New records, rare and noteworthy species of the genus *Nowakowskiella* (Nowakowskiellaceae, Chytridiomycota) from India

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Abstracts

Six species of the genus *Nowakowskiella* (Nowakowskiellaceae, Chytridiomycota), specifically *Nowakowskiella elegans*, *N. hemisphaerospora*, *N. profusa*, *N. multispora*, *N. ramosa* and *N. macrospora* as well as one variety *N. multispora* var. *longa* are described herein from India. *N. hemisphaerospora* and *N. macrospora* are reported as new records for India. This increases the number of *Nowakowskiella* species known from India to seven. *Nowakowskiella multispora* var. *longa* is rarely reported in the world and in the past has been recorded only from Poland and India. All these species along with a variety are illustrated with the help of light micrographs and compared with other similar species. Morphological descriptions, illustrations, distribution and comments of these examined species are presented. Besides, we analysed nu-rRNA gene sequences (partial LSU) of six *Nowakowskiella* isolates from Indian aquatic and soil samples. A synopsis of the characters to all these investigated species based on morphological characters are provided in this study to differentiate them.

Key words: Chytridiomycota, LSU, molecular phylogeny, systematics, taxonomy, zoosporic-true fungi

Introduction

Chytridiomycota is a phylum of the kingdom Fungi presently comprised of twelve orders: Cladochytriales Mozley-Standridge, Chytridiales Cohn, Gromochytriales Karpov and Aleoshin, Lobulomycetales Simmons, Mesochytriales Karpov and Aleoshin, Polychytriales Longcore and Simmons, Rhizophlyctidales Letcher, Rhizophydiales Letcher, Spizellomycetales Barr, Synchytriales Doweld, Zygorhizidiales Seto and Zygodiplonchidiales Seto (Powell, 2017a; Seto et
Recently, based on molecular monophyly and zoospore ultrastructural analyses, one monophyletic clade in the diversified order Chytridiales was phylogenetically analysed and taxonomic revision resulted in the elevation of a new order, Cladochytriales, represented by four families: Cladochytriaceae Schröt., Endochytriaceae Sparrow ex Barr, Nowakowskiellaceae Sparrow ex Mozley-Standridge and Septochytriaceae Mozley-Standridge (Mozley-Standridge et al., 2009). The chytrids comprising this clade possess diverse range of thallus development (epibiotic or endobiotic, eucarpic, monocentric or polycentric), sporangium structure (inoperculate or operculate) and rhizoidal organization (non-apophysate or apophysate, catenulate rhizoids, tapering, or isodiametric axis). Further, many of its representatives with the families have often been observed growing primarily as saprotrophs on decomposed plant tissue such as vegetable debris and other cellulosic materials in aquatic habitats and terrestrial moist soils. Over the period of time, many of its members have been commonly isolated and reported from grass leaves, cellophane, hemp, the epidermis of Allium cepa, corn (Zea mays) straw/leaves, fibrin film, filter paper and lens paper (Sparrow, 1960; Mozley, 2005; Marano and Steciow, 2006; Marano et al., 2007; Mozley-Standridge et al., 2009).

