40 µm) macronuclear nodules were found present to the left of the cell's median line. Two spherical compact micronuclei of 3.4 µm (3.3 – 3.5 µm) diameter were also found close to macronuclear nodules at variable positions. Morphometric characters are presented in Table 1.

The AZM, occupying ~30% of the body length of the cells, was shaped like a 'question mark' (**?**) symbol, and consisted of 19 membranelles. The buccal cavity was flat and narrow. The undulating membranes (UMs) were arranged in a typical *Oxytricha* pattern. The ventral ciliature consisted of 17 FVT cirri ($F_{1-8}V_{1-5}T_{1-4}$). The eight frontals (F_{1-8}) were hypertrophied to a similar lev-

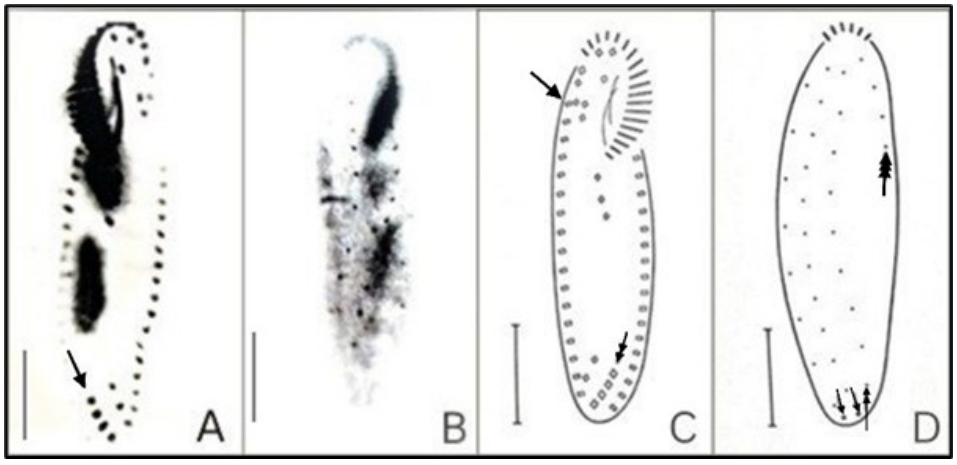

Fig. 1. *Photographs (A, B) and Line diagrams (C, D) of protargol impregnated cells of H. linea n. sp. showing vegetative surface. A. Vegetative ventral surface: linearly arranged four transverse cirri (arrow). B. Dorsal surface: showing three dorsal kineties. C. Vegetative ventral surface: Beginning of RMC row (arrow), linearly arranged four transverse cirri (double arrow). D. Presence of caudal cirrus at the end of $DK_{1\&2}$ (arrows), absence of caudal cirrus at the end of DK_3 (double arrow), presence of only one DM (triple arrow). Bar represents 20 µm*

Table 1. Morphometric data of *Hemiurosomoida linea* n. sp.

Character		Mean	Min	Max	SD	CV	N
Body length		81.20	75.50	86.40	3.80	4.70	20
Body width		17.90	16.70	20.70	1.12	6.24	20
Body length/ body width ratio		4.60	3.80	4.90	0.33	7.22	20
Number of macronuclear nodules		2.00	2.00	2.00	0.00	0.00	20
Length of macronuclear nodules		14.90	12.40	16.70	1.20	8.00	20
Width of macronuclear nodules		7.40	6.30	8.40	0.54	7.29	20
Number of micronuclei		2.00	2.00	2.00	0.00	0.00	20
Diameter of micronucleus		3.40	3.30	3.50	0.10	0.03	20
Number of AZM		19.30	16.00	24.00	1.66	8.60	20
Length of adoral zone		24.60	22.20	28.80	1.74	7.06	20
Adoral length/body length ratio		0.30	0.30	0.30	0.02	6.67	20
Number of frontal cirri,		8.00	8.00	8.00	0.00	0.00	20
Number of ventral cirri,		5.00	5.00	5.00	0.00	0.00	20
Number of transverse cirri		4.00	4.00	4.00	0.00	0.00	20
Number of LMC		17.30	16.00	20.00	1.26	7.28	20
Number of RMC		16.90	14.00	20.00	1.71	10.12	20
Number of DKs		3.00	3.00	3.00	0.00	0.00	10
Number of DMs		1.00	1.00	1.00	0.00	0.00	10
	DK_1	8.20	8.00	9.00	0.45	5.49	10
	DK_2	11.00	10.00	12.00	0.71	6.45	10
Number of Dorsal bristles	DK_3	9.00	8.00	10.00	0.71	7.89	10
	DM_1	4.40	4.00	5.0	0.55	12.50	10
Number of caudal cirri		2.00	2.00	2.00	0.00	0.00	10

