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Original Research Article

Describing a New Freshwater Ciliate, *Aponotohymena botrinucleata* from Delhi, India

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ABSTRACT

Ciliates (Phylum Ciliophora) are one of the most diverse groups of protozoa which are present in a variety of habitats. Their taxonomic diversity is immense, encompassing thousands of species. Understanding the biodiversity of ciliates is essential for unraveling the intricate web of interactions that shape ecosystems. Consequently, the number of studies on the diversity and distribution of ciliates have increased globally. Ciliates are very important as they play roles in nutrient cycling, microbial food webs, and symbiotic associations, etc. to maintain the balance of ecosystem. In the present study, a new species of genus *Aponotohymena* (*Aponotohymena botrinucleata*) is being reported, from a pond located near 'Garden of Five Senses,' Saket, Delhi, India. This genus was created by Foissner in 2016. The type species of this genus, *A. australis* was first named as *Oxytricha austalis* and later redesignated as *Notohymena australis*. However, recently it is further redesignated as *Aponotohymena australis*. The identifying feature of this genus include: flexible body; 18 Frontal-ventral-transverse (FVT) cirri; more than 3 caudal cirri associated with dorsal kineties 1, 2 and 4 and splitting of DP_3 (third dorsal primordium). According to Foissner (2016), the genus *Aponotohymena* differs from the genus *Notohymena* only in the number of caudal cirri. Only three species of this genus are reported till date namely *A. australis*, *N. apoaustralis* and *A. isoaustralis*. In the present study, a new species of this genus, *Aponotohymena botrinucleata*, is being reported from a freshwater pond.

Keywords: *Aponotohymena*, Oxytrichidae, protargol, morphology & morphogenesis

1. INTRODUCTION

Ciliates, which are amongst the most diverse groups of Protozoa, inhabit a diverse range of environments, which includes free-living, parasitic, commensal, and symbiotic forms (Corliss & Coats, 1976; Corliss, 1979). The free-living forms of the ciliates have been reported from a number of varied water bodies such as lakes, ponds, underground pools, rivers, estuaries and oceans. Additionally, their existence has also been recorded in a variety of terrestrial environments, such as soil, desert sands and forest litter (Corliss, 1979; Lynn & Small, 1985; Foissner, 1987; Patterson et al., 1989; Foissner, 1994; Foissner et al., 1995; Foissner, 1998; Cheng et al., 2019; Bai et al., 2020).

The taxonomic diversity of ciliates is immense, encompassing thousands of species with distinct morphologies, behaviours, and ecological niches. Gradually, the number of studies on diversity and distribution of ciliates have increased globally (Dolan & Marrasé, 1995; Johansson et al., 2004; Yang et al., 2020).

Oxytrichidae (Ehrenberg, 1830) is a species-rich family within the subclass Hypotrichia (Berger, 1999, 2018; Foissner, 2016). Recently, some new genera have been established (Foissner, 2016). *Aponotohymena*, whose species were originally placed in the genus *Oxytricha*, and were later redesignated as *Notohymena*, and further, redesignated as *Aponotohymena*. The identifying features of this genus include: flexible body; 18 FVT cirri; more than 3 caudal cirri associated with dorsal kineties 1, 2 and 4, and splitting of third dorsal primordium (DP_3). Only three species of this genus are reported till date, viz. *A. australis* (Foissner & O'donoghue, 1990; Berger, 1999; Voss, 2008; Hu & Kusuoka 2015; Foissner, 2016), *Notohymena apoaustralis* (Lv et al., 2013) and *A. isoaustralis* (Gupta et al., 2017). According to Foissner (2016), the genus *Aponotohymena* differs from the genus *Notohymena* only in the number of caudal cirri. In the present study, a new species of the genus *Aponotohymena* is being identified and reported, which was isolated from a fresh water pond located in the 'Garden of Five Senses,' Saket, Delhi, India.