The family Nowakowskiellaceae at present contains only a single genus Nowakowskiella Schröt. represented by N. elegans (Nowak.) Schröt. as the type species3,8. The presence of an epibiotic or endobiotic, eucarpic, polycentric thallus consisting of allegedly non-septate rhizoidal swellings along the rhizomycelium of varying widths, exo- or endo-operculation of reproductive bodies and conspicuous resting spores are the main general characteristics of Nowakowskiella4,9. To date based on Mycobank10 and Index Fungorum11 database, under this generic concept, around 22 taxa have been reported; nevertheless, many names are believed to be synonyms. To date, this large genus in terms of the number of species comprises 15 valid species and one variety11,12 across the globe; they are mostly saprophytic in nature and commonly found primarily on cellulosic substrates (especially cellophane bait) in both aquatic and land-based environments with two exceptions: N. keratinophila and N. pitcairnensis that were found to prefer insect wings and hemp seeds as bait, respectively4,5,13. The extensive scrutiny of the literature of literature revealed that out of 15 valid species, the molecular data is available for only 5 species, particularly, N. elegans which has molecular sequence data from collections around the world (Jerônimo et al. 2019). In India, the genus is widely distributed and represented by 6 species14. Historically, this species-rich genus is considered to be cosmopolitan in distribution and has been investigated more intensively
in temperate areas (Palearctic and Nearctic regions or Holarctic) than in tropical areas\textsuperscript{5,7}. However, although \textit{Nowakowskiiella} been \textit{ubiquitous genus in the order}, only a small fraction of these species have been isolated, cultured, and included in molecular phylogenies, while most were described based on gross culture characteristics, like microscopic morphology, keeping their interspecific relationships, and indeed the monophyly of the generic concept, uncertain. In general, morphological and ultrastructural features examination combined with molecular phylogeny is the most preferable taxonomic approach for determining a meaningful and comprehensive classification of the \textit{Nowakowskiiella} species. As far as their taxonomic and phylogenetic diversity is concerned, \textit{Nowakowskiiella} spp. have received much attention and were continuously revised in the last decade\textsuperscript{3,15}. The most recent taxonomic work involved a phylogenetic framework for the genus and detailed morphological descriptions of species shown by DNA sequence data analysis, include the work of Jerônimo et al.\textsuperscript{16} for Brazilian \textit{Nowakowskiiella} species. This study was based on morphological and phylogenetic approaches and discovered \textit{N. crenulata} as a new or potential undescribed species alongside establishing the new genus, \textit{Karlingiella} for a single species \textit{N. elongata}.

During a continuing survey of zoosporic fungi in India, some species of the genus \textit{Nowakowskiiella} associated mainly with cellulosic substrates were discovered. Based on morphological features, and phylogenetic inferences from nuLSU sequence data, some of these species were determined to represent an undescribed species from India. Thus, this study aims to enhance the knowledge of the genus \textit{Nowakowskiiella}, describing and illustrating \textbf{two new records} for India along with their distributional range and habitat associations. Descriptions are accompanied by illustrations of morphological characters and a discussion of related taxa is presented. Further, in this study, we for the first time analysed a DNA sequence-based phylogeny of Indian \textit{Nowakowskiiella} spp. based on an nuLSU sequence dataset to establish the species boundaries and relationship within the family. Also, newly obtained DNA sequence data are reported in this paper.

\textbf{Material and Methods}

\textit{Sample collection, morphological examination and photomicrography}

Collections were made in Chandra Prabha Wildlife Sanctuary (24°55’59.9” N; 83°10’47.6” E), Chandauli district, Uttar Pradesh, India from 2014 to 2015. The water and soil samples were collected at random in sterile polythene bags and collection details were noted. The samples were
brought to the laboratory for further studies. Baiting technique was employed for the processing of samples within 24 h of collection by following the protocol outlined by Sparrow\textsuperscript{3}. Cellulosic materials in the form of lens papers, filter papers, leaves and corn straw were used as baits. Overall, the samples were placed with 20 ml of deionized (DI) water in sterilized Petri plates. Each recipient Petri plates were kept at room temperature (25°C) in the dark for two weeks. In this entire period, the baits were routinely examined under the light microscope and when optimal growth was observed on the baits, small bits of colonized baits were introduced in a test tube containing 8 mL of DI water. The test tube was kept undisturbed for a period of 30–40 min to initiate the discharge of zoospores. After this incubation period, a small drop of DI water was picked from the interface of water and tube to harvest the zoospores before streaking on Peptone–Yeast–Glucose (PYG) agar plates supplemented with streptomycin sulphate and ampicillin (40 mg/1000 mL DI water each). The plates were incubated for growth at 25°C for a week. Subsequently, the colonies appeared after a week were further purified by dissecting out a block of agar (5 mm in diameter) using a core borer from the advancing or growing edge of the old colony aseptically and repeated reculturing on new PYG culture plates. The pure colonies were photographed and characters were noted. The pure cultures were routinely sub-cultured after every 1 month and preserved for long term storage on culture tube slants sealed with parafilm in a refrigerator at 4–8°C.

