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Taxonomic and Morphogenetic Description of the Freshwater Ciliate *Aponotohymena isoaustralis* n. sp. (Ciliophora; Oxytrichidae) Isolated from Sanjay Lake, Delhi, India

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Abstract. Morphology, morphogenesis and molecular phylogeny of a freshwater oxytrichid ciliate, *Aponotohymena isoaustralis* n. sp. collected from Sanjay Lake (28°36′51″N, 77°18′14″E), Delhi, India, were studied. The described species is characterized by a flexible body, with body size (*in vivo*) of about 148 × 46 µm and yellowish green cortical granules. Morphological characters exhibit: undulating membranes in *Notohymena*–pattern; two macronuclei and absence of micronucleus (amicronucleate); about 36 adoral membranelles; 18 frontoventral-transverse (FVT) cirri; one right and one left marginal row separated posteriorly; 6 dorsal rows; 7 caudal cirri arranged in 2 + 2 + 3 pattern (constant). In the present study, a detailed description of all the developmental stages is also provided. Prominent distinguishing features of the new species are the absence of micronucleus, 7 caudal cirri (constant), yellowish green cortical granules aligned along the margins and irregularly distributed throughout the cell. They may also be randomly concentrated as clusters along the left margin and posterior end of the cell. Molecular phylogeny based on small subunit rDNA sequence data suggests sister relationship of *Aponotohymena isoaustralis* n. sp. with *Notohymena apoaustralis* and *Aponotohymena australis* (*Notohymena australis*) which cluster in a clade with *Paraurostyla weissei* and *Paraurostyla coronata*. Further analysis of nucleotide sequence of SSU rDNA also suggests that *A. isoaustralis* n. sp. is distinct from the type species *A. australis*.

Key words: *Aponotohymena*, morphogenesis, morphology, oxytrichid, phylogeny, SSU rDNA

INTRODUCTION

The genus *Notohymena* was established by Blatterer and Foissner (1988) distinguishing it from the genus *Oxytricha* as it has a distinct hook shaped distal end of paroral membrane (PM), recognizable only after protargol impregnation (Berger 1999). Since the establishment of the genus, nine species have been assigned to it: *N. selvatica* (Hemberger 1985, Blatterer and Foissner 1988), *N. rubescens* (Blatterer and Foissner 1988), *N. australis* (Foissner and O' Donoghue 1990, Berger 1999, Voss 2008, Hu and Kusuoka 2015), *N. antarctica* (Foissner 1996), *N. pampasica* (Küppers *et al.* 2007), *N. saprai* (Kamra and Kumar 2010), *N. apoaustralis*

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(Lv *et al.* 2013), *N. quadrinucleata* (Foissner 2016) and *N. limus* (Naqvi *et al.* 2016). *Notohymena australis* has been redesignated as *Aponotohymena australis* by Foissner (2016) on the basis of: flexible body; 18 FVT cirri; more than 3 caudal cirri associated with dorsal kineties 1, 2 and 4; splitting of DP_3 (third dorsal primordium).

Present investigation describes the morphology, morphogenesis and phylogenetic relationship of a new freshwater species of *Aponotohymena* genus isolated from Sanjay Lake, Delhi, India. Morphological, morphometric and molecular analysis indicates that *A. isoaustralis* n. sp. is a new species. The systematic and phylogenetic position of this species within the family Oxytrichidae is also assessed by using SSU rDNA as molecular marker gene.

MATERIALS AND METHODS

Sampling site and cultivation

Water samples were collected in large beakers along with the root system of water hyacinth and mixed planktonic cultures from the sides of the lake on September 12, 2013 from Sanjay Lake, which is an artificial freshwater lake in Trilokpuri (28°36′51″N, 77°18′14″E), East Delhi, India. Its surface area is about one square kilometer and has an extensive growth of water hyacinth. It is mainly a rain water lake but occasionally receives inputs from sewage. Water temperature and pH at the time of collection were 22.5°C and 7.2 respectively. A clonal culture of *Aponotohymena isoaustralis* n. sp. was maintained in the laboratory at 22–23°C in Pringsheim's medium (Chapman–Andresen 1958). Boiled cabbage was added to the medium to promote the growth of bacteria which served as the primary food source.

Morphology and morphogenesis

In vivo observations were done using stereomicroscope, phase contrast microscope and differential interference contrast microscope. Protargol impregnation (Wilbert 1975, Kamra and Sapra 1990) was used to reveal the infraciliature. Nuclear morphology was observed from Feulgen stained cells (Chieco and Derenzini 1999). Counts and measurements of the impregnated specimens were performed at magnification of 1000 ×. Terminology is mainly according to Berger (1999, 2008); Foissner and Stoeck (2011) and Küppers *et al.* (2011). Numbering system used is according to Borror (1972) and Wallengren (1900).

DNA extraction, amplification and sequencing

Cell culture (50 ml) was taken and filtered using 120 micron Nytex mesh to remove cabbage from the medium. Cells were then centrifuged to obtain a cell pellet with minimal volume of medium and washed to remove any potential contamination. Genomic DNA was extracted as per protocol for cultured animal cells using DNeasy Blood and Tissue Kit (QIAGEN, India).

Primers for SSU rDNA gene amplification were 18S-F(5'–AAC CTG GTT GAT CCT GCC AGT–3') and 18S-R (5'–TGA TCC TTC TGC AGG TTC ACC TAC–3') (Lv *et al.* 2013, Medlin *et al.* 1988). Additionally, two internal primers: one forward (5'–CGG-TAATTCCAGCTCCAATAG–3') and one reverse (5'–AACTA-AGAACGGCCATGCAC–3') were used. PCR conditions were as follows: 1st cycle with denaturation at 95°C for 5 min, annealing at 48°C for 1 min and elongation at 72°C for 1 min, followed by 30 cycles with denaturation at 95°C, annealing at 48°C and elongation at 72°C for 45 s each and last cycle with denaturation at 95°C for 45 s, annealing at 48°C for 45 s and elongation at 72°C for 10 min. PCR product was eluted using QlAquick Gel Extraction kit (QIAGEN, India). The eluted product was sequenced using Applied Biosystems 3130xl Automated DNA Sequencer.

SSU rDNA analysis and phylogenetic tree construction

SSU rDNA sequence was submitted to BLAST search (http://www.ncbi.nlm.nih.gov) to find closely related sequences. SSU rDNA sequences of a total of 39 other taxa were retrieved. Sequences were aligned using CLUSTAL X2 sequence analysis software (Larkin *et al.* 2007). The resulting alignments were checked and corrected manually to remove ambiguous nucleotide positions at the beginning and end of the fragments in the BioEdit editor (Hall 1999). Also, the SSU rDNA sequences of *N. apoaustralis* and *A. australis* were selected and separately aligned with *A. isoaustralis* n. sp. by using BioEdit software and were simultaneously analyzed to calculate the differences in nucleotide sequences. Phylogenetic tree using Maximum Likelihood method was constructed with the MEGA 5 (Molecular Evolutionary Genetics Analysis) software package (Tamura *et al.* 2011). Also, Bayesian Inference tree was constructed using MrBayes 3.2.4 program under GTR + I + G model where 1,00,000 Markov Chain Monte Carlo generations were sampled with a 25% burn-in for tree construction. 1000 bootstrap replicates were used to calculate supporting values. The final BI tree was viewed using TreeView software.

