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ORIGINAL ARTICLE

On the phylogenetic position of *Nuclearia leuckarti* (Frenzel 1897) Patterson 1984 (Opisthokonta: Holomycota)

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Summary

Nuclearia leuckarti was isolated from a freshwater lake in Leningrad Region (Russia) and studied using light microscopy and SSU rDNA sequencing. The cells were spherical, floating and rarely in the amoeboid attached state. Cell size varied from 4.9 to 14.5 µm. Pseudopodia were straight, sometimes bending or branching, and exceeded the cell diameter 2–3 times. All the observed cells contained a single nucleus with a spherical distinctive nucleolus. Extracellular polymeric substances were not evident. The obtained sequence occupied a derived position inside the genus *Nuclearia* in the cluster, containing *N. thermophila, N. delicatula, N. moebiusi* and *Nuclearia* sp. (strains A5, B3). The problems of the criteria for species distinction in the genus *Nuclearia* were discussed.

Key words: light microscopy, SSU rDNA, Rotosphaerida, nucleariids

Introduction

Nucleariids (formally known as the Rotosphaerida Rainer, 1968) are the clade of amoeboid eukaryotes, representing the deepest branch in Holomycota/Nucletmycea lineage, which also contains Fungi and several parasitic/parasitoid groups (Galindo et al., 2019; Gabaldyn et al., 2022). The pseudopodia of nucleariids are typical filopodia (Zlatogursky, 2021) and the flagella are lost completely (Torruella et al., 2015). The group includes external skeleton-covered forms (*Pompholyxophrys, Lithocolla*), tiny naked amoeboid *Parvularia*, cellular slime mould *Fonticula*, freshwater mucous-coated *Nuclearia* and potentially a broad range of other not yet molecularly characterised genera (reviewed in Gabaldyn et al., 2022). Members of the genus *Nuclearia* Cienkowski, 1865 are ubi-

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quitous freshwater organisms, consuming a broad range of food sources, e. g. bacteria (Pozdnyakov et al., 2023), filamentous cyanobacteria (Dirren et al., 2017) and flour granules (Yoshida et al., 2009). They are important as predators of toxic bloom-forming cyanobacteria (Dirren et al., 2017) and epizoic agents (Dyková et al., 2003). For several species, a close association with both ecto- and endosymbiotic bacteria was shown (Dirren et al., 2014; Dirren and Posch, 2016; Galindo et al., 2019). At the same time, much greater is the importance of this genus for addressing the evolutionary questions due to its unique phylogenetic position, representing one of the most ancient clades in Opisthokonta (Liu et al., 2009). Meanwhile, taxonomy of the genus *Nuclearia*, despite being carefully revised by David Patterson (Patterson, 1984) is still problematic, because most of diagnostic features used for species discrimination are too variable and mostly do not help to recognize entities revealed by molecular phylogeny (Dirren and Posch, 2016). Thus, obtaining molecular data from morphologically studied strains is crucially important. Here we report morphology and SSU rDNA sequence of the strain LU21.6, which we identified as *Nuclearia leuckarti* (Frenzel, 1897) Patterson 1984. To our knowledge, this is the first report of *N. leuckarti* since the initial description and first molecular survey of this species phylogenetic position.

Material and methods

Cultures and samples

The sample was collected from the Poddubskoye Lake, Russia (58°46'41.2"N, 30°10'22.4"E) on 17 August 2021 by gathering bottom sediments from the shore with a 15 ml plastic tube. The clonal culture was obtained by isolation of single cells in PJ medium (Prescott and James, 1955; Page, 1988) with addition of 0.025% cerophyl (Weizengras, Sanatur GmbH, Germany). In detail, culturing conditions were described in the previous paper (Pozdnyakov et al., 2023). Initially, based on the length and identity percent of the resulting alignments, the isolated organism was identified as *Nuclearia thermophila* as reported by Pozdnyakov and co-authors (2023). Now the strain is kept in culture at the Zoological Institute of the Russian Academy of Sciences.