Identification using morphological features
The evaluation of microscopical features for identification of each isolate was performed based on its morphological traits using vegetative (shape and size of the hyphae) and asexual reproductive characteristics such as shape, size and development of sporangium and their zoospores, patterns of discharge/liberation and germination on baits under a light microscope. Sections were mounted in DI, in which also all measurements were taken and the permanent mounts were prepared by fixing with formalin-acetic-alcohol and mounting in lactophenol\textsuperscript{17,18}. The morphological features were studied under a Dewinter microscope and measurements were recorded and photographed. Standard monographs, descriptions and keys containing original descriptions of taxa were used as reference material for morphological identification\textsuperscript{3,7,9,13,14}. Finally, the specimens were allotted accession numbers, preserved and deposited at the culture collection of the Banaras Hindu University, Centre of Advanced Study in Botany, Laboratory of Mycopathology and Microbial Technology, Varanasi, India and/or in its Herbarium.

DNA extraction, PCR amplification, sequencing and phylogenetic analyses
Genomic DNA of the isolates was extracted from mycelium grown in 100 mL PYG broth incubated under stationary conditions at 25°C temperature for 14 days following the cetyl trimethyl ammonium bromide (CTAB) method\textsuperscript{19} with slight modifications. PCR was carried out to amplify the LSU (Large Subunit) regions of the nuclear rDNA, using primer pair LR0R\textsuperscript{20} with LR5\textsuperscript{21}. Each PCR reaction condition, as well as the procedure for the purification of amplified PCR products, cycle sequencing reaction and their purification were performed according to the conditions described previously by Dubey \textit{et al}..\textsuperscript{22} All newly generated sequences in this study were assembled, edited, and finally the obtained sequences data were deposited with the NCBI GenBank database.

The generated sequences were compared to data in GenBank with BlastN. The BLAST searches were run excluding uncultured/environmental sample sequences in the database. LSU data sets were generated based on the blast output and available literature. The relationship of the isolates was established by constructing a phylogenetic tree along with other nucleotide sequences of LSU of representative fungi that were acquired from the NCBI GenBank database. ClustalW was used to carry out multiple nucleotide sequence alignments. Moreover, the Neighbour-joining (NJ) method with the Kimura 2-parameter (K2P) distance model and 1000 bootstrap replicates was used to perform phylogenetic analysis. The percent nucleotide identity values were calculated as pairwise p-distances. Using a complete deletion option all the positions containing gaps and missing data were eliminated. The topology of the tree was confirmed by the Hasegawa-Kishino-Yano model and the Maximum Likelihood (ML) method. The initial tree for the heuristic search was built by applying NJ and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. To model evolutionary rate differences among sites, a discrete Gamma distribution was used. The rate variation model allowed for some sites to be evolutionarily invariable. The evolutionary analysis was carried out using MEGA X software\textsuperscript{23}.

\textbf{Results}

1. \textit{Nowakowskia elegans} (Nowak.) Schroeter, Engler and Prantl, \textit{Naturlichen Pflanzenfam.} 1(1): 82. 1892/1893. Figure 1 A–I