Data is based on protargol-impregnated specimen observations; All measurements are in µm. CV = coefficient of variation (%); DK = dorsal kineties; Mean = arithmetic mean; Max = maximum; Min = minimum; n = number of specimens examined; SD= standard deviation

el, with V_1-V_3 of five ventral cirri arranged at equidistance from each other, and the transverse cirri (T_{1-4}) in a linear row (Fig. 1A- arrow, Fig. 1C- double arrow). The right marginal row was observed to start at the level of F_6 and terminate near the third transverse cirrus (T_3), while the left marginal row was 'J' shaped and ended at the midline.

On the other hand, the dorsal ciliature (DK) consisted of 4 rows, identified as DK_{1-4}. It was further observed that the DK_{1-3} ciliary rows were extended along the entire body length, with a curve in the posterior half of the cell. There was only one, very short, dorso-marginal row consisting of only 4-5 bristles. There were two caudal cirri, one at the end of DK_1 and DK_2.

Developmental morphogenesis (Figs. 2 and 3)

The *de novo* appearance of kinetosomes between the left marginal cirral row and post-oral ventral cirri (V_1-V_3) marked the beginning of the morphogenesis. Further proliferation of kinetosomes led to the formation of Oral Primordium (OP). The gradual disaggregation of six parental cirri, including 3 frontals ($F_{1, 7 \text{ and } 8}$) and 3 ventrals (V_1-V_3), led to the formation of two sets of six ciliary streaks each, one for proter and the other for opisthe. In proter, the disaggregating UMs were observed to function as streak I, whereas streak II was of composite in origin, formed from OP and F_1. Streaks III and IV originate from disaggregating parental F_8 and F_7. Streaks V and VI of the proter are formed from the anterior migration of the V and VI streaks of the opisthae.

In the opisthe, on the other hand, streaks I and II originated from the OP. Streak III was formed from V_1, while streaks IV, V and VI were formed from the disintegrating V_2 and V_3. Differentiation of 17 cirri for the two daughter cells followed the typical pattern of 1,2,3,3,4,4. It was further observed that the marginal rows were formed from within the row proliferation of kinetosomes.

The dorsal ciliature developed by the within-row proliferation of kinetosomes in DK_{1-3} in proter and opisthe. The three dorsal primordia formed the three long dorsal kineties, however, the DK_4 did not develop due to the characteristic absence of a split in the third dorsal primordia. The dorso-marginal row was developed close to the RMC primordia on the ventral surface and then shifted to the dorsal surface. Finally, the appearance of the caudal cirri marked the end of morphogenesis as the posterior-most kinetosome of DK_1 and DK_2 proliferated into one caudal cirrus each.

Etymology

The species name "*linea*" was given based on the fact that the transverse cirri of this species were observed to

be arranged in a linear fashion.

DISCUSSION

Comparison with related species

A comparison of morphological, morphometric and morphogenetic features of all the species of the genus *Hemiurosomoida* is shown in Table 2.The genus *Hemiurosomoida* displays typical characteristic features such as a reduced number of FVT cirri, 3 dorsal kineties, one dorso-marginal row, two caudal cirri and UMs in *Oxytricha* patterns. All these features were observed in the new species under investigation.

Hemiurosomoida comprises four known species to date, *viz. H. longa* (Gelei and Szabados, 1950; Singh and Kamra, 2015); *H. warreni* (Chen *et al*., 2021); *H. tibetensis* (Kouser *et al*., 2022); *H. koreana* (Omar *et al*., 2024), and our study reports the fifth, *H. linea* n. sp. It differed from the other described species of this genus in its shape, size, ratio of body length to body width, presence or absence of granules, number of macronuclei and micronuclei, and number and arrangement of transverse cirri.