RESULTS

Morphology of *Aponotohymena isoaustralis* n. sp.

Live cell: Average body size *in vivo* 148 × 46 µm (n = 25), length: width ratio 4:1, elongated body; dorsoventrally flattened about 2:1, flexible, colourless cytoplasm with a greenish appearance at the margins when observed at low magnifications. Yellowish green cortical granules about 0.8 µm in diameter aligned along the margins and irregularly distributed throughout the cell; they may also be randomly concentrated as clusters along the left margin and posterior end of the cell. Single contractile vacuole about 15 µm in diameter located below the AZM near the left margin. Cell division every 11 h. Encystment generally takes place within 24 h of starvation; the average diameter of the resting cyst in life is 64.53 µm (n = 12) with smooth ectocyst covered

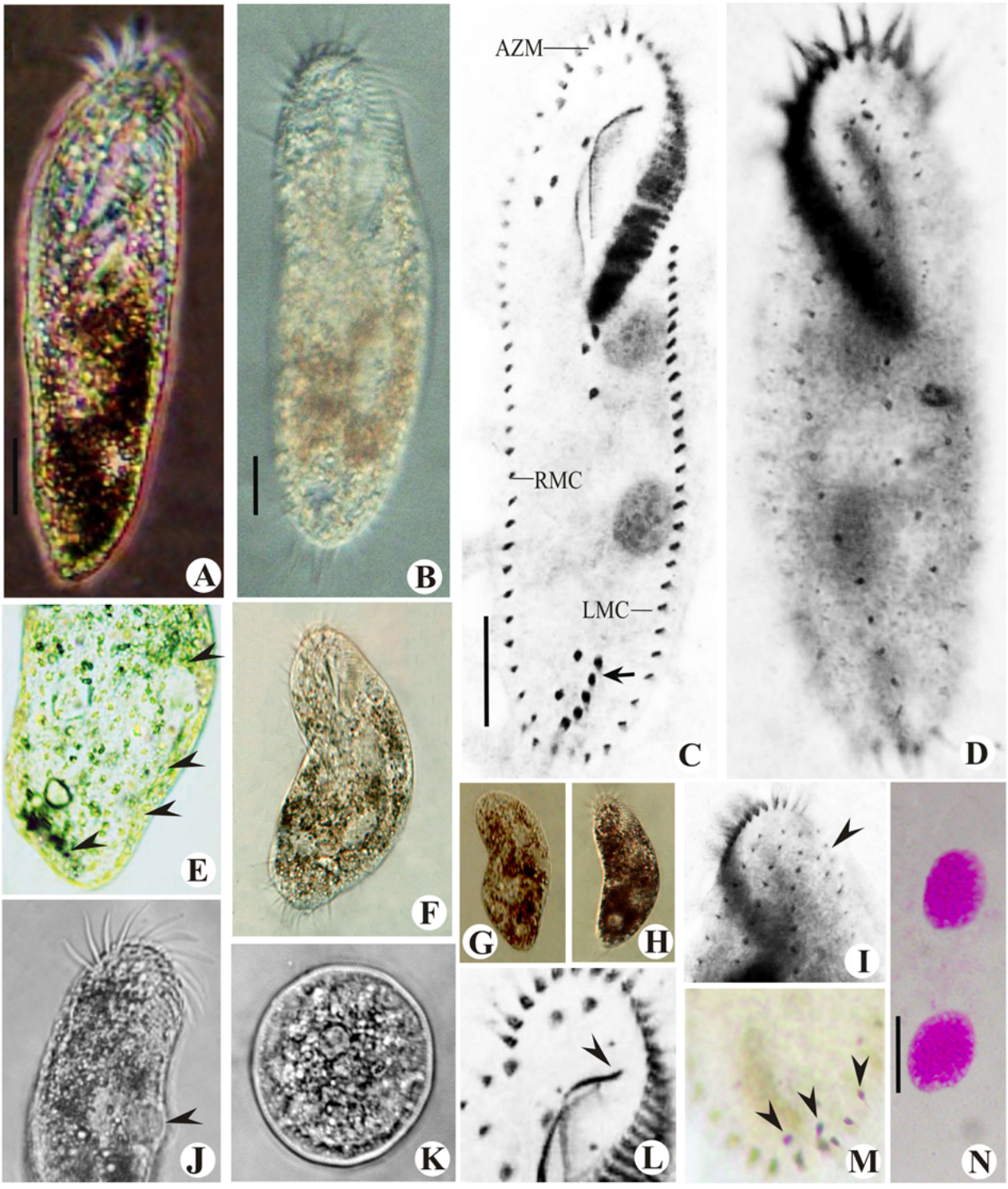


Fig. 1. Photomicrographs of live (A, B, E, F, G, H, J, K), protargol impregnated (C, D, I, L, M) and Feulgen stained (N) cells of *Aponotohymena isoaustralis* n. sp. **A, B** – cells in ventral view; **C** – ventral view of a vegetative cell with 5 transverse cirri arranged in a pseudo row (arrow); **D** – dorsal view of a vegetative cell; **E** – ventral view to show the arrangement of cortical granules (arrowheads) and colouration; **F, G** and **H** – ventral view of different cells showing flexible body; **I** – anterior portion of the dorsal surface showing dorsal rows (arrowhead); **J** – anterior portion of the cell showing contractile vacuole (arrowhead); **K** – cyst; **L** – anterior hook (arrowhead) of paroral membrane; **M** – dorsal view showing caudal cirri (2 + 2 + 3) (arrowheads); **N** – two macronuclei. AZM – adoral zone of membranelles, LMC – left marginal cirri, RMC – right marginal cirri. Scale bars: 20 μm.

with mucus layer and granulated cytoplasm (Fig 1A, B, E–H, J, K).

Nuclear morphology: Two macronuclear nodules each about 20 × 14 µm in size (in protargol preparations), spherical to ellipsoidal, located along the cell mid line, one about 19 µm away from the anteriormost part and the other about 16 µm away from the posteriormost part of the cell; distance between the two macronuclei is about 22.2 µm; micronucleus absent (Fig. 1N).

Infraciliature characteristics: Adoral zone of membranelles (AZM) occupies about 34% of the body length in protargol preparations and is composed of 32–40 membranelles. Undulating membrane (UM) with a characteristic hook at the distal end (*Notohymena*-pattern); 18 FVT cirri with 3 frontal cirri, 4 frontoventral cirri, 1 buccal cirrus, 3 postoral ventral cirri, 2 pretransverse ventral cirri and 5 transverse cirri; transverse cirri equidistant to each other and usually arranged in a pseudo row. IV/2 and V/4 are closely placed whereas V/3 is distantly placed (distance between IV/2 and V/4 is 3.10 µm and between V/4 and V/3 is 5.14 µm). Marginal rows are separated (nonconfluent) posteriorly

Table 1. Morphometric characterization of protargol impregnated cells of *Aponotohymena isoaustralis* n. sp.