\textit{Morphology}: Thallus eucarpic, polycentric, extra-intramatrical, epibiotic and endobiotic. Zoosporangia operculate, usually exo-operculate, occasionally endo-operculate, generally apical
position or subapical, 7–10 μm in diameter; hyaline, smooth, thin-walled, usually terminal, occasionally intercalary, frequently apophysate or non-apophysate, pear-shaped, extremely variable, spherical or subspherical, 15–50 μm in diameter, pyriform, ovoid, ampulliform, obclavate, rarely cylindrical or elongated or irregular; often clustered, occasionally in a linear fashion or scattered; sometimes with a short, broad tubular outlet/exit tube or a papilla for discharge, usually one, rarely two, with the apex often closed by a hyaline plug of mucous material; internal proliferation absent. Rhizomycelium extensive, hyaline, coarse or delicate, long or short, dendriform, profusely or sparingly branched, sparse or dense, usually with ellipsoidal, fusiform, or subglobose, non-septate, intercalary swellings, extremely variable in diameter, usually 15–30 μm in diameter, occasionally with septate intercalary swellings. Zoospores, minute, spherical or oval, 3.5–7 μm in diameter, with a single large refractive lipid droplet, containing a single posteriorly directed flagellum; Zoospores either already cleaved prior or cleaved after release, emerge embedded in a gelatinous matrix from exit pore or orifice, remain motionless for a short period after discharge, before becoming motile and drifting apart. Resting spore hyaline, rarely formed, terminal or intercalary, subspherical, ovoid, ellipsoidal, or fusiform, rarely irregular, 10–25 μm in diameter, smooth and thick-walled, without a space between the wall and cytoplasmic content, formed by enlargement, or maturation of intercalary swellings in the rhizomycelium, with a large bright central lipid globule; germination not observed.

Intercalary smooth-walled resting spore.


*Distribution:* Poland, Argentina, Brazil, USA, UK, Germany, Australia, Equatorial Africa, New Zealand, India, Canada, Egypt, Japan, Thailand, Mexico, Romania, South Africa, Argentina, China, and Iceland.

*Comments:* The examined specimen showed features that agree with the description of Sparrow and Karling. *N. elegans* is considered synonymous with *Cladochytrium elegans* Nowakowski, *N.
endogena Constantineanu, N. delica Whiffen, and N. crassa Karling. Similar to the previous reports our specimen never forms pseudo-parenchymatous tissue and rarely, if ever, produces resting bodies. It is ubiquitous in distribution at all the sampling sites, being saprophytic on decaying leaves, plant debris, vegetable debris and floating organic matter, obtained from samples of water, agricultural land, and forest. It can be easily cultivated on fragments of cellulosic material and isolated into an axenic culture.

2. *Nowakowskiella hemisphaerospora* Shanor, Am. J. Bot. 29: 174. 1942. Figure 2 A–F

*Morphology:* Main thallus polycentric, eucarpic, variable development and mostly intramatrixal. Zoosporangia operculate, hyaline, smooth, thin-walled, usually terminal, in short lateral branches or infrequently intercalary, variable in shape and size, ovoid, ellipsoid or pyriform, generally 15–26 μm in diameter, often non-apophysate, with a cross wall delimiting the rest of the rhizomycelium from zoosporangia, and occasionally with or without one to several discharge papilla. Rhizoidal system extensive, delicate, much-branched, hyaline, with individual strands or filaments of variable diameter; non-septate intercalary swellings, numerous or very much scattered. Zoospore discharge similar-like *N. elegans*. Zoospores minute, spherical, 4.5–6.5 μm in diameter, with a single hyaline lipid droplet and single posteriorly inserted long flagellum. Resting bodies abundant, usually terminal or occasionally intercalary, develop from either intercalary or terminal swellings of the rhizomycelium, smooth, often elliptical, 12–28 μm in diameter, containing one-four hyaline thickened wall resting spores or resting bodies, accompanied by some hemispherical cells without content or empty cells (accompanying cells), usually somewhat 8–14 μm in diameter, at maturity with large lipid droplet and several smaller ones commonly surrounding it; germination not seen.

*Material examined:* India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, recovered on cellophane, grass leaves, corn seedling leaves, filter paper, and lens paper bait from water and soil samples. May 2014, saprobic on cellulosic materials, accession numbers BHU–BOT–172 and BHU–BOT–176 with GenBank accession numbers – MH685382 and MH685386, respectively.

*Distribution:* Japan, Mexico, USA, UK, Cuba, New Zealand, Cook Islands, Brazil, Poland, Argentina and Singapore.
Comments: The presence of abundant thick-walled two-celled hemispherical resting spores and the manner of their formation are a striking feature of this species. This is the first citation of *N. hemisphaerospora* from India.