The body length of two described species of the genus *Hemiurosomoida* was comparable to *H. linea* n. sp., however, at 76 – 86 mm, its size is smaller than that of *H. warreni* (85 - 138µm) (Chen *et al*., 2021) and larger than *H. koreana*. Further, three of the four previously described species and the newly discovered species contain 2 macronuclear nodules, while *H. warreni* (Chen *et al*., 2021) has 4 - 8 macronuclear nodules. Similarly, the number of micronuclei was 2 in the case of *H. linea* n. sp., *H. tibetensis* (Kouser *et al*., 2022) and *H. longa* (Gelei and Szabados, 1950; Singh and Kamra, 2015), while 1-2 micronuclei were reported in *H. koreana* (Omar *et al*., 2024). However, the presence of any micronucleus has not been reported in the case of *H. warreni* (Chen *et al*., 2021). Four transverse cirri were reported in three of the earlier reported species and also in the *H. linea* n. sp., however, only 3 were reported in *H. warreni* (Chen *et al*., 2021). In most of the reported species, the transverse cirri are arranged in a V-shaped pattern except for *H. warreni* (Chen *et al*., 2021) and *H. linea* n. sp., where they are arranged linearly. Cortical granules are reported in three earlier reported species except *for H. longa* (Gelei and Szaba-

Table 2. Comparison of different species of the genus Hemiurosomoida

Characters	*H. linea* n. sp.	*H. koreana* (Omar *et al*., 2024)	*H. tibetensis* (Kouser *et al*., 2022)	*H. warreni* (Chen *et al*., 2021)	*H. longa* (Gelei & Szabados, 1950; Singh & Kamra, 2015
Body shape	Narrowly elongated; rounded anterior end and tapering posterior end	Elliptical with narrow posterior ends	Elongate elliptical with anterior and posterior ends broadly rounded	Slender, oval to elliptical; posterior end slightly narrow	Elliptical; both ends narrowly to broadly rounded
Body length	76 - 86	41 - 52	53 - 86	85 - 138	51 - 69
Body width	18 - 21	10 - 16	20 - 48	22 - 42	16 - 21
Body length-to-width ratio	3.8 - 4.9:1	3 - 4.4:1	2.2:1	3 - 5:1	3.5:1
Macronuclear number	2	2	2	4 - 8	2
Micronuclear number	2	1 -2	2	Not observed	2
AM, number	16 - 24	18 - 20	17 - 24	25 - 38	18 - 22
LMC, number	16 - 20	16 - 20	16 - 23	26 - 35	14 - 21
RMC, number	14 - 20	16 - 21	16 - 23	29 - 39	17 - 23
Number of transverse cirri	4	4	4	3	4
Arrangement of transverse cirri	Linear	V-shaped pattern	V-shaped pattern	Linear	V-shaped
Dorsal kineties	4	4	4	4	4
Caudal cirri	2	2	2	2	2
Cortical granules	Absent	Present in irregular patches on the ventral surface and randomly scattered on the dorsal surface	Colourless; grouped along cirri on ventral surface and along dorsal cilia and between dorsal kineties	Present	Absent