Character*	Mean	Min	Max	Median	SD	SE	CV
Body, length	132.3	115.5	161.2	129.4	12	2.4	9
Body, width	34.8	26.7	52.8	33.60	6.0	1.2	17.3
Adoral zone of membranelles, length	44.6	33.8	52.3	44.2	4.1	0.8	9.1
Adoral membranelles, number	36.4	32	40	36	2.1	0.4	5.7
Frontal cirri, number	3	3	3	3	0	0	0
Frontoventral cirri, number	4	4	4	4	0	0	0
Buccal cirri, number	1	1	1	1	0	0	0
Postoral ventral cirri, number	3	3	3	3	0	0	0
Pretransverse ventral cirri, number	2	2	2	2	0	0	0
Transverse cirri, number	5	5	5	5	0	0	0
Caudal cirri, number	7	7	7	7	0	0	0
Left marginal cirri, number	34.4	30	38	35	1.9	0.4	5.6
Right marginal cirri, number	32.9	30	38	32	2.1	0.4	6.4
Dorsal kineties, number	6	6	6	6	0	0	0
DK_1, dikinetids number	20.4	18	22	21	1.8	0.4	8.8
DK_2, dikinetids number	20.4	18	22	21	1.8	0.4	8.7
DK_3, dikinetids number	16.9	16	18	17	0.9	0.2	5.5
DK_4, dikinetids number	8	8	8	8	0	0	0
DM_1, dikinetids number	8	8	8	8	0	0	0
DM_2, dikinetids number	6	6	6	6	0	0	0
Macronuclei, number	2	2	2	2	0	0	0
Micronucleus, number	0	0	0	0	0	0	0
Anterior macronucleus, length	20.8	16.1	38.4	19.5	5.1	1.0	24.5
Anterior macronucleus, width	14.3	10.1	17.5	14.6	2.1	0.4	14.8
Posterior macronucleus, length	19.4	13.8	26.4	19.5	2.6	0.5	13.4
Posterior macronucleus, width	13.6	7.6	18	13.9	2.4	0.5	17.4
Distance between the two macronuclei	22.2	9.3	41.6	18.8	9.0	1.8	40.8
CC in kinety 1, number	2	2	2	2	0	0	0
CC in kinety 2, number	2	2	2	2	0	0	0
CC in kinety 4, number	3	3	3	3	0	0	0

*All measurements are in µm (n – sample size – 25). CC – caudal cirri; CV – coefficient of variation (%); DK – dorsal kineties; DM – dorsomarginal; Max – maximum; Min – minimum; SD – standard deviation; SE – standard error.

with about 30–38 cirri in right and left marginal row (Figs 1C, L, 2A; Table 1).

The dorsal ciliature is of typical oxytrichid pattern with 4 dorsal kineties ($DK_{1–4}$) and 2 dorsomarginal rows ($DM_{1,2}$). Three dorsal kineties ($DK_{1–3}$) encompass the entire length of the cell whereas DK_4 commences near midline of the cell and extends to posterior end. DM_1 and DM_2 start at the anterior end of the cell and terminate at 1/3rd of the cell length. Length of the dorsal bristles varies from 0.7 to 2.15 µm (average bristle length in DK_1 = 2.15 µm, DK_2 = 1.82 µm, DK_3 = 1.04 µm, DK_4 =1.65 µm, DM_1 = 1.74 µm and DM_2 = 0.7 µm; n = 10 cells). Seven caudal cirri (CC) are arranged in 2 + 2 + 3 pattern at the end of $DK_{1, 2 \& 4}$ respectively; caudal cirri are about 6.25 µm long (Figs 1D, I, M, 2B; Table 1).

Morphogenesis

Stomatogenesis: The first morphogenetic event is the occurrence of a group of scattered basal bodies for oral primordium (OP) which arises apokinetally on the ventral surface between the postoral ventral cirri (POVC) and the left marginal row. There is no contri-

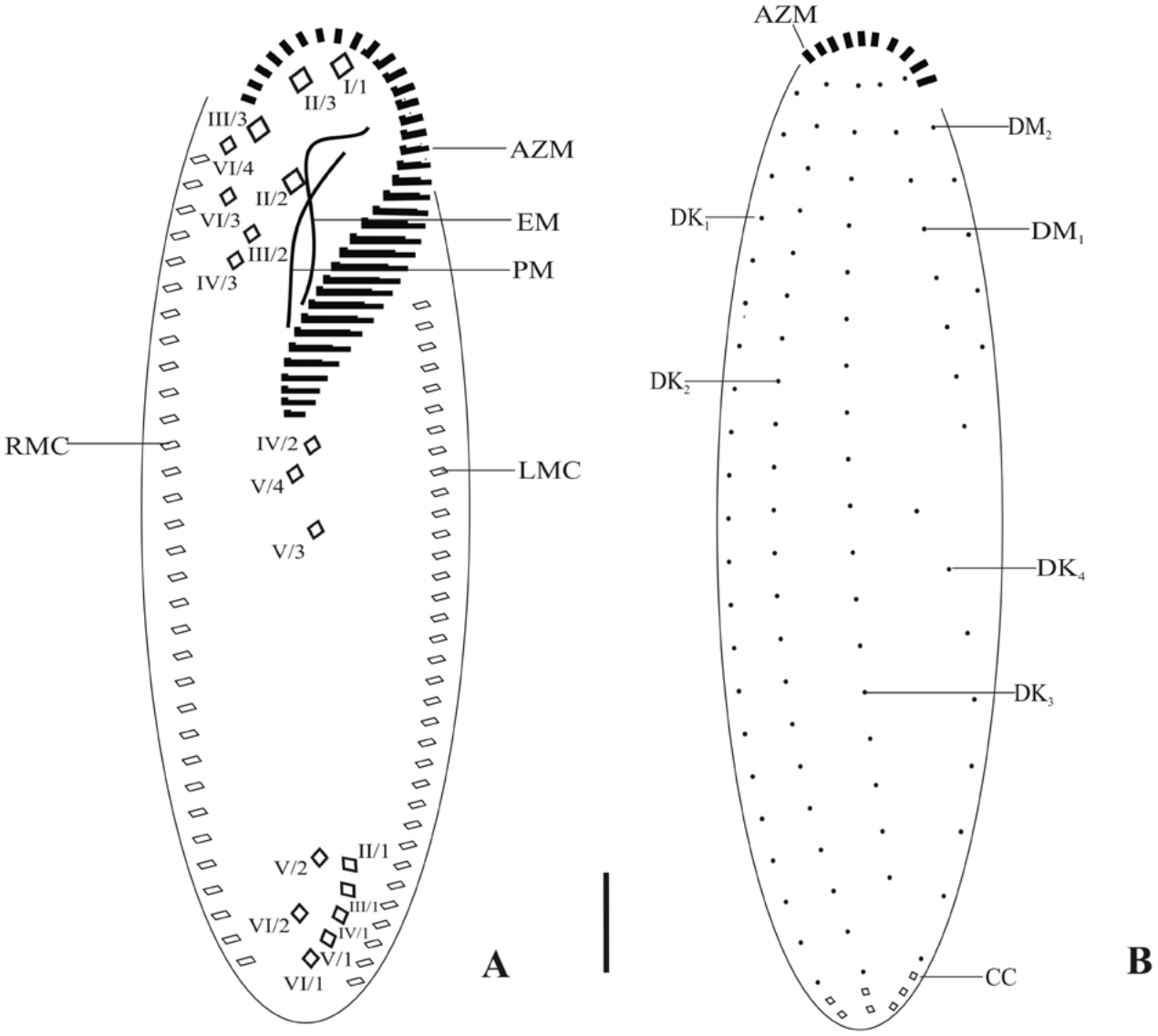


Fig. 2. Line diagrams showing protargol impregnated vegetative cells of *Aponotohymena isoaustralis* n. sp. **A** – ventral surface; **B** – dorsal surface. AZM – adoral zone of membranelles, CC – caudal cirri, $DK_{1–4}$ – dorsal kineties, $DM_{1,2}$ – dorsomarginals, EM – endoral membrane, LMC – left marginal cirri, PM – paroral membrane, RMC – right marginal cirri, II/2 – buccal cirri, I/1, II/3, III/3 – frontal cirri, VI/4, VI/3, IV/3, III/2 – frontoventral cirri, – IV/2, V/4, V/3 – postoral ventral cirri, V/2 and VI/2 – pretransverse ventral cirri, II/1, III/1, IV/1, V/1, VI/1 – transverse cirri. Scale bar: 20 µm.

