The Journal of Veterinary Medical Science

NOTE

Parasitology

Phylogenetic position of *Nyctotherus teleacus* isolated from a tortoise (*Astrochelys radiata*) and its electron microscopic features

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J. Vet. Med. Sci.
82(6): 699–703, 2020
doi: 10.1292/jvms.20-0004

Received: 5 January 2020
Accepted: 14 April 2020
Advanced Epub:
24 April 2020

ABSTRACT. A commensal ciliate was isolated from the stool of a tortoise (*Astrochelys radiata*). The ciliate was classified as *Nyctotherus teleacus*, according to its basic morphological features. Electron microscopic observations using cultured *N. teleacus* (NictoT1 strain) revealed many spherical hydrogenosomes and methanogen-suspected bacteria, together with a characteristic triangular macronucleus containing many spherical chromosomes in the cytoplasm of NictoT1. The results of phylogenetic analysis showed that NictoT1 was included in the cluster of *Nyctotheroides* spp. (family Nyctotheridae). *Nyctotheroides* spp. commonly infest amphibians, which are taxonomically closely related to reptiles, including the tortoises evaluated in the present study.

KEY WORDS: *Astrochelys radiate*, electron microscopic feature, genetic phylogeny, *Nyctotheroides* sp., *Nyctotherus teleacus*

The genus *Nyctotherus* was established by Leidy (1849) [10] after the discovery of *Nyctotherus velox* from the millipede *Julus marginatus*. Grassé (1928) [9] suggested a redefinition of *Nyctotherus* based on its karyophore and introduced the generic name *Nyctotheroides* to include members lacking or having an indistinct karyophore. Albaret (1975) [1] proposed that *Nyctotheroides* can be distinguished from *Nyctotherus* based on the presence of one (*Nyctotherus*) or two (*Nyctotheroides*) kinetal suture systems in the anterior part of the cell, and restricted *Nyctotheroides* to amphibian hosts and *Nyctotherus* to invertebrates and non-amphibian vertebrates (fishes and reptiles). *Nyctotherus teleacus* has a distinct karyophore from tortoise (reptile) [8]. However, these morphological classification criteria do not necessarily agree with the results of genetic classification among Nyctotheridae members [11]. To date, genetic information for *Nyctotheroides* spp. registered in GenBank is limited to that from frogs and toads (an amphibian), with information for *Nyctotherus* spp. limited to that from cockroaches (an insect) and millipede (a myriapod) [11].

N. teleacus was firstly isolated from three giant Galapagos tortoises (*Testudo hoodensis*, *T. elephantine*, and *T. vicina*) [8]. These common ciliate species have been suggested to be commensal organisms in the gastrointestinal tract of tortoises [3, 4, 6, 8, 16] and aid in the digestion of cellulose [7].

Accordingly, in the present study, we succeeded in cultivating *N. teleacus* isolated from *Astrochelys radiata*. We then analyzed *N. teleacus* using genetic methods and electron microscopy.

Stool samples from three tortoises (*A. radiata*) (7 years old, gender unknown) that were bred and kept isolated from other