2. MATERIALS AND METHODS

2.1. Water Sampling and Processing

Water samples were collected from a small perennial nutrient-rich man-made Water Lilly Pond located in the Garden of Five Senses, Saket, New Delhi, India, (Latitude: N 28° 30' 47.7252", & Longitude: E 77° 11' 53.2248"). The surface water samples were collected from an accessible corner. Along with the water samples, the roots of water hyacinth, which are 'free-floating' perennial aquatic plants, not more than 5 - 12 inches in length and were widely dispersed throughout the pond, were also collected into the sample bottles. Thus, the ciliates in our study were not benthic forms. All the samples were transported to the laboratory, and processed further. Large crustaceans and other macroscopic debris were removed using various Nitex nylon mesh filter screens with pore sizes ranging between 60 and 120 µm. Mixed cultures were grown at room temperature and fed with *Chlorogonium elongatum* (green alga) and periodically examined for ciliates.

2.2. Culturing and Maintenance of Cells

Live ciliates were examined under a microscope, and separated in order to produce clonal cultures of the species under investigation. Cells were cultured in Pringsheim's medium (Chapman-Andersen, 1958; Pringsheim, 1964). The Pringsheim's medium was made up of 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 [$Na_2HPO_4.2H_2O$]. The cells were fed with a green alga, *Chlorogonium elongatum*. The temperature of the cells was maintained at 23 ± 1 ºC in B.O.D.

2.3. Staining of the Cells

Protargol impregnation technique was used to investigate the organism's infraciliature and nuclear apparatus, following the previously mentioned Wilbert method (Wilbert, 1975), as well as slightly altered Kamra and Sapra (Kamra & Sapra, 1990) method. In brief, the cells were fixed in regular Bouin's fixative for 10 minutes. The cells were then washed, coated with Mayer's glycerinated albumin, and bleached in 0.6% sodium hypochlorite (NaOCl) solution. Then, the cells were stained using freshly prepared 2% Protargol stain. Post-staining, the slides were dipped in "slow developer" solution (1.4 g Boric acid, 0.3 g Hydroquinone, 2 g Sodium sulphate, and 15 ml acetone, and the final volume was made up to 100 ml with double distilled water), which was a slight departure from the originally established protocol, to allow the stain to develop the desired colour intensity after impregnation. Following that, the slides were

dipped in 5% sodium thiosulphate ($Na_2S_2O_3.5H_2O$) solution to fix the silver stain. Finally, the slides were dehydrated in alcohol grades, cleared with xylene, and mounted in D.P.X.

2.4. Morphometry and morphogenesis

The infraciliature of protogol impregnated cells was examined in accordance with the previously mentioned techniques of Wilbert (1975) and Kamra and Sapra (1990). After the silver staining, a total of twenty protargol-impregnated cells were observed and measured under oil immersion objective lens (1000X), photographed, and camera lucida drawings were made. The present study used the same terminology as standard reference studies, including Berger (2008), Foissner and Stoeck (2011), and Küppers et al. (2011) to describe the species; however, the cirri numbering system used was the same as that developed by Wallengren (1901), Borror (1972), Martin (1982), and Hemberger (1985).

2.5. Statistical Analysis

The morphometric data were subjected to statistical analysis. MS Excel software was used for calculating various parameters such as minimum and maximum values, mean of values, standard deviation (SD) etc., and coefficient of variation (CV) was calculated by using the formula: CV = SD/Mean X 100.

3. RESULTS

3.1. Etymology

The ciliate species identified in the present study has been named as *botrinucleata* (*Aponotohymena botrinucleata*), after '*botrus*' a Latin noun for 'cluster' or 'bunch', referring to the clustery arrangement of the micronuclei, which is unique to this species (Figure 1D).

3.2. Distribution

The cells of *A. botrinucleata* n. sp. were collected and identified from a pond located in the Garden of Five Senses, Saket, New Delhi, India.