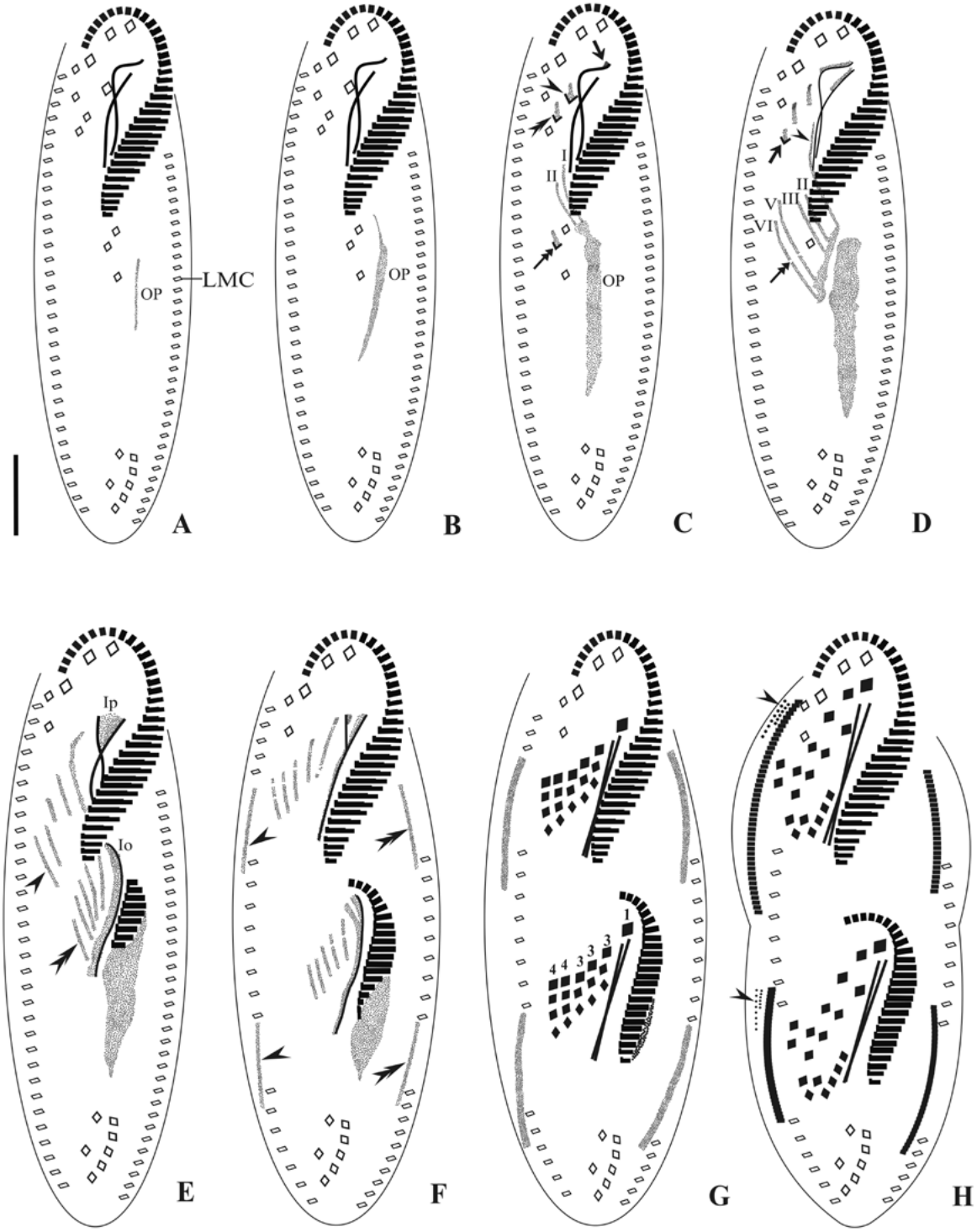


Fig. 3. Line diagrams showing morphogenetic stages on ventral surface of protargol impregnated cells of *Aponotohymena isoaustralis* n. sp. **A, B** – origin of OP apokinetally between the LMC and POVC for the opisthe; **C** – reorganization of parental UM (arrow), disaggregation of II/2 (arrowhead), III/2 (double arrowhead) and V/4 (double arrow) to form primordia IIp, IIIp and Vo respectively, kinetosomes from OP form primordia Io and IIo; **D** – dissagregation of IV/3 to form primordium IVp (arrow); kinetosomes from OP move anteriorly (arrowhead); the two primary primordia, one each formed from disaggregation of V/4 and V/3 split transversely (double arrow) to form primordia V and VI for proter and opisthe; **E** – full complement of 6 FVT primordia Ip to VIp (arrowhead) and Io to VIo (double arrowhead); **F** – within-row marginal primordia formation for RMC (arrowheads) and LMC (double arrowheads); **G** – differentiation of cirri in 1, 3, 3, 3, 4, 4 pattern; **H** – late divider showing formation of new dorsomarginals (arrowheads) close to newly formed RMC. LMC – left marginal cirri; OP – oral primordium. Scale bar: 20 µm.

bution of the transverse cirri and POVC in the formation of the OP, indicating *de novo* origin of OP (Figs 3A, B, 4A, B). The primordium then elongates, widens and the adoral membranelles begin to organize end forming new AZM for the opisthe. The parental AZM is retained intact during the morphogenetic process for the proter.

Development of the cirral primordia:

Opisthe. Streaks I–III arise from the developing oral primordium (OP). The ventral cirri IV/2, V/4 and V/3 disaggregate independently giving rise to streaks IVo, Vo and VIo respectively. Cirrus V/4 disaggregates first giving rise to the streak Vo followed by V/3 to form streak VIo and lastly IV/2 disaggregates to form the streak IVo. Simultaneously, the two undulating membranes are formed from Io (Figs 3C, D, 4C, D).

Proter. The anterior portion of the parental paroral membrane (PM) reorganizes partially to form the streak Ip. The buccal cirrus (II/2) and the frontoventral cirri (III/2 and IV/3) form the streaks IIp, IIIp and IVp respectively. Kinetosomes from OP move anteriad and also contribute in the formation of IIp (Figs 3C, 4D, E). Streak Vo and VIo extend anteriorly and split transversely (splitting of primary primordia) giving rise to streaks Vp and VIp for the proter. Thus, 6 parental cirri (II/2, III/2, IV/3, IV/2, V/4 and V/3) are involved in primordia formation (Figs 3C–E, 4C–E).

The two sets of 18 FVT cirri arise in typical oxytrichid pattern of 1, 3, 3, 3, 4, 4 (Figs 3G, H, 4I–K).