Data is based on protargol-impregnated specimens; All measurements are in µm

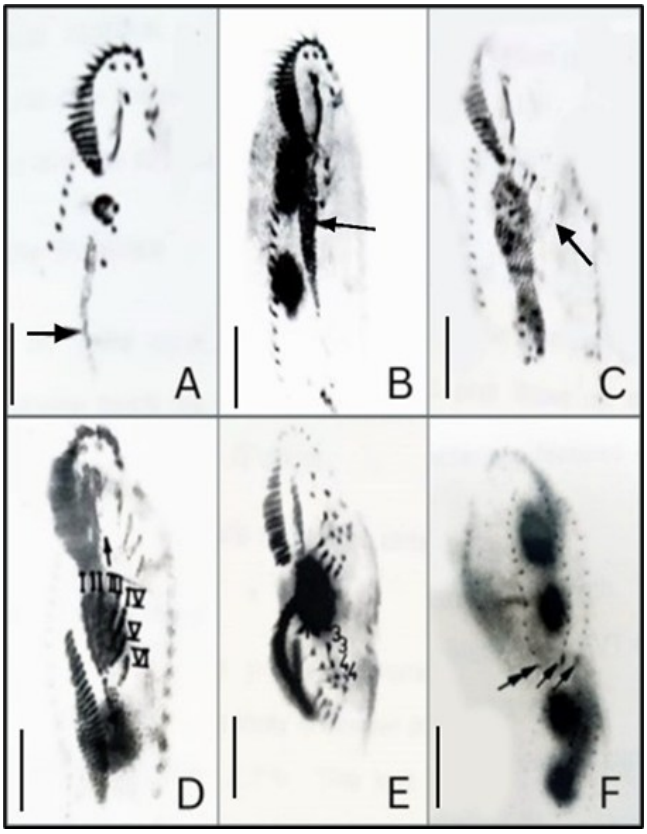

Fig. 2. *Photomicrographs of protargol impregnated cells of H. linea n. sp. showing division morphogenesis on ventral (A-E) and dorsal (F) surface. A. Proliferation of OP (arrow) B. Anarchic field of OP (arrow) C. Formation of streaks for opisthe (arrow) from OP and V_1-V_3. D. Full set of six (I-VI) streaks. Presence of kinetosomes from OP lying posterior to disaggregated F□ (arrow). E. Differentiation pattern of 1, 2, 3, 3, 4, 4. I Proliferation of CC at the ends of $DK_{1\&2}$ (arrows) and absence of CC at the end of DK_3 (double arrow). Bar represents 20 µm*

dos, 1950; Singh and Kamra, 2015). However, they are absent in the case of *H. linea* n.sp.

Conclusion

Based on the morphological and morphogenic observations and a comparative discussion of similarities and differences between the type species and different closely related species of the genus, the study concluded that *Hemiurosomoida linea* is a new species. Further, it also added a new species to the Indian ciliate biodiversity, expanding its base.

ACKNOWLEDGEMENTS

Our profound gratitude to Prof. G.R. Sapra for his continuous assistance, support, and direction with this research project. Sincere thanks to Late Prof. Renu Gupta for her constant guidance throughout this research. The authors would also like to extend heartfelt gratitude for the technical support of Dr. Prakash Borgohain and Ms. Insha Naqvi with the text and figures in this manuscript. And we further like to acknowledge the support extended by the Head, Department of Zoology, University of Delhi, and Principal, Zakir Husain Delhi College (University of Delhi), by providing the infrastructure and a congenial & conducive atmosphere to work. Our departmental colleagues' constant support and encouragement are the driving force behind our success. Our cordial acknowledgements are due to them.

Conflict of interest

The authors declare that they have no conflict of interest.

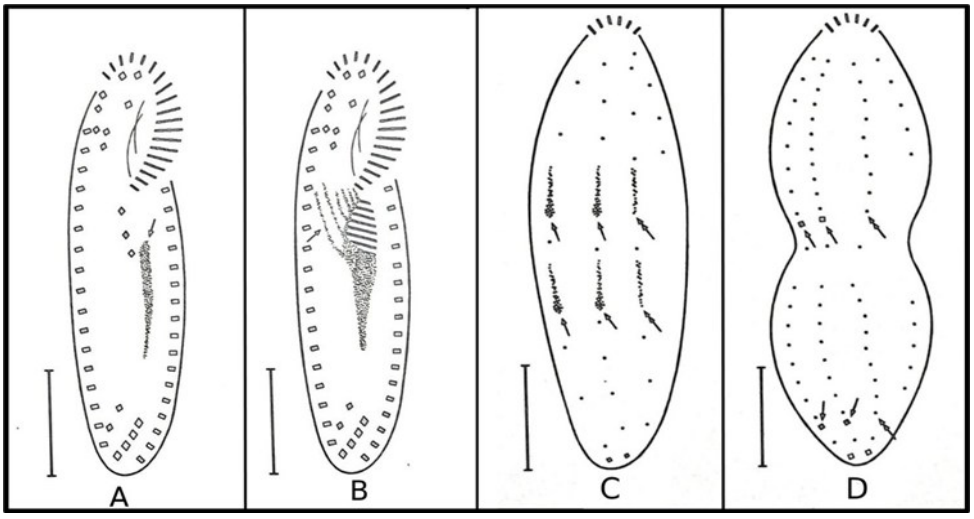

Fig. 3. *Line diagrams of protargol impregnated cells of H. linea n. sp. showing division morphogenesis on ventral (A, B) and dorsal (C, D) surface. A. Origin of OP (arrow). B. Anterior movement of streaks V & VI of opisthe (arrow). C. Localized proliferation of kinetosomes at the end of $DP_{1\&2}$ (arrows), absence of proliferation of kinetosomes at the end of DK_3 (double arrows). D. Formation of new caudal cirri at the end of $DK_{1\&2}$ (arrows) and absence of caudal cirrus formation at the end of DK_3 (double arrow). Bar represents 20 µm*

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