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

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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 the country. This increases the number of *Nowakowskiella* species known from India to seven. *N. multispora* var. *longa* is a rare species that has previously only been found in Poland and India. All these species along, with the variety, are illustrated with the help of light photomicrographs and compared with other similar species. Morphological descriptions, illustrations, distribution and comments of the species examined are presented. Besides, we analysed nu-rRNA gene sequences (partial large subunit) of six *Nowakowskiella* isolates from Indian aquatic and soil samples. In addition, a summary of unique traits based on the morphological features of all these species are provided in this study to differentiate them.

Keywords: Gene sequences, molecular phylogeny, *Nowakowskiella* species, systematics, taxonomy.

CHYTRIDIOMYCOTA is a phylum of the kingdom Fungi presently comprised of 12 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 Zygophlyctidales Seto[1,2]. 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[3]. The chytrids comprising this clade possess a 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 a 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[3–7].

The family Nowakowskiellaceae at present has only a single genus *Nowakowskiella* Schröt. represented by *N. elegans* (Nowak.) Schröt. as the type species[3,8]. 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 *Nowakowskiella*[4,9]. To date, based on Mycobank[10] and Index Fungorums[11] databases, under this generic concept, around 22 taxa have been reported; nevertheless, many names are considered to be synonyms. At present, this large genus, in terms of the number of species, comprises 15 valid species and 1 variety across the globe[11,12]; 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, viz. *N. keratinophila* and *N. pitcairnensis* were found to prefer insect wings and hemp seeds as bait respectively[4,5,13–16]. Extensive scrutiny of the literature revealed that among the 15 valid species, molecular data are available for only 5, particularly *N. elegans*, which has molecular sequence data from collections around the world. In India, the genus is widely distributed and represented by six species[17]. Historically, this species-rich genus is considered to be cosmopolitan in distribution and has been studied more intensively in temperate areas (Palearctic and Nearctic

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regions or Holarctic) than in tropical areas[5,7]. However, although *Nowakowskiella* is a 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, examining morphological and ultrastructural features combined with molecular phylogeny is the preferable taxonomic approach for determining a meaningful and comprehensive classification of the *Nowakowskiella* species. With respect to their taxonomic and phylogenetic diversity, *Nowakowskiella* species have received much attention and were continuously revised in the last decade[3,15]. The most recent taxonomic work involved a phylogenetic framework for the genus. Detailed morphological descriptions of the species shown by DNA sequence data analysis include the work of Jerônimo *et al.*[17] for Brazilian *Nowakowskiella* species. This study was based on morphological and phylogenetic approaches and discovered *N. crenulata* as a new or potentially undescribed species alongside establishing the new genus, *Karlingiella* for a single species *N. elongata*.

During a continuing survey of zoosporic fungi in India, some species of the genus *Nowakowskiella* associated mainly with cellulosic substrates were discovered. Based on morphological features and phylogenetic inferences from nu-large subunit (LSU) sequence data, some of these were determined to represent an undescribed species from India. Thus, this study aims to enhance knowledge of the genus *Nowakowskiella*, describing and illustrating two new records for India along with their distributional range and habitat associations. Illustrations of morphological characters accompany descriptions and a discussion of related taxa is presented. Further, we have analysed a DNA sequence-based phylogeny of Indian *Nowakowskiella* species based on a nu-LSU sequence dataset to establish the species boundaries and relationships within the family. Also, newly obtained DNA sequence data are reported here.

Material and methods

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 randomly in sterile polythene bags and collection details were noted. The samples were brought to the laboratory for further studies. The baiting technique was employed to process samples within 24 h of collection by following the protocol outlined by Sparrow[4]. Cellulosic materials such as lens paper, filter paper, 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 plate was kept at room temperature (25°C) in the dark for two weeks. During the entire period, the baits were routinely examined under a light microscope and when optimal growth was observed on them, small bits of colonized baits were introduced in a test tube containing 8 ml of DI water. The test tube was kept undisturbed for 30–40 min to initiate the discharge of zoospores. After this incubation period, a small drop of DI water was collected 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 that appeared after a week were further purified by dissecting 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 subcultured after every 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 done 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 the light microscope. Sections were mounted in DI water, in which also all measurements were taken and the permanent mounts were prepared by fixing with formalin–acetic–alcohol and mounting in lactophenol[18,19]. 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[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 and incubated under stationary conditions at 25°C for 14 days following the cetyl trimethyl ammonium bromide (CTAB) method[20], with slight modifications. PCR was carried out to amplify the LSU