Development of marginal rows: The streaks for the new marginal cirri are formed within the parental marginal rows. The marginal primordia elongate with disaggregation of 10–12 parental cirri which differentiate into new marginal rows replacing the old ones. Rest of the parental marginal cirri are resorbed in later stages of development (Figs 3F–H, 4I, J).

Development of dorsal ciliature: The dorsal ciliature is formed as in most other oxytrichids. Three dorsal primordia DP_{1-3} are formed at two levels within the parental DK_{1-3}. DP_4 is formed by an unequal fragmentation of DP_3. The two dorsomarginals originate on the ventral surface, close to the anterior end of the right marginal primordia (Figs 3H, 4J). Caudal cirri differentiate in the 2 + 2 + 3 pattern at the posterior ends of the new dorsal kineties $DK_{1, 2 \& 4}$ (Fig. 5).

As cytokinesis commences, the new cirri arrange themselves according to their species-specific pattern while the remaining parental ciliature undergoes resorption.

Molecular analysis

The length of the SSU rDNA sequence of *A. isoaustralis* n. sp. was 1,703 bp and GC content was 44.74% and the sequence is available in GenBank with accession number KP336402. The percentage differences in nucleotide sequence of SSU rDNA between *A. isoaustralis* n. sp. and *A. australis* is 2.92% and between *A. isoaustralis* n. sp. and *N. apoaustralis* is 4.24%.

Phylogenetic analysis based on SSU rDNA sequence

Phylogenetic analysis was done including 39 taxa from GenBank (26 Sporadotrichida, 6 Stichotrichida and 7 Urostylida) and *A. isoaustralis* n. sp. In the analysis, *A. isoaustralis* n. sp., *N. apoaustralis* and *A. australis* clustered together with high support (98% ML and 100% BI value). This group formed a clade with *Paraurostyla weissei* and *Paraurostyla coronata* with a moderate support (63% ML and 57% BI value) (Fig. 6).

DISCUSSION

Justification of new species *Aponotohymena isoaustralis* n. sp.

Aponotohymena isoaustralis n. sp. is distinct from *A. australis* and *N. apoaustralis* on the basis of morphology, morphometry and SSU rDNA gene (Table 2).

(1) Micronuclei: The most distinguishing feature is the absence of micronucleus in *A. isoaustralis* n. sp. whereas, in *A. australis* the number of micronuclei varies from 3–8 and in *N. apoaustralis* one micronucleus is reported. Existence of amicronucleate species of *Stylonychia, Oxytricha, Paraurostyla* and *Tetrahymena* have been reported earlier as well (Prescott 1994, Doerder 2014).

(2) Caudal cirri: The number of caudal cirri is 7 (constant) in *A. isoaustralis* n. sp. whereas the number of caudal cirri varies from 5–10 (variable) in *A. australis* and 8–10 (variable) in *N. apoaustalis* (Table 2).

(3) Cortical granules: The colour of cortical granules is yellowish green in all the three species whereas the position of granules varies which may be one of the diagnostic features in species identification. In *A. isoaustralis* n. sp. granules are aligned along the margins and also irregularly distributed throughout the cell. They may also be randomly concentrated as clusters along the left margin and posterior end of the cell. In *A. australis*, granules group around cirri and dorsal cilia