3. *Nowakowskiiella profusa* Karling, Bull. Torrey Bot. Club 68: 386. 1941. Figure 3 A

*Morphology*: Main thallus polycentric, eucarpic, mostly intramatrical. Zoosporangia operculate, usually exo-operculate, smooth, thin-walled, terminal, or mostly intercalary, obpyriform, 20–38 μm in diameter, generally ellipsoidal or ovoid, globose or subglobose, hyaline, predominantly non-apophysate; with a prominent one or rarely two long discharge tubes, the orifice of each papilla enclosed by a hyaline mucilaginous plug, usually laterally but often apically placed. Rhizoidal system extensive, moderately branched, stout, hyaline, non-septate, hypha-like rhizomycelium with ellipsoidal, fusiform, or subglobose, irregular expansion or swellings, 2.5–7.5 μm in diameter, which may give rise either zoosporangia or resting spores, few slender lateral rhizoids at irregular intervals. Zoospores microscopic, spherical to oval, 5–5.5 μm in diameter, contains a unique hyaline lipid globule, usually smaller than that in *N. elegans*, with single posteriorly inserted long flagellum; discharge similar-like other species of *Nowakowskiiella*. Resting spores abundant, generally intercalary, occasionally terminal, spherical or subspherical, infrequently ellipsoidal or ovoid, elongated, with truncated edges, smooth, double-walled, 15–25 μm in diameter, filled with granular content, golden-yellow, arranged in chain occasionally; germination not seen.


*Distribution*: Poland, Germany, Argentina, Egypt, Brazil, USA, UK, Australia, New Zealand, India, South Africa, France Canada and Iceland.

Comments: *N. profusa* differs principally from the other known species of this genus in several aspects by having comparatively smaller zoospores, more minute refractive lipid globules, the rare occurrence or even lack of well-defined spindle organs, and the presence of golden-yellow resting spores.

4. *Nowakowskiiella ramosa* Butler, Mem. Dep. Agr. India, Bot. Ser. 1: 141. 1907. Figure 3 B
**Morphology:** Thallus eucarpic, polycentric and extra-intramartical. Zoosporangia hyaline, generally apically operculate, terminal, or intercalary, apophysate or non-apophysate, smooth, thin-walled, spherical, or pyriform, 15–60 µm long × 12–35 µm wide, cylindrical, with swollen apex, curved or coiled, or slightly irregularly oval, elongated flask-shaped, usually with inflated or swollen apical ends and occasionally with a long spout. Rhizoidal system hyaline, extensive, coarse, profuse, richly branched; rhizomycelium filaments 1–6 µm in diameter, occasionally anastomosing, broad, septate occasionally forming a seudoparenchyma. Zoospore minute, globose, 5–8.5 µm in diameter, with a large hyaline noticeable refractive lipid droplet and single posteriorly inserted long flagellum; discharge similar-like other species of *Nowakowskiella*. Resting spores usually intercalary, hyaline, smooth wall or verrucose, formed by mycelial expansions (intercalary enlargements) into the fairly thickened wall structure, spherical or slightly angular, 15–28 µm in diameter; germination not observed.

**Material examined:** India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, saprophytic on cellophane, corn leaves, onion skin and lens paper from the soil. November 2014, accession number BHU–BOT–319.

**Distribution:** India, Egypt, Brazil, USA, South Africa, Hungary and Argentina.

**Comments:** The most recognizable characteristics of this species are the presence of rhizomycelium seudoparenchyma and the resting spores having verrucose wall.