3.3. Diagnosis

Size: The Protargol stained cells measured approximately 86-109 x 31-39 µm (avg. size: 93 x 36 µm); *Body shape*: Rounded anterior and tapering posterior ends; *Nuclei*: 2 macronuclear nodules and 8-10 micronuclei; *Location of micronuclei*: Micronuclei were present in the form of a cluster between the 2 macronuclei; *Infraciliature*: 18 frontal ventral transverse (FVT) (F_{1-8}, V_{1-5}, T_{1-5}) cirri; six dorsal rows (DK_{1-4} & DM_{1-2}); 7 caudal cirri; on an average 41 adoral membranelles (AM); 36 each right (RMC) and left marginal cirri (LMC); *Granules*: Two types of granules were present *i.e.* yellow and green; *Buccal Cavity*: Large and deep; and the undulating membranes (UMs) were in typical *Notohymena* pattern (Figure 1A; Table 1).

3.4. Description of the species

These are usually benthic forms. The morphological and morphometric features of *A. botrinucleata* pertains to the Protargol-stained non-dividing cells (Figure 1), which are summarised in Table 1. The average size of the cells was around 93 x 36 µm (Range: 86-109 µm x 31-39 µm), with a body length to width ratio of 2.6:1 (Table 1). Anterior end of the cells was broad and round, while the posterior was tapering and round. The left and right margins of the cells were parallel to each other. The two macronuclear nodules were ellipsoidal in appearance, and measured 14x10 µm. They were located to the left of the median line of the

cell. In addition, around 8-10 spherical and compact micronuclei were observed, measuring ~3.5 µm. In most of the cells, 1 or 2 micronuclei were seen very close to the macronuclei, and rest were present as a botrus/ cluster (based on which the new species is named as *A. botrinucleata*) in the space between the 2 macronuclear nodules (Figure 1). It is a unique feature which was observed only in this particular species under investigation.