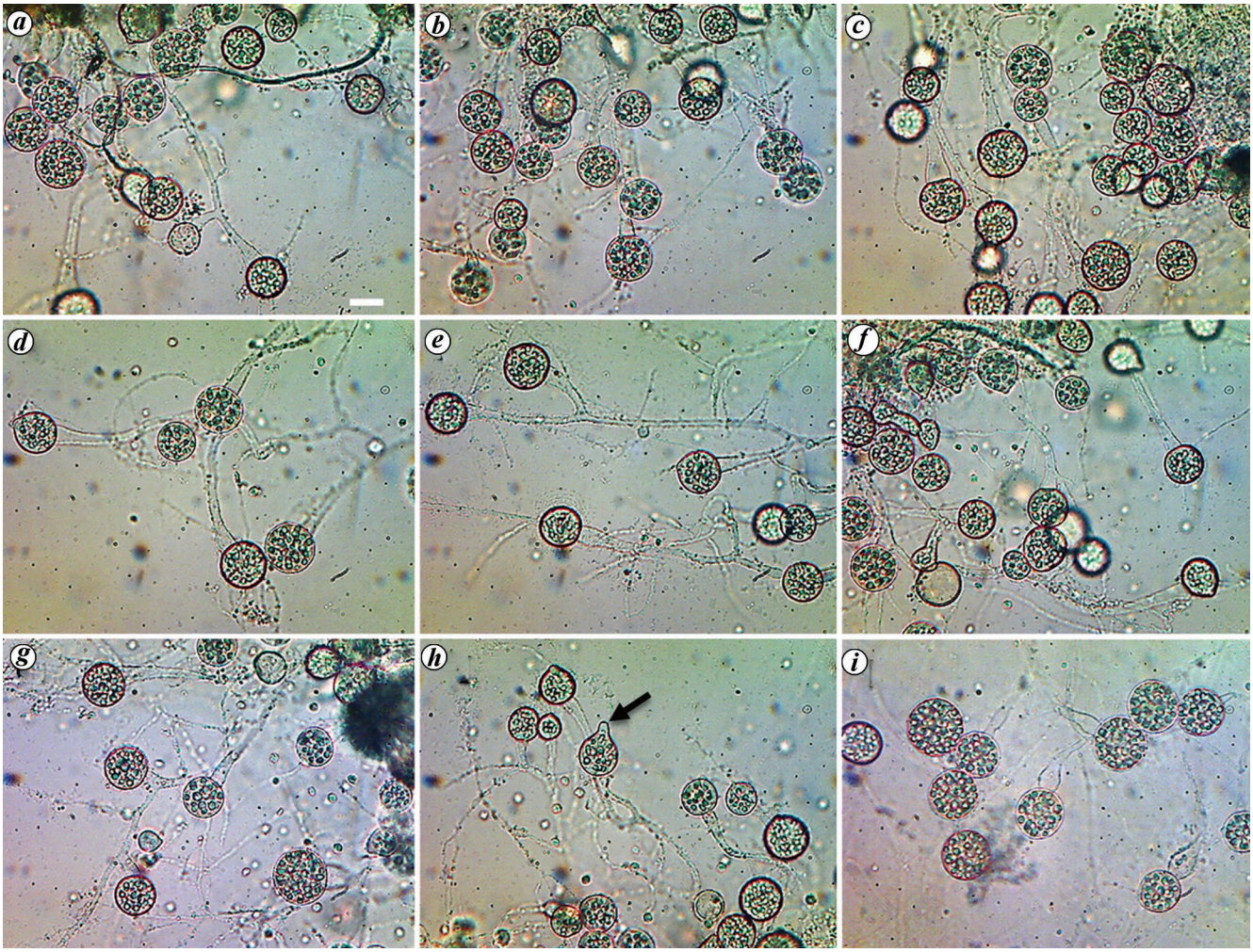

Figure 1 ***a–i.*** Eucarpic, polycentric and epibiotic thallus of *Nowakowskiella elegans*; apical exit tube or papilla indicated by arrow. Bar = 20 µm.