DNA amplification and sequencing

To determine the species identity of the isolated organism, single cell PCR amplification of the SSU rRNA gene fragment was performed using universal eukaryotic primers S12.2 and RibB (Fahrni et al., 2003). The PCR products were Sanger sequenced and a BLAST search in the NT database was performed for the obtained reads using the web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Microscopy and morphometry

Light micrographs were taken using the temporary preparation on a microscope slide with Leica DM2500 (100× planapochromate objective) microscope, equipped with differential interference contrast (DIC) and phase contrast and a Nikon DS-Fi1 camera with accompanying software (Nis-Elements, Nikon Corporation, Japan). The morphometry was performed using the photos taken with Leica DMI3000 inverted microscope (63× objective) equipped with phase contrast optics and with the same camera. All measurements of living cells and scales were made using ImageJ Ver. 1.46r (Abramoff et al., 2004). Scatterplot for Fig. 2 was built using R v. 4.2.0 (R Team, 2021).

Molecular phylogeny

Sequences were aligned using MAFFT v. 7 (Katoh et al., 2018) with manual adjustments using SeaView v. 4.3.5. (Gouy et al., 2010). Maximum likelihood reconstruction was done using RAxML v. 8 (Stamatakis, 2014) with 1,794 unambiguously aligned positions selected by TrimAl v. 1.2.59 (Capella-Gutiérrez et al., 2009), GTR substitution model for 66 taxa (45 nucleariids and 21 fungi as outgroup). The model of evolution was suggested by the program Modeltest (Posada and Crandall, 1998). In the analysis, 4 among-site rate categories were used, assessment of clade stability was performed with a bootstrap using 1,000 resamplings. TrimAL and RAxML were run using CIPRES science gateway (Miller et al., 2010). SSU rDNA sequence of *N. leuckarti* strain LU21.6 was deposited in Gen Bank under accession number OR854351.

A Bayesian phylogenetic tree for the above sequences was performed using MrBayes 3.2.7a (Ronquist et al., 2012) with TIM2+I+G substitution