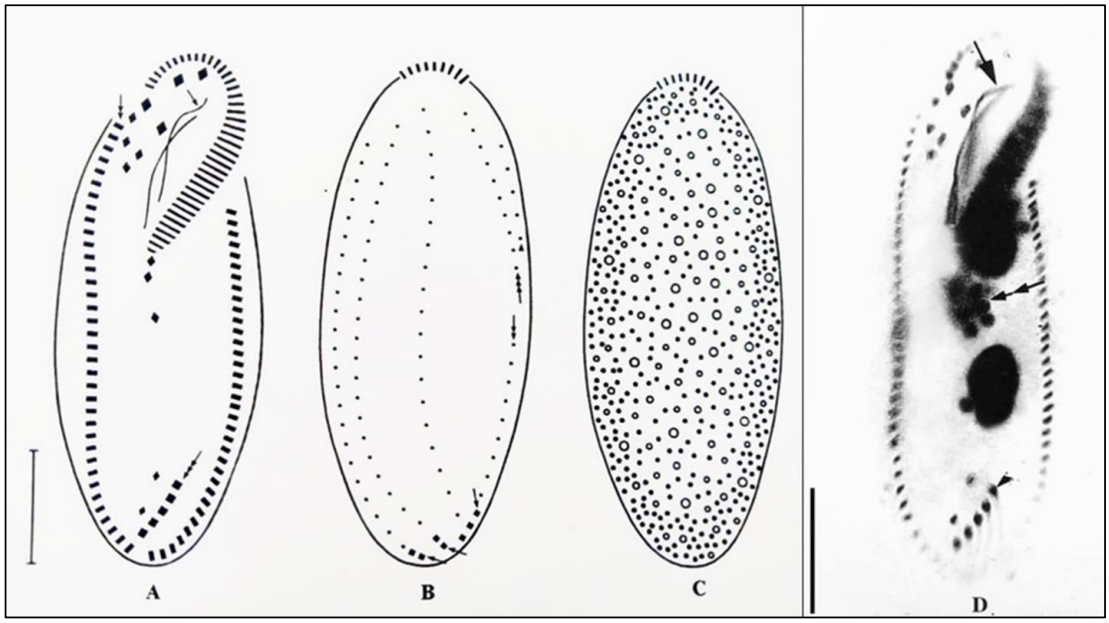

Figure 1: Line diagrams (A-C) and photomicrograph (D) of Protargol impregnated vegetative cells of *A. botrinucleata* n. sp. **A.** Ventral surface: UMs in *Notohymena* pattern (arrow), beginning of RMC row (double arrow), and five linearly arranged transverse cirri (triple arrow). **B.** Vegetative dorsal surface: 2, 2 & 3 caudal cirri are present at the end of $DK_{1,2\&4}$ (arrows), Shortened DK_4 (double arrow), long DM_1 (triple arrow), small DM_2 (arrow head). **C.** Dorsal surface showing arrangement of sub-pellicular granules as observed under phase contrast microscope. **D.** Vegetative ventral surface: UMs in *Notohymena* pattern (arrow), micronuclei present in cluster in between macro-nuclear nodules (double arrow), 1 micronucleus attached to each macro-nuclear nodules (triple arrow), transverse cirri arranged in a linear row (arrow head). Bar represent 20 µm.

The AZM was markedly well-developed and occupied 38% of the body length. This species contained about 41 membranelles. The buccal cavity was large and deep, and the undulating membranes (UMs) were in a typical *Notohymena* pattern. A contractile vacuole was observed on the left half of the body.

The ventral ciliature consisted of 18 frontal-ventral-transverse (FVT) cirri (Figure 1A). Three post-oral ventral cirri (V_1-V_3) appeared near the cytostome, with V_1 and V_2 appearing as a pair followed by V_3. The pre-transverse ventrals were present near the transverse cirri. Five to six transverse cirri were seen arranged in a linear row. The right marginal row of cirri ended near the last cirrus, whereas the left row ended at the mid line.

Table 1: Morphometric analysis of *Aponotohymena botrinucleata* n.sp.

Character		**Min***	**Max***	**Mean ± SD**	**CV (%)**	**n**
Body length		85.50	108.80	92.60 ± 5.53	5.98	20
Body width		31.40	39.20	35.70 ± 2.47	6.91	20
Body length/ width ratio		2.30	3.50	2.60 ± 0.29	11.1	20
Macronuclear, number		2.00	2.00	2.00 ± 0.00	0.00	20
Macronuclear, length		11.90	15.30	13.50 ± 0.98	7.27	20
Macronuclear, width		8.40	10.60	9.40 ± 0.55	5.84	20
Micronuclear, number		8.00	10.00	8.70 ± 0.82	9.43	20
Micronuclear diameter		3.10	3.50	3.40 ± 0.14	4.08	20
AM, number		37.00	44.00	40.80 ± 2.40	5.89	20
Adoral length		33.10	39.20	35.40 ± 1.80	5.09	20
Adoral length/ Body length		0.30	0.40	0.40 ± 0.02	5.26	20
Frontal cirri, number		8.00	8.00	8.00 ± 0.00	0.00	20
Ventral cirri, number		5.00	5.00	5.00 ± 0.00	0.00	20
Transverse cirri, number		5.00	7.00	5.40 ± 0.59	11.03	20
LMC number		34.00	38.00	36.30 ± 1.25	3.45	20
RMC number		34.00	39.00	36.10 ± 1.64	4.55	20
DKs, number		4.00	4.00	4.00 ± 0.00	0.00	20
DMs, number		2.00	2.00	2.00 ± 0.00	0.00	20
Total number of bristles in	DK_1	22.00	24.00	22.80 ± 0.92	4.04	10
	DK_2	20.00	25.00	22.00 ± 1.57	7.14	10
	DK_3	17.00	21.00	18.80 ± 1.39	7.39	10
	DK_4	7.00	11.00	9.30 ± 1.25	13.44	10
	DM_1	8.00	12.00	9.70 ± 1.57	16.19	10
	DM_2	4.00	07.00	4.60 ± 0.97	21.09	10
Total number of bristles		78.00	93.00	86.40 ± 4.22	4.88	10
Caudal cirri, total number		7.00	7.00	7.00 ± 0.00	0.00	10

*All dimensional measurements are in µm; Data based on protargol-impregnated cells.