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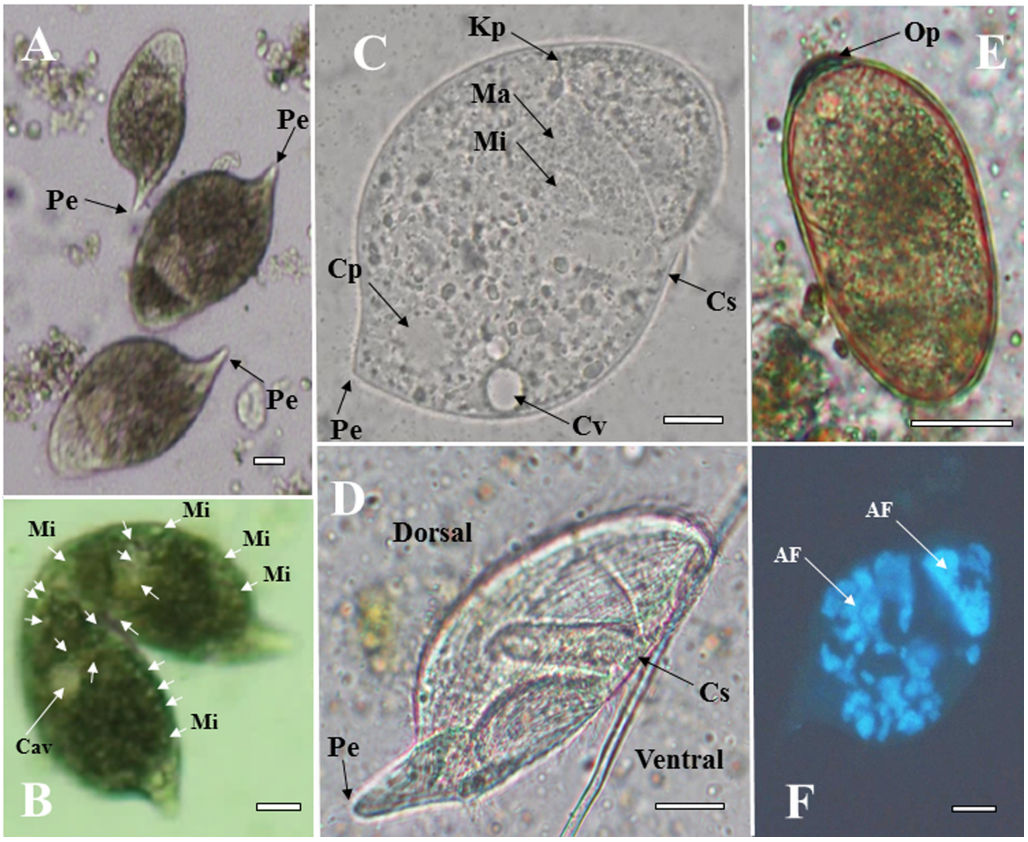

Fig. 1. Light and fluorescence microscopy images of the ciliate isolate (NictoT1). A: Proliferated trophozoites in culture medium. B: Conjugating trophozoites in culture medium. C, D: An extended trophozoite between slide- and cover-glasses by moisture evaporation. E: A cyst. F: A trophozoite with autofluorescence (excitation at 365 nm). Scale bar=20 μm. AF, autofluorescence; Cav, cavity after disappearance of the macronucleus; Cp, cytopyge; Cs, cytostome; Cv, contractile vacuole; Kp, karyophore; Ma, macronucleus; Mi, micronucleus; Op, operculum; Pe, posterior end.

reptiles and amphibians in a zoo in Kanto region, Japan, were collected as soon as possible after defecation and provided by the zoo. A stool sample (approximately 10–200 mg) from a tortoise containing cysts (approximately 2,400–3,400/g) of *Nyctotherus* sp. (identified morphologically) was inoculated into 1.5 m*l* of modified BR medium containing 4% bovine serum replaced with modified R medium [19] immediately before the primary culture or subculture.

The cultured ciliates (NictoT1) were partially purified by centrifugation at 400 × *g* for 30 min after layering the cultured suspension (4 m*l*) on 3 m*l* of 30% Percoll PLUS (GE Healthcare Bio-Sciences AB, Little Chalfont, UK) and observed by scanning electron microscopy (JSM5600LV; JEOL Ltd., Tokyo, Japan) [17].

Ultrathin sections of the specimens were observed under a HITACHI H-7600 electron microscope (Hitachi High-technologies Corp., Tokyo, Japan) after staining with 1% uranyl acetate and lead citrate.