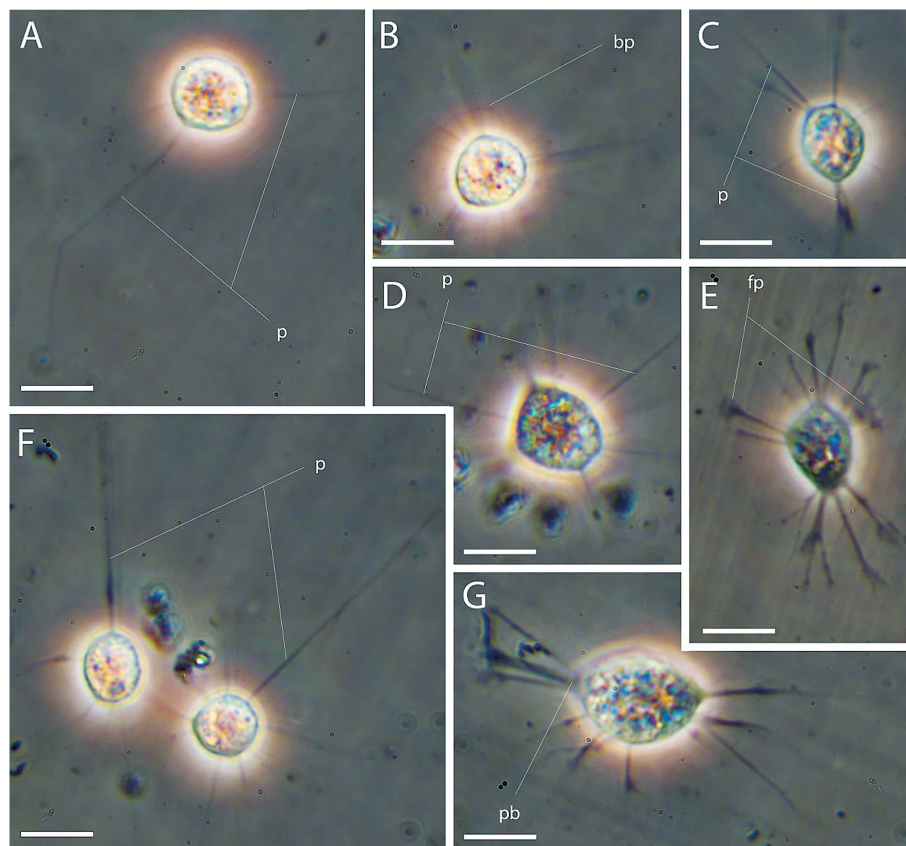

Fig. 1. Light microscopy of *Nuclearia leuckarti* strain LU21.6. Undisturbed cells photographed in the Petri dish; DIC. A, B, D, F – Cells in a spherical form; C, E, G – cells in an amoeboid form. *Abbreviations*: bp – bending pseudopodium, fp – flattened pseudopodia, p – pseudopodia, pb – pseudopodia bundle. Scale bars: 10 μm.

model. The substitution model was proposed using jModelTest 2.1.10 (Darriba et al., 2012). For Mr Bayes two chains, four independent runs, burn in 25% and 2000000 generations were used.

Results

Light microscopy

The cells from the strain LU21.6 were mostly more or less spherical (Fig. 1, A, B, D, F), floating in the culture medium, sometimes demonstrating walking movement and rarely acquiring the amoeboid substratum-associated form (Fig. 1, C, E, G). The observed diameter of the cell body was 4.9–14.5 μm; 8.9±1.87 [n=84] (minimum value-maximum value μm; arithmetic mean ±standard error [n= number of measurements/one measurement per individual]), as measured in October 2023 after 15 months of cultivation. For most of the studied cells, size varied between 6 and 12 μm (Fig. 2). In spherical forms, numerous more or less straight (Fig. 1, A, D, F), but sometimes bending (Fig. 1, B) pseudopodia were radiating in all directions; their length typically exceeded the cell diameter 2-3 times. In the substratum-associated forms, pseudopodia were often forming a bundle on the leading pole of the cell (Fig. 1, G). When inoculated in a fresh plastic Petri dish, pseudopodia of the cells were forming the flattened areas, when contacting with the substratum (Fig. 1, E) pseudopodia sometimes could be branching (Fig. 3, D). All the observed cells contained a single nucleus with a spherical distinctive nucleolus (Fig. 3, A–C). The cytoplasm contained multiple optically translucent vacuoles and numerous granules of unknown nature (Figs 1, 3). The contractile vacuoles were not recognizable. Extracellular polymeric substances (EPS) were not evident, but no specific staining was applied in order to visualise them. At the same time, the typical empty areas, devoid of bacteria, otherwise numerous in the medium were always observed. Cysts were never observed.

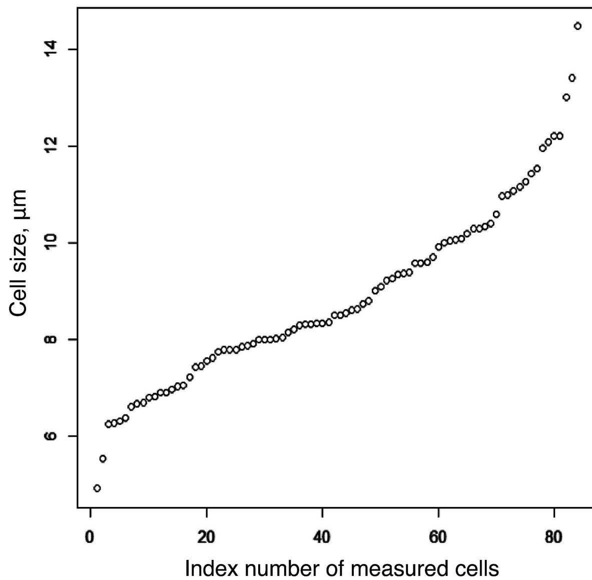

Fig. 2. Scatterplot visualizing the difference in cell size between the cells of LU21.6 strain.