regions of the nuclear rDNA using primer pair LR0R[21] with LR5 (ref. 22). Each PCR reaction condition, as well as the procedure for purification of the amplified PCR products, cycle sequencing reaction and their purification were performed according to the conditions described previously by Dubey *et al.*[23]. All newly generated sequences in this study were assembled, edited and finally the obtained sequence 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 datasets 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 the representative fungi acquired from NCBI GenBank. 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 were used to perform phylogenetic analysis. The per cent 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 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 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[24].

Results

(1) *Nowakowskiella elegans* (Nowak.) Schroeter, Engler and Prantl, *Naturlichen Pflanzenfam*. 1(1): 82. 1892/1893 (Figure 1 *a–i*).

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 the exit pore or orifice and 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.

Material examined: India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, on water and soil samples rich in plant debris baited with lens paper, grass leaves, cellophane, corn straw, corn leaves and onion skin. June 2015, saprobic on cellulosic materials, accession numbers BHU–BOT–177, BHU–BOT–217, BHU–BOT–248, BHU–BOT–271 and BHU–BOT–272 with GenBank accession numbers MH685389, MH685388, MH685390, MH685910 and MH685387 respectively.

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[4] and Karling[9]. *N. elegans* is 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[4]. 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 intramatrical. 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 is similar to *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 striking features of this species. This is the first citation of *N. hemisphaerospora* from India.

(3) *Nowakowskiella 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 one prominent or rarely two long discharge