5. *Nowakowskiella multispora* Karling, Sydowia 17: 314-316. 1964. Figure 4 A–D

**Morphology:** Thallus eucarpic, polycentric and extra-intramartical. Rhizomycelium profuse, extensive, richly branched, tenuous filaments, 2–5 µm in diameter; numerous and frequent non-septate, intercalary swellings/ enlargements in tandem, narrowly ovoid, or fusiform, spindle-shaped, 10–15 × 15–30 µm in diameter, or elongate, 6–12 × 15–25 µm in diameter. Zoosporangia hyaline, smooth, operculate, either exo-operculate or endooperculated, non-apophysate, usually terminal, occasionally intercalary, predominantly fusiform, 10–35 µm in diameter, fusiform, 25–45 µm in diameter, narrowly oval or spherical, 18–25 µm in diameter, or elliptical, 10–28 µm in diameter, frequently elongate, or almost cylindrical; with the long discharge/exit tubes. Zoospores, minute, 3–4 µm in diameter, with a small lipid droplet; discharge similar-like other species of *Nowakowskiella*. Resting spores abundant, usually intercalary, hyaline, smooth, formed by direct
transformation of intercalary swellings into the fairly thickened wall structure, broadly to narrowly oval, 10–18 μm in diameter, some spherical, 12–15 μm in diameter with truncate ends, containing numerous lipid globules; germination not observed.

**Material examined:** India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, samples of water with leaf litter using cellophane and lens paper. May 2014, saprobic on cellulosic materials, accession number BHU–BOT–173 with GenBank accession number – MH685383.

**Distribution:** India, Egypt, Argentina, and Brazil

**Comments:** The sporangia of this species vary markedly in size and shape, and the majority of them develop long necks on cellophane. It is characterized primarily by a minute zoospores and unusually abundant production of resting spores. The structure and appearance of the tenuous portions of the rhizomycelium, intercalary enlargements, and sporangia are specific characteristics for *N. multispora*.


**Morphology:** Thallus eucarpic, polycentric and extra-intramatrical. Zoosporangia hyaline, predominantly exo-operculate, terminal, or intercalary, occasionally internally proliferating, rarely apophysate, smooth, thin-walled, spherical, ovoid, pyriform, oblong or elongate, 10–36 μm in diameter; often with 1–6 long, less often branched, discharge tubes per zoosporangium. Rhizoidal system extensively developed; very branched, filaments 2–6 μm in diameter, bearing numerous non-septate, intercalary swellings, narrowly ovoid, or fusiform, 8–25 μm in diameter, with fine rhizoids. Zoospores minute, oval, 6–7 μm in diameter, with a single lipid droplet and a posteriorly directed long flagellum; discharge similar-like most species of *Nowakowskiella*. Resting spores not observed.

**Material examined:** India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, on insect wings, lens paper and cellophane from pond water and soil samples. November 2014, saprobic on keratinous and cellulosic materials, accession number BHU–BOT–202.

**Distribution:** India and New Zealand
Comments: Kiran’s variety differs as it produces exoooperculate zoosporangia with the extremely long, and branched discharge tubes\(^2\). As per literature consulted, this is the second report of this variety from India and probably third from the world.

6. *Nowakowskia macrospora* Karling, Am. J. Bot. 32: 29. 1945. Figure 6 A–D

Morphology: Thallus eucarpic, polycentric and extra-intramatrical. Zoosporangia hyaline, predominantly exo-operculate, terminal and intercalary, occasionally internally proliferating, usually apophysate, smooth, thin-walled, often slightly flattened and elongated transversely, spherical, 12–38 µm in diameter, or oval, 15–30 µm in diameter, pyriform, 10–40 µm in diameter, or elongate, 15–55 µm in diameter, often with an elongate neck, 10–40 µm long × 5–8 µm wide; apophysis oval or nearly spherical, 9–18 µm in diameter, oblong, clavate or elongate. Rhizoidal system extensively developed, richly-branched, fairly coarse, filaments 2–6 µm in diameter, bearing numerous non-septate intercalary swellings, narrowly oval, 8–15 µm in diameter, broadly spindle-shaped, elongate and fusiform, or slightly irregular with fine rhizoids. Zoospores relatively large, oval, 10–12 µm in diameter, with a single large lipid globule, up to 3–5 µm in diameter and multiple minute lipid globules lying near the posterior end, and a single posteriorly inserted long flagellum; discharge similar-like most species of *Nowakowskia*. Resting spores not observed.


Distribution: Brazil, Poland and New Zealand

Comments: This species differs markedly in the large size of its zoospores which is the primary distinguishing characteristic, and because the zoospores surpass in size those of all other species of this genus. This is the first report of *N. macrospora* from India.