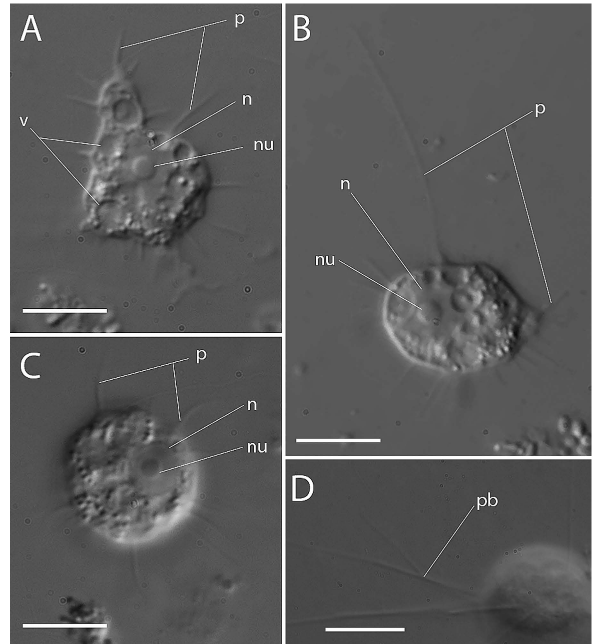

Fig. 3. Light microscopy of *Nuclearia leuckarti* strain LU21.6. Cells photographed on the temporary preparation under a cover slip; DIC. A – Cell in an amoeboid form; B – cell with a long pseudopodium; C – cell in a spherical form; D – cell with branching pseudopodia. *Abbreviations*: n – nucleus; nu – nucleolus; p – pseudopodia; pb – pseudopodia branch; v – vacuoles. Scale bars: 10 µm.

Molecular phylogeny

The obtained SSU rDNA sequence of *N. leuckarti* occupied a derived position inside the genus *Nuclearia* and formed a sister lineage to *N. thermophila* clade on a maximum likelihood tree (Fig. 4) or to *N. moebiusi* clade on a Bayesian tree, based on the same dataset (Fig. 5). Inside the genus, it was a part of the fully supported cluster, containing four other species: (1) *N. thermophila*, (2) *N. delicatula*, (3) *N. moebiusi* and (4) fully supported clade of three sequences (strains B3, A5; clone Alchichica AQ1w5E06), most probably representing an undescribed *Nuclearia* species. The position of *N. leuckarti* inside this cluster was unresolved and both of the contradicting positions in two analyses (sister to *N. thermophila* or sister to *N. moebiusi*) had negligible support. The position of the abovementioned *Nuclearia* sp. clade was also different depending on the analysis method and not supported (Figs 4, 5). The distinction of *N. leuckarti* SSU rDNA sequence from both *N. moebiusi* and *N. thermophila* was also evident from p-distance comparison: it had p-distance of 0.146–0.152 from *N. moebiusi* (with distances between *N. moebiusi* sequences of 0.003–0.111) and p-distance 0.111–0.118 from *N. thermophila* (with distances between *N. thermophila* sequences of 0–0.068).

Discussion

Interpretation of morphological characters and morphology-based species identification in *Nucleria* became challenging in the light of the recent combined morphological and molecular studies (Dirren et al., 2014; Dirren and Posch, 2016). Most of the cells of the LU21.6 strain were in a spherical form, rarely adopting a flattened substratum-associated amoeboid form (Fig. 1). Contrary to the Patterson's *Nuclearia* identification key (Patterson, 1984), which stated that *N. leuckarti* was "not adopting a rounded form when free from compression" (p. 136), original description of this species (Frenzel, 1897) reported mostly spherical form: "Die Grundgestalt ist auch hier mehr oder weniger eine Kugel, die sich nun in verschiedener Weise, hauptsдchlich nach der Formirung der Strahlen umbildet" (p. 60). Thus, this feature is in good accordance with the original diagnosis of