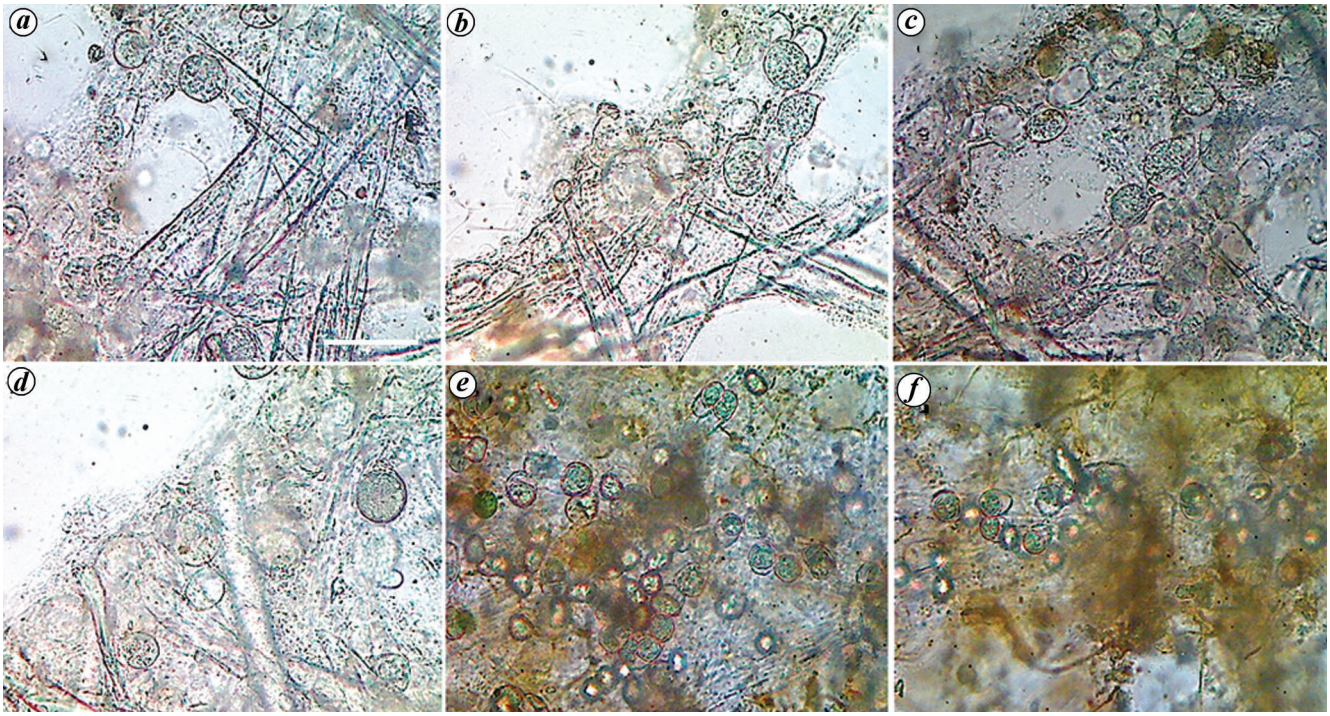

Figure 2. ***a–d***, Polycentric thallus of *Nowakowskiella hemisphaerospora*. ***e–f***, Resting spores. Bar = 50 µm.

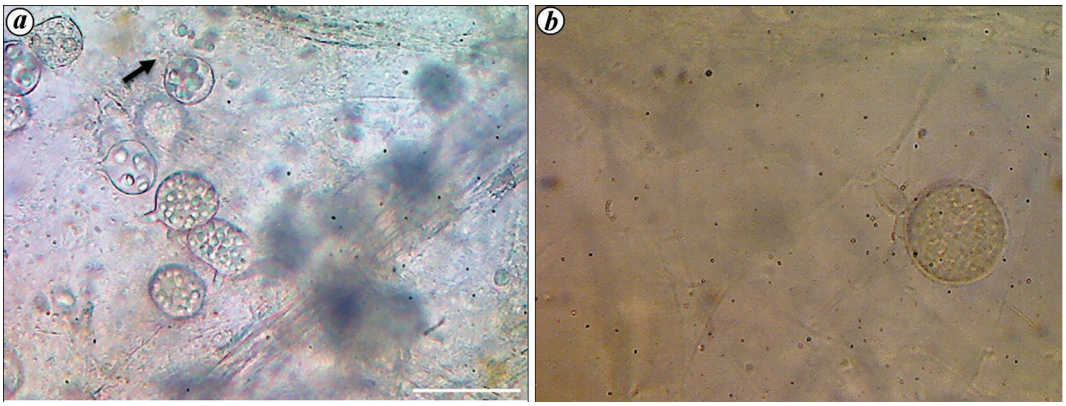

Figure 3. ***a***, Zoosporangia of *Nowakowskiella profuse*; arrow indicates discharged sporangia and released zoospores. ***b***, Resting spore of *Nowakowskiella ramosa*. Bar = 50 µm.

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 to either zoosporangia or resting spores, few slender lateral rhizoids at irregular intervals. Zoospores microscopic, spherical to oval, 5–5.5 µm in diameter, contain a unique hyaline lipid globule, usually smaller than that in *N. elegans*, with single posteriorly inserted long flagellum; discharge similar to other species of *Nowakowskiella*. 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.

Material examined: India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, water samples baited with lens paper. May 2014, saprobic on cellulosic materials, accession number BHU–BOT–175.