On the dorsal side, six rows of dorsal bristles were observed, which include four dorsal kineties (DK_1 – DK_4) and two dorso-marginals (DM_1 & DM_2). Of the four DKs, DK_{1-3} were seen extending along the entire body length, however, the DK_4 was relatively shorter, and terminates in the mid of the body (Figure 1B). Two dorso-marginal rows ($DM_{1\&2}$) of cirri were observed in the anterior half of the body; the 2 DMs were unequal in size, where DM_2 was smaller, with 4-7 bristles only. In most of the cells, presence of 7 caudal cirri have been noticed in the posterior ends of $DK_{1, 2 \& 4}$. $DK_{1 \& 2}$ bear two caudal cirri each at their respective posterior ends. Rests of the cirri were present at the posterior end of DK_4 (Figure 1).

The live cell observations of *A. botrinucleata* have revealed that the body is flexible and dorso-ventrally flattened, with moderately rapid locomotory movements; also observed that during these movements the cells change their direction with a brief pause. Intra-clonal conjugation was observed, but it was not a very frequent phenomenon though. Similarly, encystment was not very frequent either.

The pellicle is flexible and appeared dark greenish in live cells. Two types of sub-pellicular granules were observed in live cells. Yellow coloured granules are smaller in size and are present throughout the pellicle while green-coloured granules are rounded in shape with variable size and are present in the middle of the body. Granules do not get impregnated with the protargol, hence not visible in the photographs of silver-stained cells (Figure 1C).

3.5. Divisional morphogenesis

Stomatogenesis begins with the *de novo* appearance of a few kinetosomes in the region between the left marginal row and the post oral ventral cirri. Later on, these kinetosomes form of a long anarchic field of Oral primordium (OP) (Figure 2A).

During morphogenetic development, it has been observed that, in opisthe, a group of basal bodies dissociate from the anterior right side of the OP. Simultaneously, the postoral ventral cirri V_1 and V_2 also began to disaggregate. A part of kinetosomal streak, which was formed from the OP, observed to move anteriorly. Later, kinetosomes from the V_1 and V_2 and the oral primordium were aligned to form the primordial streaks I to V for the opisthe. The disaggregating third ventral cirrus (V_3) contribute its kinetosomes for the formation of primordial streak VI. All these six sets of streaks remain connected with OP (Figure 2).

Morphogenesis of the proter starts much later, with disaggregation of F_1, which, together with the kinetosomes from the OP formed the streak II. The parental UMs functions as streak I for the proter. The parental F_8 and F_7, disaggregate to form streaks III and IV respectively. A part of the streak V of the opisthe moves anteriorly, later accompanied by a part of the streak VI. Thus, the streaks V and VI of the proter originate from the streaks V and VI of the opisthe.

In all, two sets of six FVT ciliary streaks have been formed. The cirri differentiate in these streaks in the typical oxytrichinae pattern of 1,3,3,3,4,4, ultimately forming 18 FVT cirri each for each of the daughter cells (Figure 2).