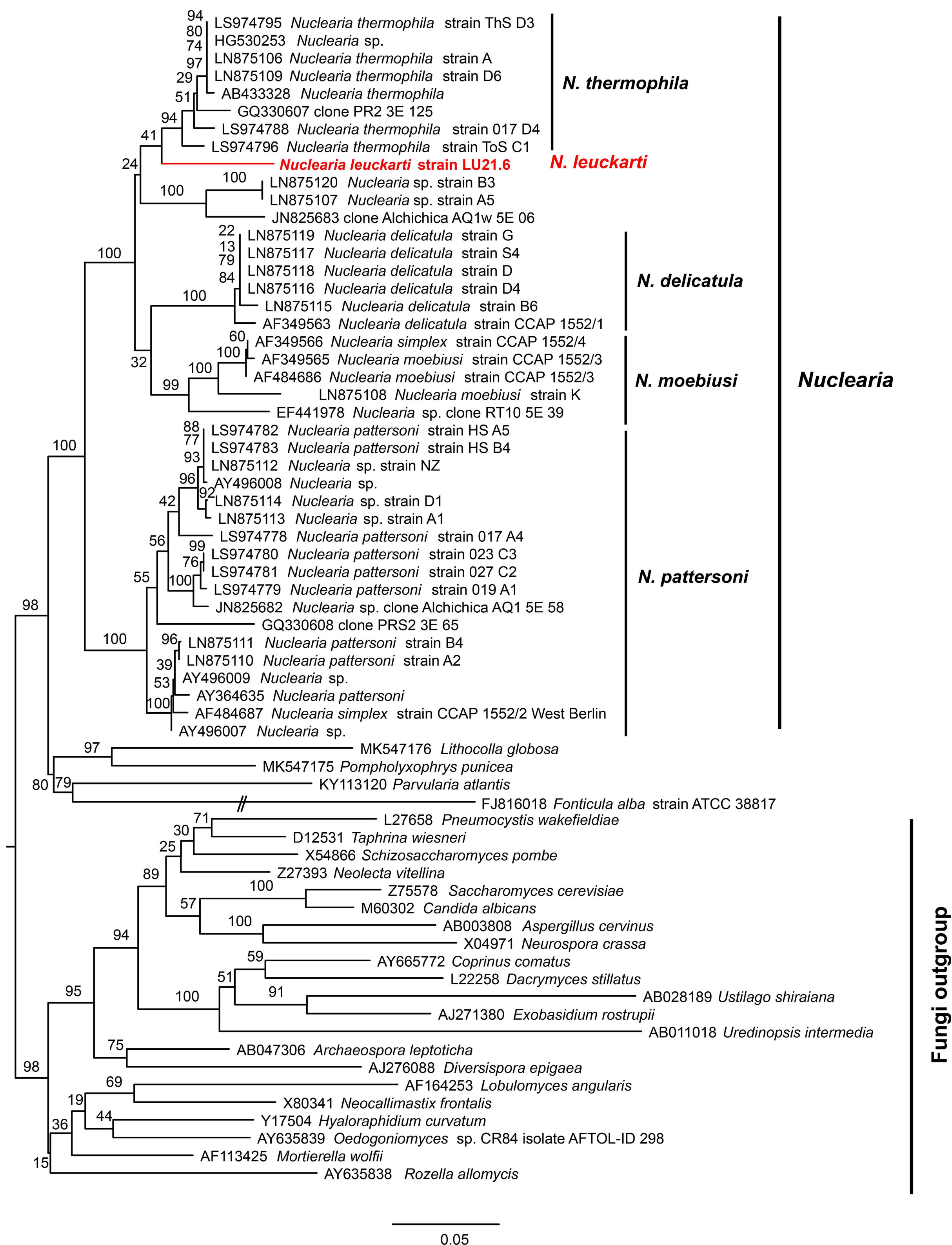

Fig. 4. Maximum likelihood tree for SSU rDNA of 45 nucleariids and outgroup of 21 fungal sequences (1,794 sites; GTRCAT; bootstrap 1,000 iterations; 4 rate classes). The branch of *Fonticula alba* is shortened twice.