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

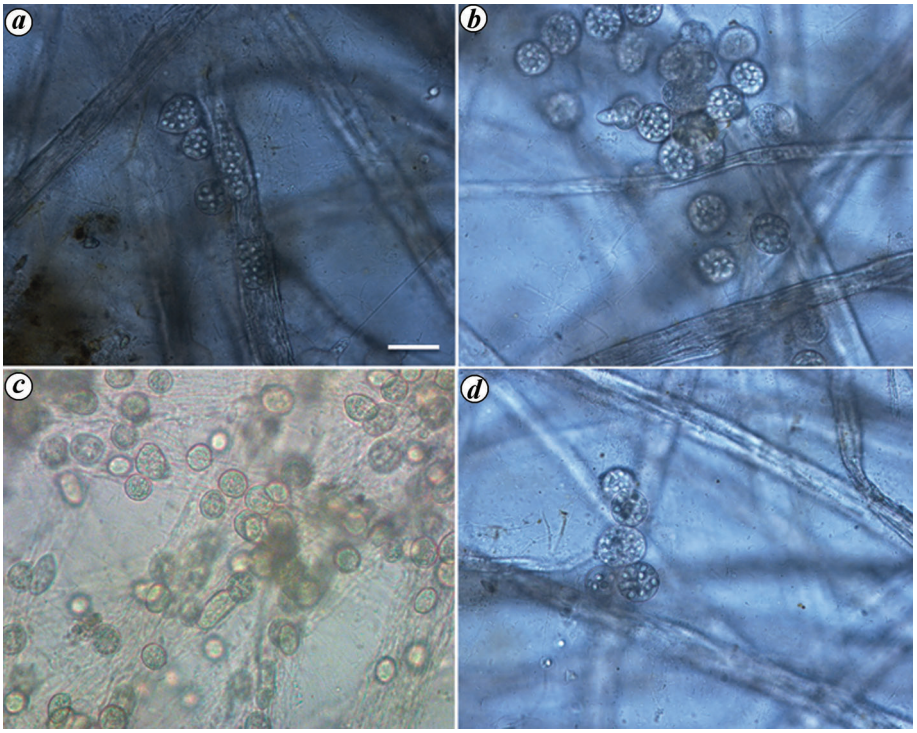

Figure 4. ***a–b***, Sporangia of *Nowakowskiella multispora* with delicate rhizomycelium. ***c***, Resting spores. ***d***, Relatively smaller zoospore with a single lipid globule. Bar = 50 µm.

lipid globules, the rare occurrence or even lack of well-defined spindle organs and the presence of golden-yellow resting spores.

(4) *Nowakowskiella ramosa* Butler, *Mem. Dept. Agric. India, Bot. Ser.*, 1: 141. 1907 (Figure 3 *b*).

Morphology: Thallus eucarpic, polycentric and extra-intramatrical. 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 pseudoparenchyma. 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 to other species of *Nowakowskiella*. Resting spores are usually intercalary, hyaline, smooth-walled 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 pseudoparenchyma 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-intramatrical. 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 endooperculate, 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 to other species of *Nowakowskiella*. Resting spores abundant, usually intercalary, hyaline, smooth, formed