Phylogenetic analyses

A phylogenetic tree for selected *Nowakowskia* isolates was constructed along with different representative molds of Cladochytriales to determine the exact species placement of our isolates. LSU data of other representative molds was obtained from the NCBI GenBank database. Figure 7 shows the optimal tree in which 39 nucleotide sequences were involved. The evolutionary
distances were calculated by using the K2P method and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All the ambiguous positions containing gaps and missing data were removed using a complete deletion option. In all, a total of 234 positions were there in the final dataset. MEGA X was used for evolutionary analyses.

Discussion

This article contributes to the knowledge of Indian zoosporic-true fungi with nationally or regionally new species, and records of rare species. *N. hemisphaerospora* and *N. macrospora* are newly reported from India whereas other reported species, specifically, *N. multispora*, *N. multispora* var. *longa*, *N. ramosa* and *N. macrospora* are globally very rare in distribution. In the present study, the addition of these newly reported species for India has raised the tally of *Nowakowskiiella* species known from India to seven. The present study provides morphological descriptions of seven *Nowakowskiiella* species and their comparison with morphologically similar taxa. All these reported species are new to Chandra Prabha Wildlife Sanctuary. Moreover, notes on substrata and habitats of each record are given, and the ecology and distribution of some species are discussed. Although some *Nowakowskiiella* spp. have been collected from terrestrial habitats, most have been isolated from streams, rivers, or ponds. The identity of some of the species was further supported by molecular analysis of the large subunit (LSU) of the ribosomal RNA gene through phylogenetic DNA sequence analyses. In phylogenetic analysis, a distinction between strains of same species of *Nowakowskiiella* was noted which may be due to the insufficient length of nucleotide sequences and also all the ambiguous positions containing gaps and missing data were removed using complete deletion option. However, phylogenetic analyses inferred from LSU sequence data support the molecular lineages for taxa of *Nowakowskiiella*, corresponding to their morphological features.

In the past decades, some taxonomic studies with *Nowakowskiiella* distributional records have been reported from India. However, the fungal species of this genus have not yet been comprehensively studied for this country. At present, five species of *Nowakowskiiella*, namely *N. elegans* (Nowak.) Schroeter, *N. ramosa* Butler, *N. multispora* Karling, *N. profusa* Karling and one variety *N. multispora* var. *longa* Kiran have been reported from India. The localities of most of these species lie in South India except, *N. multispora*, *N. granulata* and *N. profusa*, *N. elegans,
and *N. multispora var. longa* which were reported from North India\(^4\). Despite their ubiquity, the diversity, distribution, and vital roles in the ecosystem, however, since the work of Kiran\(^2\), no further species have documented from India in this genus. Moreover, to date the identification of all the reported *Nowakowskia* spp. in India was based on morphological data in gross culture (i.e. natural samples and baits), which may cause false interpretations due to morphological plasticity, leading to conceptual misunderstandings as neither of the *Nowakowskia* representatives from India has ever been subjected for phylogenetic analyses. Further, the lack of appropriate microbiological methods typically in obtaining axenic cultures and a relatively small number of researchers working in this field are likely to be considered as other important contributing factors to this problem. Thus, the status of the taxonomy of *Nowakowskia* spp. in India remains uncertain. The application of molecular approaches such as extracting, cloning and amplifying DNA from environmental samples currently allows us to explore biodiversity without the need for culturing. In this respect, the scenario has changed in the recent years, during which many traditional zoosporic fungi have been subjected to morpho-molecular analysis to explore zoosporic fungal diversity of India\(^22,31\). In this regard, the intensity of research on the distribution of *Nowakowskia* increased noticeably in other parts of the world, using new methods and technologies. As a result, many novel species can be described. Still, the geographic distribution range of *Nowakowskia* remains of course insufficiently documented.