The DNA sample (100–200 ciliate cells) was used as a PCR template for the 18S rRNA genes from NictoT1. We used primers targeting the eukaryotic 16S-like rRNA-coding regions [14]. The amplified fragments were cloned into the pANT vector (Nippon Gene Co., Ltd., Toyama, Japan), and eight positive clones were directly sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). In addition to the 18S rRNA sequences of *Nyctotherus* sp. obtained in this study, other armophorean sequences [11] were retrieved from the GenBank database (Fig. 3); the sequence of *Metopus palmaeformis* was used as an outgroup.

The sequences were aligned using ClustalW algorithm. Ambiguously aligned positions were discarded. Phylogenetic trees were constructed using the Bayesian (BI) method [15], which was implemented in MrBayes 3.2. The data were bootstrap resampled 1,000 times to estimate the relative branch support. In the Bayesian analysis, we ran four simultaneous chains (nchain=4), 2,000,000 generations, and an initial burn-in of 1,250, at which point, the likelihood values had stabilized. The general time-reversible model was used, and trees were sampled every 100 generations.

Because the ML analytical model provided the highest log likelihood, the ML tree was derived using the Tamura 3-parameter model [18] employing estimates of the proportion of invariable sites and gamma distribution with five rate categories; statistical support was evaluated by bootstrapping with 1,000 replicates.

The characteristic features of *N. teleacus* [8] and the present isolate (NictoT1) were well-matched with those of *N. teleacus* [8]. The size of NictoT1 (110–171 × 55–94 μm) frequently changed depending on its growth stage (e.g., excystation, division, and growth degree) and culture conditions (Fig. 1A).