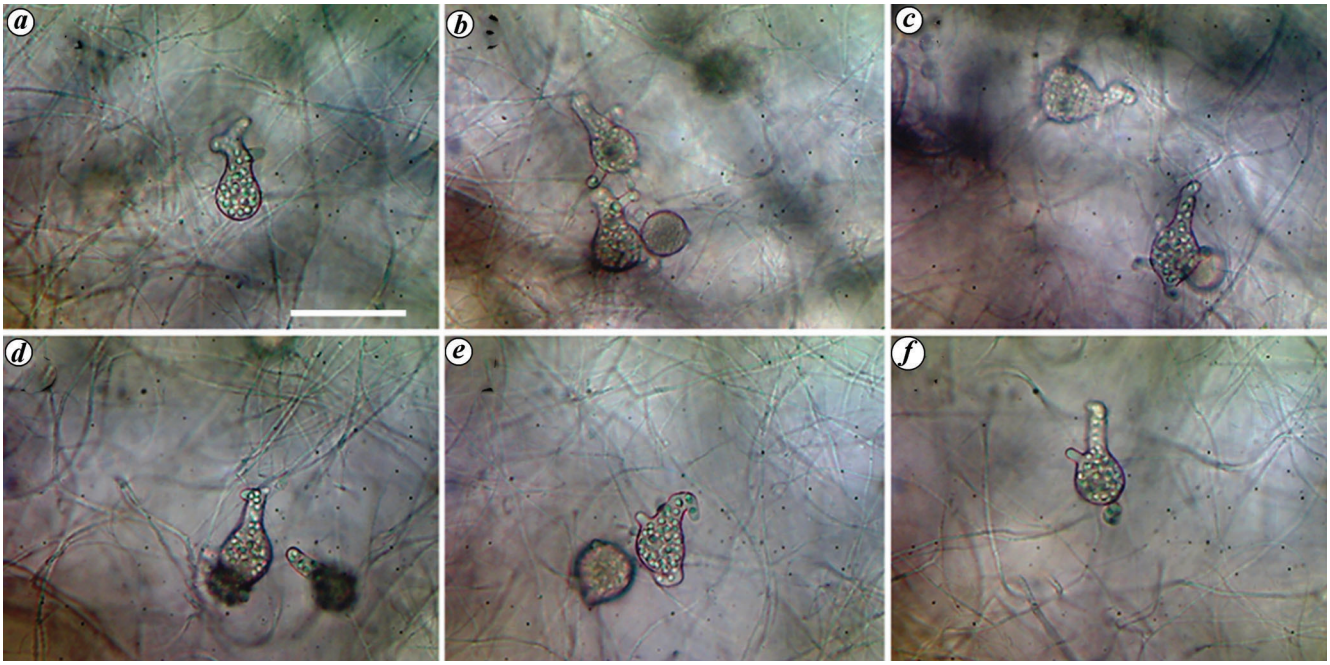

Figure 5 *a–f.* Sporangia of *Nowakowskiella multispora* var. *longa* with delicate rhizomycelium and long, branched exit tubes. Bar = 50 µm.

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 majority of them develop long necks on cellophane. It is characterized primarily by 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 of *N. multispora*.

(6) *Nowakowskiella multispora* var. *longa* Kiran, *Acta Bot. Indica*, 20: 303. 1992 (Figure 5 *a–f*).

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 to 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 exooperculate zoosporangia with extremely long and branched discharge tubes[25]. According to the literature consulted, this is the second report of this variety from India and probably the third from the world.

(7) *Nowakowskiella 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

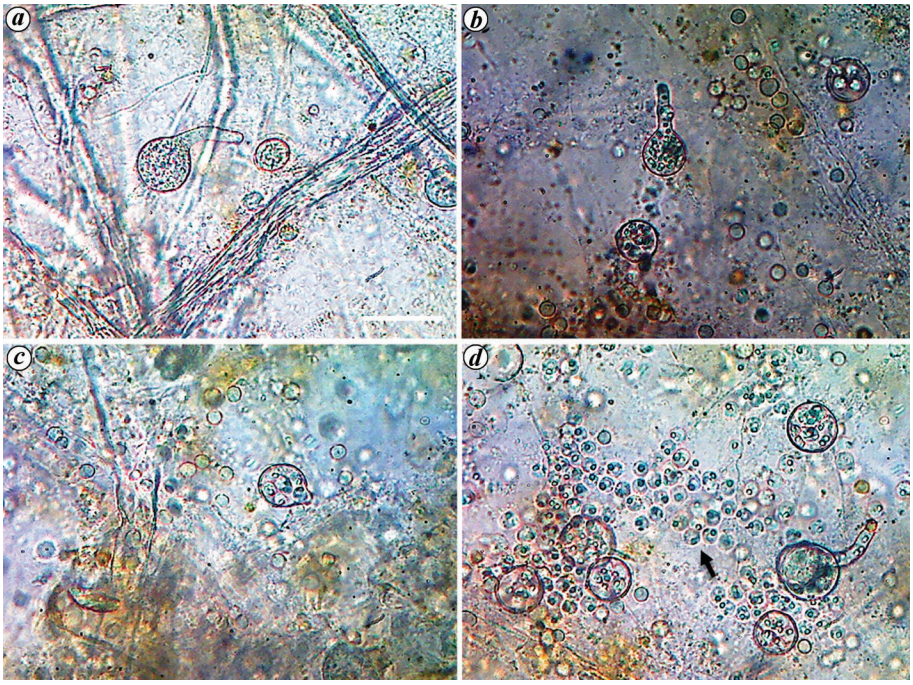

Figure 6 ***a–d.*** Sporangia of *Nowakowskiella macrospora* with exit tubes of varying lengths; arrow indicates relatively large zoospore with a single large lipid globule. Bars = 50 µm.