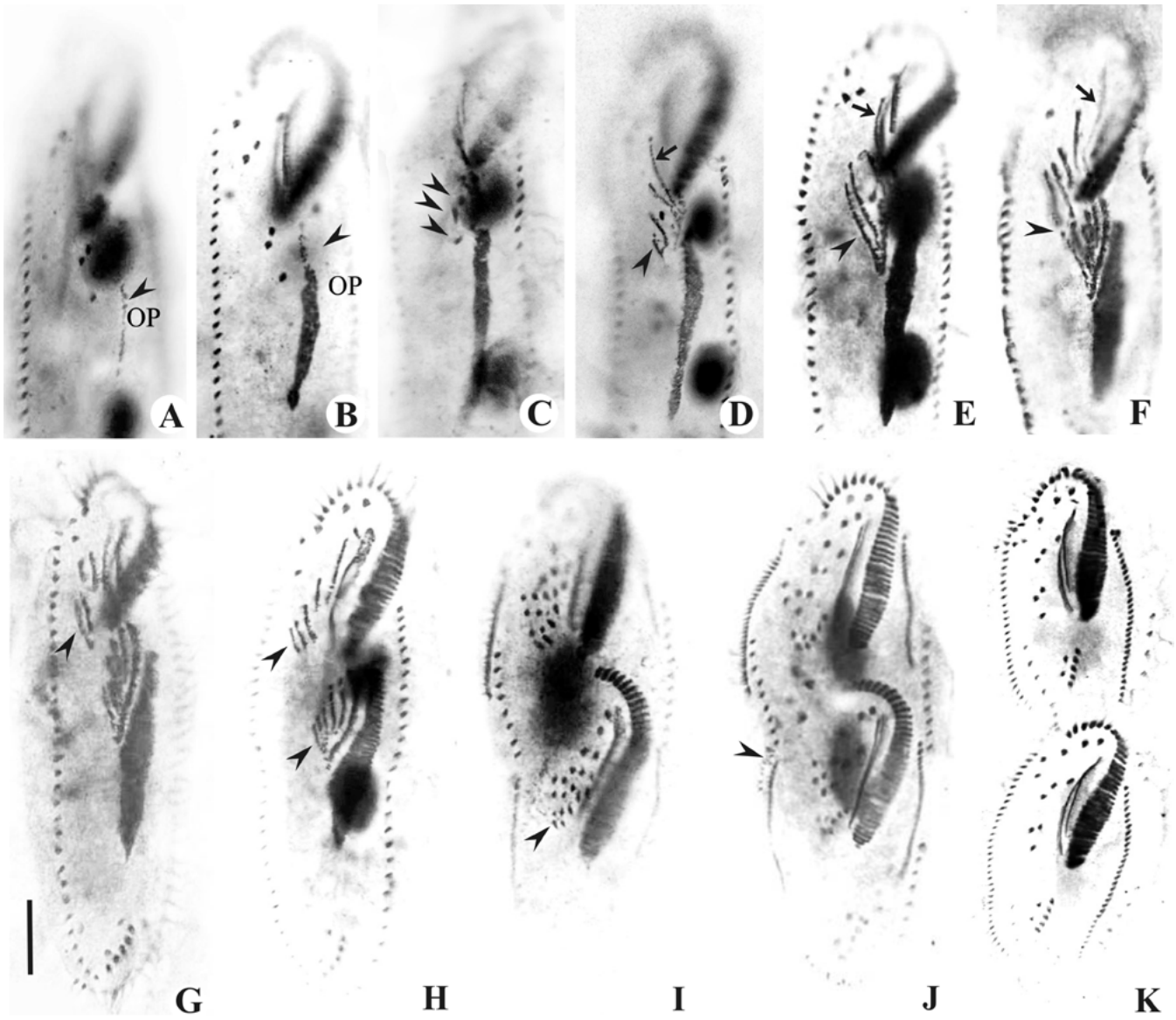


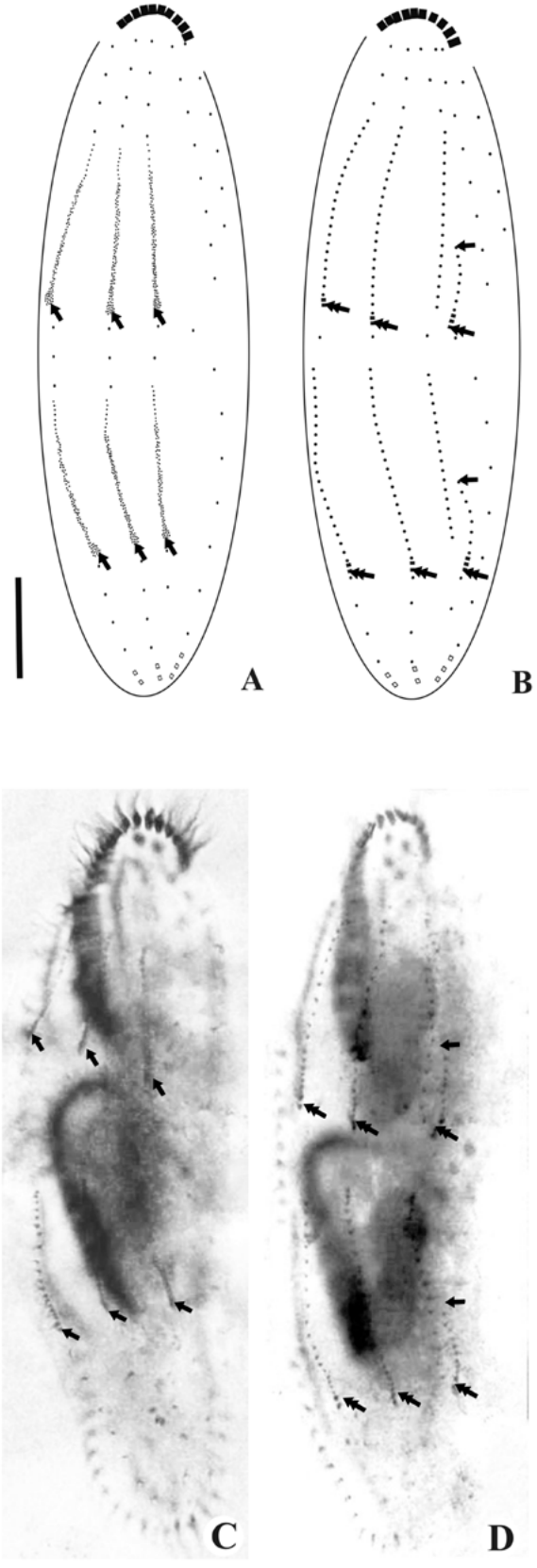
Fig. 4. Photomicrographs showing morphogenetic stages on ventral surface of protargol impregnated cells of *Aponotohymena isoaustralis* n. sp. **A, B** – *de novo* origin of OP (arrowheads); **C** – POVC (arrowheads) not contributing to OP; **D** – dissagregation of V/4 and V/3 (arrowhead), movement of kinetosomes from OP to anterior region of the cell (arrow); **E** – elongation of two primary primordia (arrowhead), kinetosomes moved from OP to contribute in the formation of IIp (arrow); **F** – splitting of primary primordia (arrowhead), composite origin of IIp from OP and cirrus II/2 (arrow); **G** – primordia Vp and VIp (arrowhead) formed from splitting of primary primordia; **H** – full complement of 6 FVT primordia (arrowheads); **I** – differentiation of new FVT cirri (arrowhead); **J** – newly formed DMs on the ventral surface (arrowhead); **K** – cell in cytokinesis. OP – oral primordium. Scale bar: 20 μm.

or align between cirral rows or dorsal kineties whereas in *N. apoaustralis*, cortical granules are grouped around marginal cirri and dorsal kineties.

(4) SSU rDNA: The SSU rDNA nucleotide sequence of *A. isoaustralis* n. sp. differs from that of *A. australis* (2.92%) and *N. apoaustralis* (4.24%) indicating that it is a distinct species.

Comparison with the genus *Notohymena*

According to Foissner (2016), the genus *Aponotohymena* is distinguishable from the genus *Notohymena* on the basis of difference in the number of caudal cirri. A detailed morphological, morphogenetic and molecular comparison suggests that there are several unique features of these two genera.



(1) Cortical granules: The cortical granules are yellowish green in colour in *A. australis, A. isoaustralis* n. sp. and *N. apoaustralis* though the position varies (Voss 2008, Lv *et al.* 2013, Hu and Kusuoka 2015). On the contrary, colour of cortical granules varies in different species of *Notohymena* [*N. rubescens* – ruby coloured granules (Berger 1999); *N. pampasica* – colourless granules (Küppers *et al.* 2017); *N. antarctica* – yellow to yellowish green granules (Berger 1999) and colourless granules in *N. limus* (Naqvi *et al.* 2016)].

(2) Number of caudal cirri: On the dorsal surface, supernumerary caudal cirri are formed by the posterior thickening at the end of $DK_{1, 2 \& 4}$ in *A. australis, A. isoaustralis* n. sp. and *N. apoaustralis* which is in contrast to formation of three caudal cirri one each at the end of $DK_{1, 2 \& 4}$ of the other species of genus *Notohymena* (Table 3).

(3) Habitat: *A. australis, A. isoaustralis* n. sp. and *N. apoaustralis* are freshwater dwellers whereas the other species of *Notohymena* are from terrestrial habitat.

(4) Oral primordium (OP): OP arises as a thin long row of kinetosomes formed apokinetally between the post oral ventral cirrus V/3 and left marginal cirri (LMC) in *A. australis, A. isoaustralis* n. sp. and *N. apoaustralis* as also reported in some of the other oxytrichids (Foissner 1983; Foissner and Adam 1983a, b; Hemberger 1985; Wirnsberger *et al.* 1985, 1986; Ganner *et al.* 1986; Voss 1991a, b; Voss and Foissner 1996; Berger and Foissner 1997; Arora *et al*. 1999; Berger 1999, 2011; Chen *et al*. 2013; Li *et al.* 2014) whereas the formation of OP in other reported species of *Notohymena* seems to be species specific: *N. rubescens* – OP is generated close to uppermost transverse cirrus II/1, *N. saprai* – OP arises *de novo* close to IV/2, V/4, V/3 and *N. limus* – OP arises *de novo* close to V/2 and transverse cirri (Table 4).

(5) FVT primordia: The OP contributes to the formation of primordium IIp in *A. isoaustralis* n. sp. and thus IIp has a composite origin (2 different sources – involvement of oral primordia and II/2) (Figs 3C, D, 4D–F) as also observed in some oxytrichids (Foissner

Fig. 5. Line diagrams and photomicrographs of *Aponotohymena isoaustralis* n. sp. showing morphogenetic stages on the dorsal surface after protargol impregnation. **A, C** – within row dorsal primordia formation for proter and opisthe with posterior thickening to form caudal cirri (arrows); **B, D** – unequal split of the third dorsal primordia (arrows); caudal cirri formed in 2 + 2 + 3 pattern (double arrows) at the ends of $DK_{1, 2 \& 4}$ for proter and opisthe. Scale bar: 20 μm.