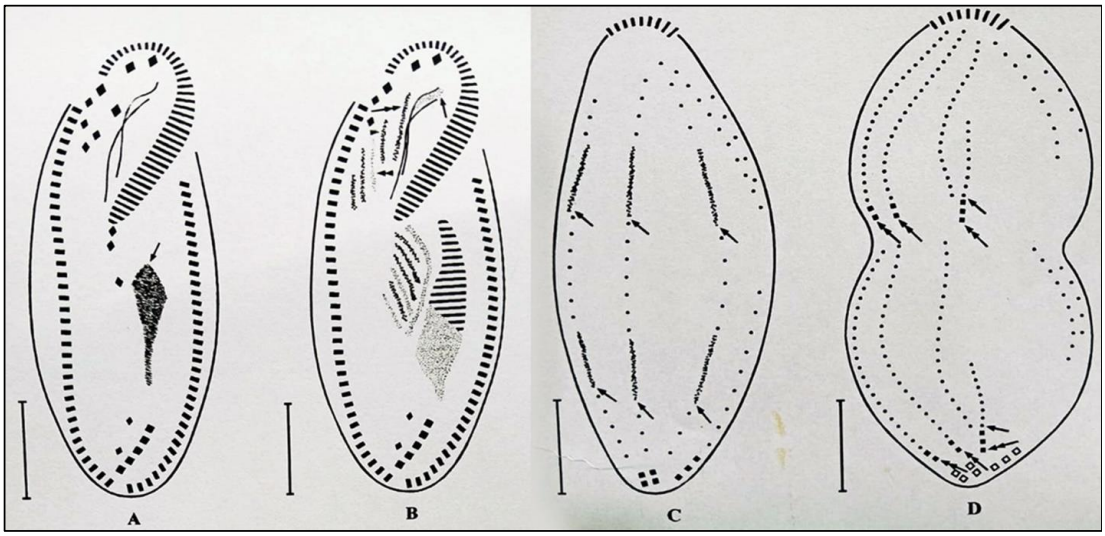

Figure 2: Line diagrams of Protargol impregnated cells of *A. botrinucleata* n. sp. showing division morphogenesis on ventral (A, B) and dorsal (C, D) surface. **A.** Origin of OP (arrow). **B.** Reorganizing UM (arrow), long streak I originated from disaggregating F_1 and joining kinetosomes of OP (double arrow), disaggregating F_8 (arrow head), disaggregating F_7 (double arrow head). **C.** Within row proliferation of 2 sets of 3DP (arrows). **D.** Unequal split of DK_3 (arrows), formation of new 2, 2 & 3 caudal cirri at the end of $DK_{1,2\&4}$ (double arrows). Bar represent 20 µm.

4. DISCUSSION

The identification and characterisation of ciliate species is mainly based on its morphology, morphometry and morphogenesis. Along with these features, the presence or absence, and the coloration of granules is also a peculiar feature for defining a new species.

Justification for making "*Aponotohymena botrinucleata*" a new species

As evident from the line diagrams as well as photomicrograph (Figures 1 & 2); morphological & morphometric data collected from the current species, and a comparison with other species (Tables 1 & 2), it is very clear that the *Aponotohymena botrinucleata* n. sp., though, has superficial resemblance to *A. australis* (Foissner & O'donoghue, 1990; Berger, 1999; Voss, 2008; Hu & Kusuoka, 2015), *N. apoaustralis* (Lv et al., 2013) and *A. isoaustralis* (Gupta et al., 2017) in its ventral ciliature, number of dorsomarginal rows and the presence of granules, it differs significantly from the above-mentioned species in several aspects, which are shown in Table 2, and discussed in detail below:

i. ***Morphometric features*****:** *A. botrinucleata* n. sp. is much smaller in size *i.e.* has body length of 92.60 µm while *A. australis* is 142 and 167 µm (reported by two groups, Table 2), *N. apoaustralis* is 161 µm and *A. isoaustralis* is 132 µm in its body length. It also varies from these three species in its body length to body width ratio *i.e.* the body length to width ratio of *A. botrinucleata* is 2.6:1, while it is 4.3:1 and 2.2:1 for *A. australis*, 2.2: 1 for *N. apoaustralis* and 4:1 for *A. isoaustralis.* All the above morphometric values are based on statistical analysis as shown in Table 1.

ii. ***Number and positioning of micronuclei*****:** The most distinguishing feature of this new species is the presence of micronuclei as a botrus (bunch or cluster) in between the macronuclei. Further, out of the 8-10 micronuclei observed in the cells of this species, 1 or 2 were noticed closer to macronuclear nodules, while the rest can be seen clustered together in the space between the two macronuclear nodules (Figure 1D). In the other three known species, however, the number of micronuclei vary significantly. For instance, in case of *A. australis*, only 3-5 micronuclei were reported, which were present closer to macronuclear nodules, whereas, in *N. apoaustralis*, only one micronucleus was present, seen located towards the posterior end of anterior macronuclear nodule. However, in *A. isoaustralis*, there is no micronucleus at all.

iii. ***Number of caudal cirri*****:** Number of caudal cirri also varies in the other reported species of the genus. In case of *A. botrinucleata* n. sp. & *A. isoaustralis*, their number is constant at 7, while in case of *A. australis* (5 -10; 5-10 & 7-10 as reported by two different groups, Table 2) and *N. apoaustralis* (8-10) the number of caudal cirri is variable (Table 2).