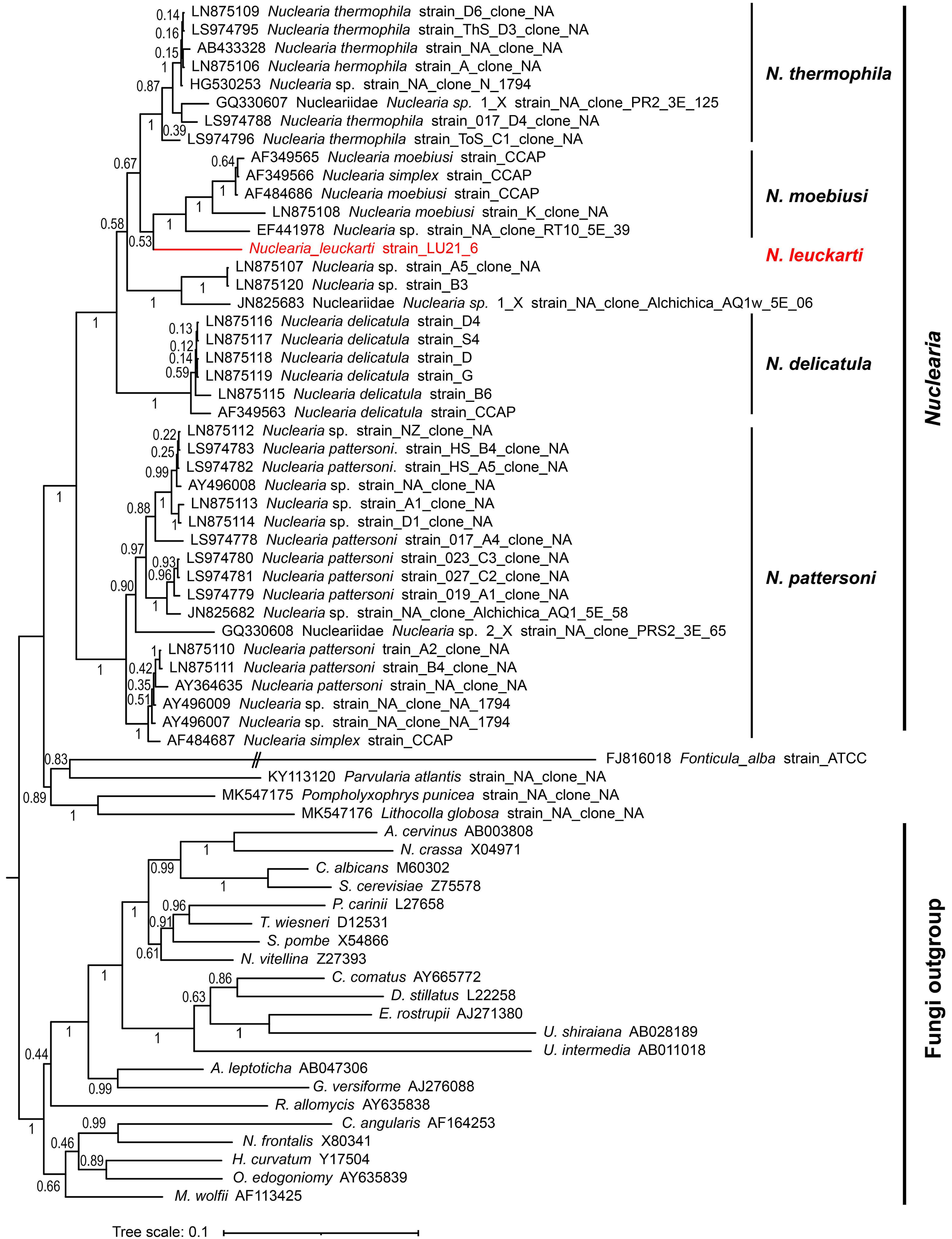

Fig. 5. Maximum likelihood tree for SSU rDNA of 45 nucleariids and outgroup of 21 fungal sequences (1,794 sites; GTRCAT; bootstrap 1,000 iterations; 4 rate classes). The branch of *Fonticula alba* is shortened twice.