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 to most species of *Nowakowskiella*. Resting spores not observed.

Material examined: India, Uttar Pradesh, Chandauli district, Chandra Prabha Wildlife Sanctuary, saprophytic on lens paper from moist soil samples. December 2015, accession number BHU–BOT–315.

Distribution: Brazil, Poland and New Zealand.

Comments: The key differentiating feature of this species is the large size of its zoospores, and hence the zoospores surpass those of all other species in this genus. This is the first report of *N. macrospora* from India.

Phylogenetic analyses

A phylogenetic tree for the selected *Nowakowskiella* isolates was constructed along with different representative moulds of Cladochytriales to determine the exact species placement of the isolates. LSU data of other representative moulds were obtained from NCBI GenBank. Figure 7 shows the optimal tree in which 39 nucleotide sequences are involved. The evolutionary distances were calculated using the K2P method and were 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. The final dataset had a total of 234 positions. MEGA X was used for evolutionary analyses[24].

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 increased the tally of *Nowakowskiella* species known from the country to seven. The present study provides morphological descriptions of seven *Nowakowskiella* 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

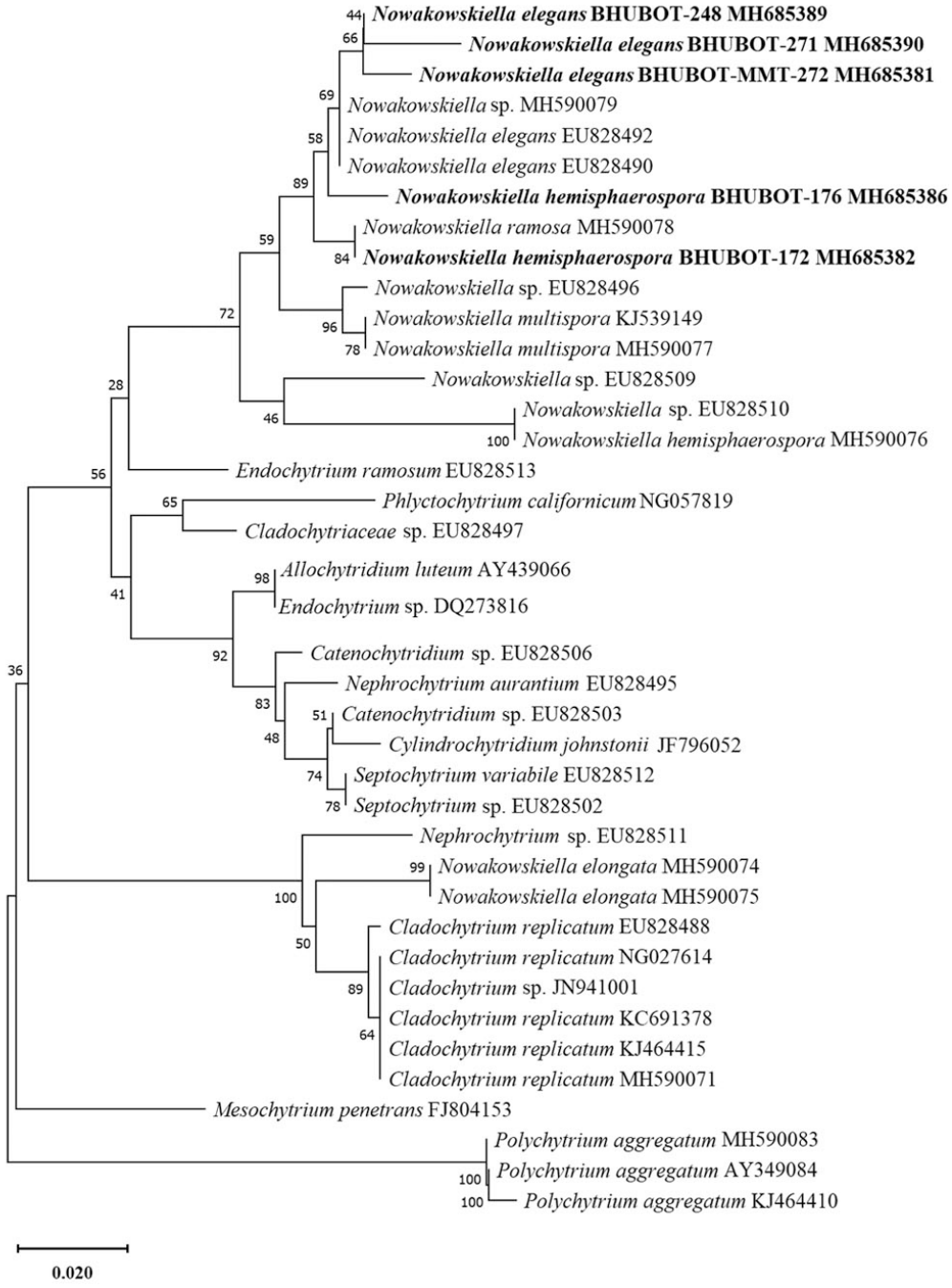

Figure 7. Phylogenetic relationship of few representative *Nowakowskiella* isolates along with members of Cladochytriales based on large subunit sequence data. The evolutionary history was established using the neighbor-joining method. The lengths of the horizontal lines are proportional to the number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

discussed. Although some *Nowakowskiella* species 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 LSU of the ribosomal RNA gene through phylogenetic DNA sequence analyses. In phylogenetic analysis, a distinction between strains of the same species of *Nowakowskiella* 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 the taxa of *Nowakowskiella*, corresponding to their morphological features.

In the past decades, some taxonomic studies with *Nowakowskiella* distributional records have been reported from India[25–31]. However, the fungal species of this genus have not yet been comprehensively studied for the country. At present, five species of *Nowakowskiella* 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[15]. 