iv. ***Colour & positioning of granules*****:** The colour of granules in all the species studied thus far is yellowish to green, however their position varies which may be a diagnostic feature of species identification. In *A. botrinucleata* n. sp. two types of sub-pellicular granules are noticed; yellow and green. Yellow granules are smaller in size, and are present throughout the pellicle, while green granules are round in shape with variable size, and are present in the middle of the body. The live cells of *A. australis* (Voss, 2008; Hu & Kusuoka, 2015) appear yellow, whereas, the current species under study, the *A. botrinucleata* n. sp. appears dark green under low magnification. However, on the ventral surface of *N. apoaustralis* (Lv et al., 2013), cortical granules are either densely arranged in short rows near marginal cirri forming belts along the cirral rows, or grouped in irregular short rows. On the dorsal side, the cortical granules are grouped in rosettes around dorsal cilia, whereas, in *A. isoaustralis*, the granules are reported to align along the margins, and also irregularly distributed throughout the cell, and sometimes, randomly concentrated as clusters along the

left margin and posterior end of the cell. The comparative features of all four species are shown in Table 2.

Table 2: Morphometric comparison of *Aponotohymena botrinucleata* n.sp. with *A. australis*, *N. aupoaustralis* and *A. isoaustralis*

Characters	***Aponotohymena botrinucleata n. sp. (Present investigation)**	***A. australis* (Voss, 2008)**	***A. australis* (Hu & Kusuoka 2015)**	***N. apoaustralis* (Lv et al., 2013)**	***A. isoaustralis* (Gupta et al., 2017)**
Body length	92.60 ± 5.53	167.00	142.00	161.00	132.00
Body width	35.70 ± 2.47	42.00	63.00	74.00	35.00
Body length/ body width	2.60 ± 0.29	4.3:1	2.2:1	2.2:1	4:1
Macronuclear length	13.50 ± 0.98	23.00	21.00	17.00	20.00
Macronuclear width	9.40 ± 0.55	12.00	14.00	12.00	14.00
Micronuclear, number	6.10 ± 2.36	3-8	3-5	1	No micronuclei
Position of micronuclei	Out of ~8, 1 - 2 were seen proximate to the macronuclei; rest were present in a cluster form in between the macronuclear nodules; no of micronuclei vary in a cluster	Nearer to both the macro-nuclear nodules	Nearer to both the macro-nuclear nodules	Located at right posterior of anterior macro-nuclear nodule	No micronucleus
AM, number	40.80 ± 2.40	42	38	39	36
Caudal cirri	7.00 ± 0.00 (constant)	5–10 (variable)	7–10 (variable)	8-10 (variable)	7 (constant)
Position of the granules	Yellow colored granules, smaller in size and are present throughout the pellicle; green coloured granules are rounded in shape with variable size and present in the middle of the body	Not described in literature	Grouped around cirri and dorsal bristles or aligned between cirral rows or dorsal kineties	i. On ventral side, the cortical granules are reported as either densely arranged in short rows near the marginal cirri, forming belts along the cirral rows; or grouped in irregular short rows. ii. On dorsal side, cortical granules are reported grouped in rosettes around dorsal cilia.	Aligned along the margins, and also irregularly distributed throughout the cell. Randomly concentrated as clusters along the left margin and posterior end of the cell

*All dimensional measurements are in µm; Data based on protargol-impregnated cells.

5. CONCLUSION

On the basis of above-discussed salient and distinctive features, we conclude that the species under investigation is in fact a new species of the genus *Aponotohymena*, and named *Aponotohymena botrinucleata* based upon the same distinctive features.

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STATEMENTS AND DECLARATIONS

All authors of this article have made substantial contributions towards the conception and design of the work, acquisition, analysis, and interpretation of data; as well as for the preparation of draft and editing of the manuscript and approved the final version for submission.

CONFLICT OF INTEREST

The authors further declare that-

i. there are no financial or non-financial interests that are directly or indirectly related to the work submitted for publication,

ii. the work was not supported by any governmental or non-governmental financial institutions, and

iii. the authors declare no conflicting interests.

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