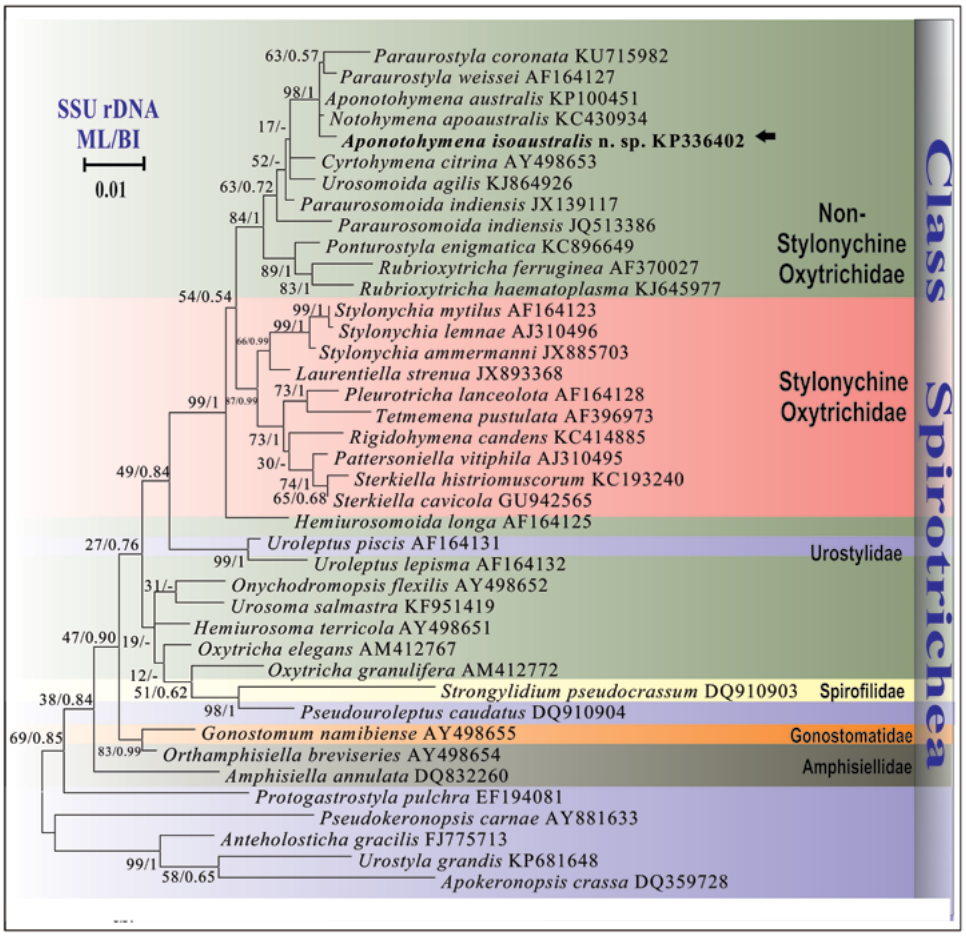


Fig. 6. Maximum likelihood (ML) phylogenetic tree based on SSU rDNA sequences showing the position of *Aponotohymena isoaustralis* n. sp. using GTR + I + G as nucleotide substitution model. The new sequence from the present study is indicated by bold font (arrow). Numbers at nodes are bootstrap values from ML and the posterior probabilities from BI. Accession numbers are provided after species names. Clades representing different orders of the subclass stichotrichia are shaded. "–" at the nodes indicate disagreement between the two methods. The scale bar corresponds to 0.01 expected substitutions per site.

and Adam 1983b, Gupta *et al.* 2006). A detailed ontogenic development of IIp primordia is not available for *A. australis* and *N. apoaustralis.* In *N. saprai* IIp originates from cirrus II/2.

In *A. isoaustralis* n. sp. POVC (IV/2, V/4 and V/3), disaggregate independently to form streaks IVo, Vo and VIo, but do not seem to merge with the developing OP. This is in contrast with *N. saprai* where disaggregating POVC merge with the developing OP and may contribute to primordia formation of both proter and opisthe (Table 4).

(6) SSU rDNA: The molecular traits though cannot be discussed for the two genera (*Aponotohymena* and *Notohymena*) in detail as SSU rDNA or any other marker genes have not been sequenced so far in any of the reported species of the genus *Notohymena.* If the molecular marker genes of all species belonging to the genus *Notohymena* are characterized and described, a more robust and precise phylogeny of these genera can be obtained in future.

Phylogenetic analyses

On the basis of molecular analysis *A. isoaustralis* n. sp. is clustered with *N. apoaustralis* and *A. australis* within a clade which is also composed of *Paraurostyla weissei* and *P. coronata* (Fig. 6). The morphological and morphogenetic features that may explain close relationship of this clade is the presence of cortical granules, *de novo* origin of OP and supernumerary caudal cirri. Analysis of UM also leads to an assumption that curved UM may be autapomorphic trait for *Notohymena* and *Aponotohymena* which may have evolved from UM of

Table 2. Morphometric comparison of three different species of *Aponotohymena, A. australis, A. isoaustralis* n. sp. and *N. apoaustralis*.

Character*	*A. australis* (Voss 2008)	*A.australis* (Hu and Kusuoka 2015)	*N. apoaustralis* (Lv *et al.* 2013)	*A. isoaustralis* n. sp. (present study)
Body size	167 × 42	142 × 63	161 × 74	132 × 35
Body length/body width	4.3:1	2.2:1	2.2:1	4:1
Cortical granules, size	–	0.8	1.0	0.8
Cortical granules, position	–	Grouped around cirri and dorsal cilia or aligned between cirral rows or dorsal kineties	On ventral side, cortical granules either densely arranged in short rows near marginal cirri forming belts along the cirral rows or grouped in irregular short rows. On dorsal side, cortical granules 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.
Macronuclear nodules, size	23 × 12	21 × 14	17 × 12	20 × 14
Micronuclear nodule, number	3–8	3–5	1	0
Caudal cirri, number	5–10 (variable)	7–10 (variable)	8–10 (variable)	7 (constant)
Adoral membranelles, number	38–45 (42)	35–42 (38)	34–42 (39)	32–40 (36)
Frontal cirri, length	–	–	15	5–6
Ventral cirri, length	–		12	3–4
Transverse cirri, length		–	20	7
Dorsal cilia, length	–	–	5	0.7–2.2
Caudal cirri, length	–	–	12	6
Marginal cirri	–	Non-confluent	Confluent	Non-confluent
Contractile vacuole, size	–	12	15	15

*All measurements are in μm.

Table 3. Morphometric comparison of *Aponotohymena isoaustralis* n. sp. with other reported species of the genus *Aponotohymena* and *Notohymena.*