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[15]. Despite their ubiquity, diversity, distribution and vital roles in the ecosystem, since the work of Kiran[25], no further species have been documented from India in this genus. Moreover, to date, the identification of all the reported *Nowakowskiella* species 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 *Nowakowskiella* representatives from India has ever been subjected to 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 *Nowakowskiella* species in India remains uncertain. The application of molecular approaches such as extracting, cloning and amplifying DNA from environmental samples allows us to explore biodiversity without needing to culturing. In this respect, the scenario has changed in recent years, during which many traditional zoosporic fungi have been subjected to morpho-molecular analysis to explore the zoosporic fungal diversity of India[23,32]. In this regard, research on the distribution of *Nowakowskiella* has 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 *Nowakowskiella* remains insufficiently documented.

Since studies on chytrid fungi started in the first quarter of the 20th century, almost 352 species have been reported from India[33]. However, none of their sequences has been uploaded to GenBank, suggesting that chytrids still need to be well-studied phylogenetically. Interestingly, 348 of them were documented from freshwater and terrestrial sources, whereas only four species were recorded from the marine environment. Dayal[15] compiled the records and provided an extensive account of comprehensive records available on chytrids. Considering the 706 described species globally[34], there is still much to be done. There are sporadic reports on chytrid fungi from Rajasthan, Odisha, Jammu and Kashmir, West Bengal, Jharkhand, Chhattisgarh, North-East India, Gujarat, Andaman and Nicobar Islands, Lakshadweep Islands, etc. Unidentified chytrids and unexplored sites demonstrate the need for further sampling in India to broaden our understanding of chytrid distribution and diversity. This study provides sequences of *Nowakowskiella* species, which should prove beneficial to the systematic molecular taxonomy of the chytrids.

Conclusion

In this study, we describe six species of the genus *Nowakowskiella* from a zone underexplored for its mycobiota, the Chandra Prabha Wildlife Sanctuary, North Central India. A contribution was made to Indian zoosporic-true fungi by the addition of two new records. The 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 studies in unexplored areas of the country. The data presented in this study are 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 the literature still shows evidence of widespread ignorance. Our study 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 the factors that influence diversity and distribution and to build stronger hypotheses about their biogeography. Further research 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 of Indian zoosporic fungi as several species of this group are still not determined from the country.

Conflict of interest: The authors declare that they have no competing interests.

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