N. leuckarti. At the same time, later studies have shown that the spherical form can be adapted by a broad variety of species, including *N. delicatula, N. meobiusi, N. pattersoni, N. thermophila* and *Nuclearia* sp. (strains B3, A5) and therefore has a little diagnostic value (Dirren and Posch, 2016). The size range of cells for LU21.6 strain (6–12 µm, Fig. 2) corresponds to the size reported in the original diagnosis. Frenzel (1897) gives a size range from 13 to 18 µm including pseudopodia (6.5 µm for a single observed extremely small cell). Taking into account the length of the pseudopodia on Frenzel's drawings (Tafel VI; Figs. 4, 8, 18), the cell size must have been in a range of 8–11 µm (or about 2.5 µm for a small cell), which is in a good accordance with our measurements. Among described species, only *N. pattersoni* has a comparable size range: 13.2 (11.0–15.7) µm for amoeboid forms and 8.2–10.8 µm for spherical forms (Dyková et al., 2003). In addition, *Nuclearia* sp. (strains B3, A5) has a size range of 5–15 µm (Dirren and Posch, 2016). Both *N. pattersoni* and *Nuclearia* sp. are distinct from LU21.6 on the SSU rDNA tree (Fig. 4), which rules out conspecificity. At the same time, again reliability of this character is doubtful in the light of the reported shrinkage of *N. thermophila* strain D6: in the course of cultivation, the size decreased from initially 24.6 µm (day 7 after isolation) to 15.7 µm (day 90; 9.4–22.7 µm) (Dirren and Posch, 2016). Our measurements were made after 15 months of cultivation and thus the shrinkage cannot be ruled out. The cells of LU21.6 strain were always uninucleate (Fig. 3, A–C), which definitely contrasts with obligately multinucleate *N. delicatula*. Nevertheless, the recent reports of occasional multinucleated stages in species *N. thermophila*, *Nuclearia* sp. (strains B3, A5), which previously were considered uninucleate (Dirren and Posch, 2016; Galindo et al., 2019), suggests that this character can be more broadly distributed and easily overlooked. The same is true for the presence of EPS. Initially they were serving a diagnostic character (Patterson, 1984; Yoshida et al., 2009), but later were shown to be present in all the studied species and unidentified strains studied by Dirren and Posch (2016). We did not perform a specific staining for the EPS presence, but its existence is strongly suggested by the observation of typical "empty zones" around the cells of LU21.6 strain. Branching of pseudopodia was not originally reported for *N. leuckarti* and clearly evident in LU21.6 (Fig. 3D), but this feature is easily overlooked by researchers. For example, absence of branching was stated for *N. pattersoni* in the original description (Dyková et al., 2003) and later was confirmed for multiple strains of this species (Dirren and Posch, 2016).

Taking into account the considerations mentioned above, the useful set of characters for reliable distinction of *Nuclearia* spp. at the light microscopy level seems to be currently missing. Previously used diagnostic features such as ability to acquire spherical form, presence of EPS and pseudopodia branching are highly likely universal for *Nuclearia*, while the size and the number of nuclei most probably vary depending not only on the species but also on the particular strain or even strain age and condition. The studies of general cell ultrastructure show that it is quite conservative not only between different *Nuclearia* species but also between different genera of nucleariids (Patterson, 1983, 1985; Cann, 1986; López-Escardó et al., 2018) and therefore hardly would be a solution for species discrimination. This leaves SSU rDNA sequence the only reliable criterion for species characterisation and distinguishing from other species. In this situation, it is not clear, what would be the best strategy with the species described only at the level of light microscopy without any molecular data, of which *N. leuckarti* is a prime example. Our solution here is to assign this name to a newly established molecularly characterised strain. The observed characters of this strain do not contradict the brief description and drawings in the original report of this species (Frenzel, 1897) and further studies will be able to rely on SSU rDNA sequence, which is now linked with a taxonomic name. Despite being arbitrary, this decision is likely the best to ensure the stability of the nomenclature and reliability of further research.

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