Since studies on chytrid fungi started in the first quarter of the 20th century, almost 352 species have been reported from India\(^32\). However, none of their sequences have been uploaded to GenBank, suggesting that chytrids are not yet well studied phylogenetically. Interestingly, 348 of them were documented from freshwater and terrestrial sources whereas only 4 species were recorded from the marine environment. Dayal\(^14\) compiled the records and provided an extensive account of comprehensive records available on chytrids. Considering the 706 described species globally\(^33\), it can easily be said that there is still much to be done. There are sporadic reports on chytrid fungi from Rajasthan, Orissa, Jammu and Kashmir, West Bengal, Jharkhand, Chhattisgarh, North-East states, Gujarat, Andaman and Nicobar Islands, Lakshadweep Islands, etc. The presence of unidentified chytrids and unexplored sites demonstrates the need for further sampling in India to broaden our understanding of chytrid distribution and diversity. This paper provides sequences of *Nowakowskia* spp., which should prove beneficial to the systematic molecular taxonomy of the chytrids.
Conclusion

The overall conclusion of this work is the report of six species of the genus *Nowakowskiella* from a zone underexplored for its mycobiota, the Chandra Prabha Wildlife Sanctuary (Chandauli district), North Central India. A contribution was made to Indian zoosporic-true fungi by the addition of two new records. The amount of interesting species discovered in our survey suggests an extraordinary diversity of *Nowakowskiella* in India, provides new data on their occurrence and indicates the need for further mycological investigations in unexplored areas of the country. The data presented in this study is an important step towards providing some nomenclatural and taxonomic notes on *Nowakowskiella*, apart from generating a checklist of zoosporic-true fungi in this region in particular and India in general for which literature still showed evidence of a general unawareness. Ours is the first study that describes *Nowakowskiella* isolates from India at the species level using morphological and molecular data. All the described species are well delimited by characters with clear diagnostic features that allow separation among species. Species distribution studies, including an exhaustive identification of *Nowakowskiella* isolates, are needed to better understand which factors influence diversity and distribution and to build stronger hypotheses about its biogeography. Further research on this matter is needed to develop a robust phylogenetic framework for *Nowakowskiella*. Overall, under the current scenario, there is a pressing need to launch a full-scale survey towards Indian zoosporic fungi as still several species of this group from India are not determined.

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Conflict of interest

The authors declare that they have no competing interests.

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Monoblepharomycota, Mortierellomycota, Mucoromycota, Neocallimastigomycota, Olpidiomyctota, Rozellomycota and Zoopagomycota), Fungal Divers., 2018, 92, 43–129.
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Figures and Legends

**Figure 1.** *Nowakowskiella elegans* A-I. Eucarpic, polycentric, and epibiotic thallus of *Nowakowskiella elegans*; apical exit tube or a papilla indicated by an arrow. Bar = 20 µm.

**Figure 2.** *Nowakowskiella hemisphaerospora* A-D. Polycentric thallus of *Nowakowskiella hemisphaerospora* E-F. Resting spores. Bar = 50 µm.

**Figure 3A.** Zoosporangia of *Nowakowskiella profusa*, arrow indicate discharged sporangia and released zoospores. **B.** Resting spore of *Nowakowskiella ramosa*. Bar = 50 µm.

**Figure 4.** *Nowakowskiella multispora* A-B. Sporangia of *Nowakowskiella multispora* with delicate rhizomycelium C. Resting spores. **D.** Relatively smaller zoospore with a single lipid globule. Bar = 50 µm.

**Figure 5.** *Nowakowskiella multispora* var. *longa* A-F. Sporangia with delicate rhizomycelium and long, branched exit tubes. Bar = 50 µm.

**Figure 6.** *Nowakowskiella macrospora* A-D. Sporangia of *Nowakowskiella macrospora* with exit tube of varying length; arrow indicates relatively large zoospore with a single large lipid globule. Bars = 50 µm.

**Figure 7.** Phylogenetic relationship of few representative *Nowakowskiella* isolates along with members of Cladochytriales based on LSU sequence data. The evolutionary history was originated by using the neighbour-joining method. The lengths of the horizontal lines are proportional to the number of nucleotide differences per site. Scale bar indicates number of nucleotide substitutions per site. All the evolutionary analysis was performed using MEGA X.