Character*	*Aponotohymena isoaustralis* n. sp. (Present study)	*A. australis* (Voss 2008)	*A. australis* (Hu and Kusuoka 2015)	*A. apoaustralis* (Lv *et al.* 2013)	*N. antartica* (Foissner 1996, Berger 1999)	*N. selvatica* (Hemberger 1985, Berger 1999)	*N. pampasica* (Küppers *et al.* 2007)	*N. saprai* (Kamra and Kumar 2008)	*N. rubescens* (Blatterer and Foissner 1988, Berger 1999)	*N. limus* (Naqvi *et al.* 2016)	*N. quadrinucleata* (Foissner 2016)
Body length	132.3	169.2	142	161.0	85.5	170–190 in life	83.8	149.2	84.1	61.5	78.8
Body width	34.8	38.8	63.3	74.7	30.4	60–70 in life	35.3	48.8	33.7	21.9	33.1
Body Length: Width	4:1	4.4:1	2.2:1	2.2:1	3:1	3:1	2.4:1	3:1	2.5:1	3:1	2.4:1
Adoral membranelle, number	36.4	42.1	38.3	38.8	30.2	–	–	52.7	26.6	25.8	27.9
Adoral zone of membranelle, length	44.6	49.6	54.4	57.9	32.4	–	32.3	–	29.2	23.6	28.4
AZM/body length %	33.7	30	37	–	37	27	–	38.5	–	40	36.4
Macronuclei number	2	2	2	2	2	2	2	4	2	4	4
Anterior macronucleus length	20.8	23.3	21.6	18.5	12.9	–	13.3	13.8	11.8	7.7	9.8
Anterior macronucleus width	14.3	11.8	13.9	12.9	7.1	–	9.7	9.1	7.3	6.5	7.1
Posterior macronucleus length	19.4	–	–	16.9	–	–	–	–	–	–	–
Posterior macronucleus width	13.6	–	–	12.4	–	–	–	–	–	–	–
Distance between macronuclei	22.2	20.4	–	–	–	–	–	–	–	–	–
Micronucleus number	0	5.6	–	1	2 (rarely 3)	2–3	2.1	3.7	2.3	2	2
Micronucleus length	–	2.5 (dia)	–	–	3–4	–	3.2	2 (dia)	3	2.7 (dia)	2
Micronuclei width	–	Not mentioned	–	–	2.3–3	–	2.8	–	3	–	1.6
Buccal cirri, number	1	1	1	1	1	1	1	1	1	1	1
Frontal cirri, number	3	3	3	3	3	3	3	3	3	3	3
Frontoventral cirri, number	4	4	4	4	4	4	4	4	4	4	4
Postoral ventral cirri, number	3	3	3	3	5	5	3.1	3	3	3	3
Pretransverse ventral cirri, number	2	2	2	2			2	2	2	2	2
Transverse cirri, number	5	5	5	5	5	4	5	5	5	5	5
Right marginal cirri, row	1	1	1	1	1	1	1	1	1	1	1
Right marginal cirri, number	32.8	39.8	34.4	38.6	16.8		17.4	43.1	18.3	15.6	15.7
Left marginal cirri, row	1	1	1	1	1	1	1	1	1	1	1
Left marginal cirri, number	34.4	40.1	33.4	36.4	17.9		17.1	43.9	17	14.5	15.1

Dorsal rows, number	6	6	6	6	6	–	6	6	6	6	6
DK_1	20.4	–	–	22.6	–	–	–	30.9	–	15.9	15.7
DK_2	20.3	–	–	–	–	–	–	31.1	–	15.1	17.8
DK_3	16.9	–	–	–	–	–	–	21	–	13.5	13.1
DK_4	8	–	–	–	–	–	–	12.7	–	14.6	11.5
DM_1	8	–	–	–	–	–	–	23.1	–	4	9.3
DM_2	6	–	–	–	–	–	–	11.5	–	1.5	5.2
Caudal cirri number.	7	8–10	7–10	9.3	3	3	3	3	3	3	3
CC, number in kinety 1	2	2.2	2.4	–	–	–	–	1	–	–	–
CC, number in kinety 2	2	2.5	2.2	–	–	–	–	1	–	–	–
CC, number in kinety 4	3	3.6	3.4	–	–	–	–	1	–	–	–
Cortical granules, colour	Yellowish Green	Yellowish Green	Yellowish Green	Yellowish Green	Yellowish	–	Colourless	Dark Green	Ruby	Colour less	Yellowish to citrine granules

*All measurements are in µm.

Table 4. Morphogenetic comparison of *Aponotohymena isoaustralis* n. sp with other reported species of *Aponotohymena* and *Notohymena.*

Character*	*Aponotohymena isoaustralis* n. sp. (present study)	*A. australis* (Voss 2008)	*A. apoaustralis (*Lv *et al.* 2013)	*N. saprai* (Kamra and Kumar 2008)	*N. rubescens* (Blatterer and Foissner 1988, Voss 1991, Berger 1999)
OP origin	*De novo*	*De novo*	*De novo*	*De novo* close to POVC (IV/2, V/4 and V/3), which are incorporated in the OP	Near transverse cirri
FVT primordia					
Proter					
I	Parental UM	Parental UM	Parental UM	Parental UM	Parental UM
II	II/2 +OP	II/2	II/2	II/2	II/2
III	III/2	III/2	III/2	III/2	III/2
IV	IV/3	IV/3	IV/3	IV/3	IV/3
V	Splitting of primary primordia	Splitting of primary primordia	*De novo*	OP	*De novo*
VI	Splitting of primary primordia	Splitting of primary primordia	*De novo*	OP	*De novo*
Opisthe					
I	OP	OP	OP	OP	OP
II	OP	OP	OP	OP	–
III	OP	OP	OP	OP	–
IV	IV/2	IV/2	IV/2	OP (POVC incorporated)	–
V	V/4	V/4	V/4	OP (POVC incorporated)	–
VI	V/3	V/3	V/3	OP (POVC incorporated)	–

Morphogenetic description of *N. antarctica, N. selvatica* and *N. pampasica* is not available.
OP – oral primordia; POVC – postoral ventral cirri; UM – undulating membrane.

Paraurostyla pattern where endoral and paroral membranes are intersecting in the middle and rejoining at the distal side without forming hook at the distal end. Therefore, it is possible that the above-mentioned characters may be significant in the phylogeny and systematics of the family Oxytrichidae.

Remarks

A comparison of different species belonging to these two genera (*Notohymena* and *Aponotohymena*) suggests that *N. apoaustralis* should be included in the genus *Aponotohymena* and redesignated as *A. apoaustralis* nov. comb.

Taxonomic summary

Order Sporadotrichida Fauré-Fremiet, 1961
Family Oxytrichidae Ehrenberg, 1830

Diagnosis of *Aponotohymena isoaustralis* n. sp.: A freshwater flexible oxytrichid measuring about 148 × 46 μm *in vivo*, elongated body, yellowish green cortical granules about 0.8 μm in diameter aligned along the margins and also irregularly distributed throughout the cell. They may also be randomly concentrated as clusters along the left margin and posterior end of the cell, adoral zone of membranelles about 34% of body length and is composed of 32–40 membranelles.18 FVT cirri with 3 frontal cirri, 4 frontoventral cirri, 1 buccal cirrus, 3 postoral ventral cirri, 2 pretransverse ventral cirri and 5 transverse cirri; one left and one right row of marginal cirri non confluent posteriorly; 4 dorsal kineties and 2 dorsomarginals; seven caudal cirri (constant) in 2 + 2 + 3 pattern. 2 macronuclei and no micronucleus (amicronucleate). *De novo* oral primordium formation.

Type location: Freshwater samples from Sanjay lake, which is an artificial lake in Trilokpuri (28°36′51″N, 77°18′14″E), East Delhi, India.

Type material: Protargol impregnated slide with holotype specimen (Fig. 1C, D) encircled in black ink is deposited in the Zoological Survey of India, Kolkata, India with accession number Pt. 3220.

Etymology: The species name *isoaustralis* refers to its similarity to *A. australis*.

Gene sequence: The SSU rDNA sequence of *A. isoaustralis* n. sp. is deposited in GenBank with accession number KP336402.

Occurrence and ecology: Till date *Aponotohymena isoaustralis* n. sp. was recorded only at the type location. Although, three other freshwater lakes were sampled around Delhi, but the novel species has been exclusively found from samples of Sanjay Lake.

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