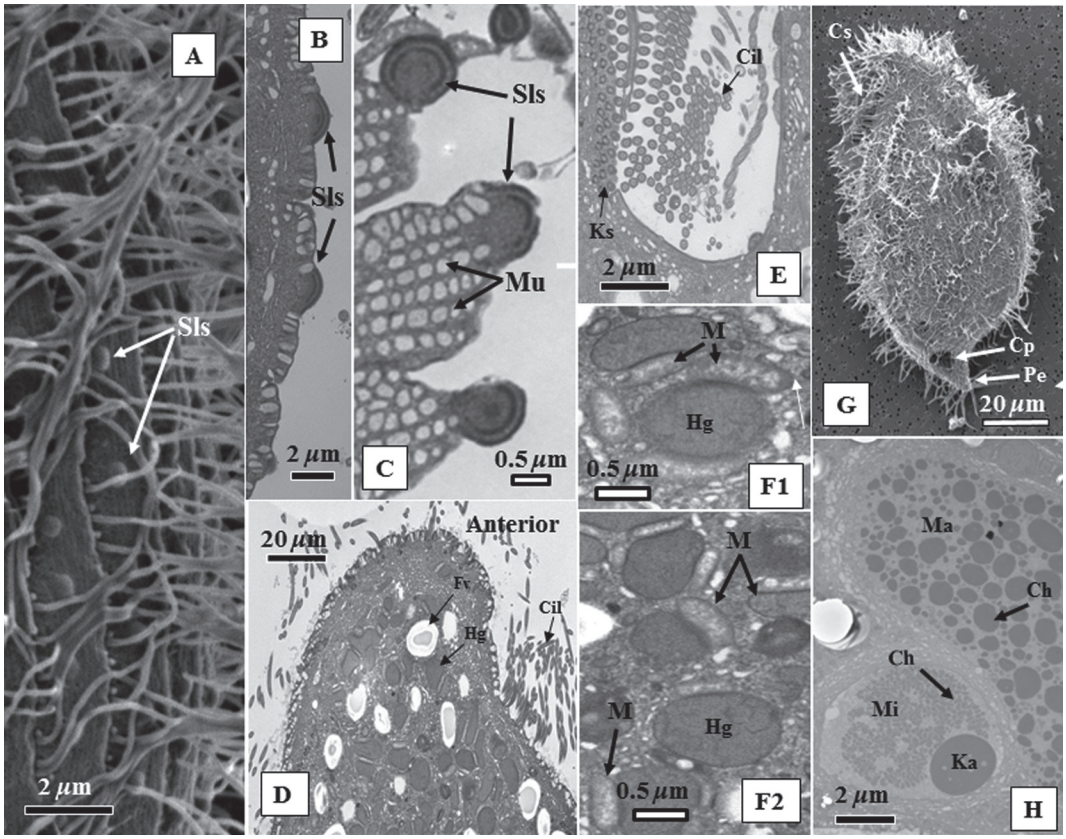

Fig. 2. Electron microscopic images of the ciliate isolate (NictoT1). A: External cortex with regularly arranged cilia and sensor-like hemispherical structures (Sls). B, C: External cortex with hemispherical (Sls) and mucocyst-like structures (Mu). D: Anterior end of the organisms. E: Fine structure of the oral cavity. F1, F2: A methanogen-like bacterium (M) in the cytoplasm. G: Whole ventral surface image. H: Fine structures of the macro- and micronucleus (Ma and Mi). Ch, chromosome; Cil, cilia; Cp, cytopyge; Cs, cytostome; Fv, food vacuole; Hg, hydrogenosome; Ka, karyosome-like structure; Ks, kinetosome; Pe, posterior end.

The SEM images showed that the external cortex of NictoT1 cells consisted of a partial pleated structure with small peaks and troughs, and the cilia appeared from the lower troughs of the plications (striations; Fig. 2A). In the TEM images, the external cortex consisted of mucocyst-like cells [2, 5]. In the front part of the cortex, some spherical or hemispherical and electron-dense structures were observed (Sls: Fig. 2B, 2C). These structures were regularly arranged on the surface of the sloped faces of plications in the SEM images (Sls: Fig. 2A). The longitudinal sections of the vocal cavities of peristomes showed many regularly arranged layers of kinetosomes on one side only, and many thin cross-sections of cilia were observed in the cavity (Fig. 2E). The cytopyge (Cp) was located at the base of the posterior end (Fig. 2G). In the TEM images, intracellular organelles [e.g., macro- and micronuclei (Ma and Mi), hydrogenosomes (Hg), and food vacuoles (Fv)] were observed (Fig. 2D, 2H). The TEM images of the endosymbiotic methanogen (M) are shown in Fig. 2F1 and 2F2.

The nucleotide sequences of the eight clones obtained by gene cloning of NictoT1 were identical. This 18S rRNA sequence consisting of 1666 nucleotides has been deposited in the GenBank/EMBL/DDBJ databases under accession number LC43448. In the Bayesian analyses tree of Clevelandellidae ciliates, NictoT1 from *A. radiate* was found to belong to the *Nyctotheroides* spp. cluster but not the *Nyctotherus* spp. cluster (Fig. 3).

In the present study, NictoT1 was identified as *N. teleacus* according to the basic morphological features of the organism. However, it is not easy to distinguish the generic difference in morphological and genetic features of *Nyctotherus* spp. from *Nyctotheroides* spp. [11].

In the Bayesian analysis tree, *Nyctotheroides* spp. and *Nyctotherus* spp. clusters were found to be sister taxa in 100% of bootstrap replicates. NictoT1 was identified as an approximate species of *Nyctotheroides* sp. based on the sequence homology and phylogenetic analyses of the 18S rRNA gene with closely related species of the order Clevelandellida. However, the DNA sequences of *Nyctotheroides* spp. from tortoises have not been registered, and the nearest registered sequence data are only from amphibians [11, 12]. In contrast, all registered sequence from *Nyctotherus* spp. is from cockroach or millipede, and those from *Clevelandella* spp. are only from wood-feeding roaches [13].

Hence, we report the sequence of the 18S rRNA gene of NictoT1 as a reference of a ciliate with consideration of validity of the genus *Nyctotheroides* from a tortoise. However, we were unable to find comparable genetic information of this family of ciliates isolated from reptiles, including tortoises, for phylogenetic classification.

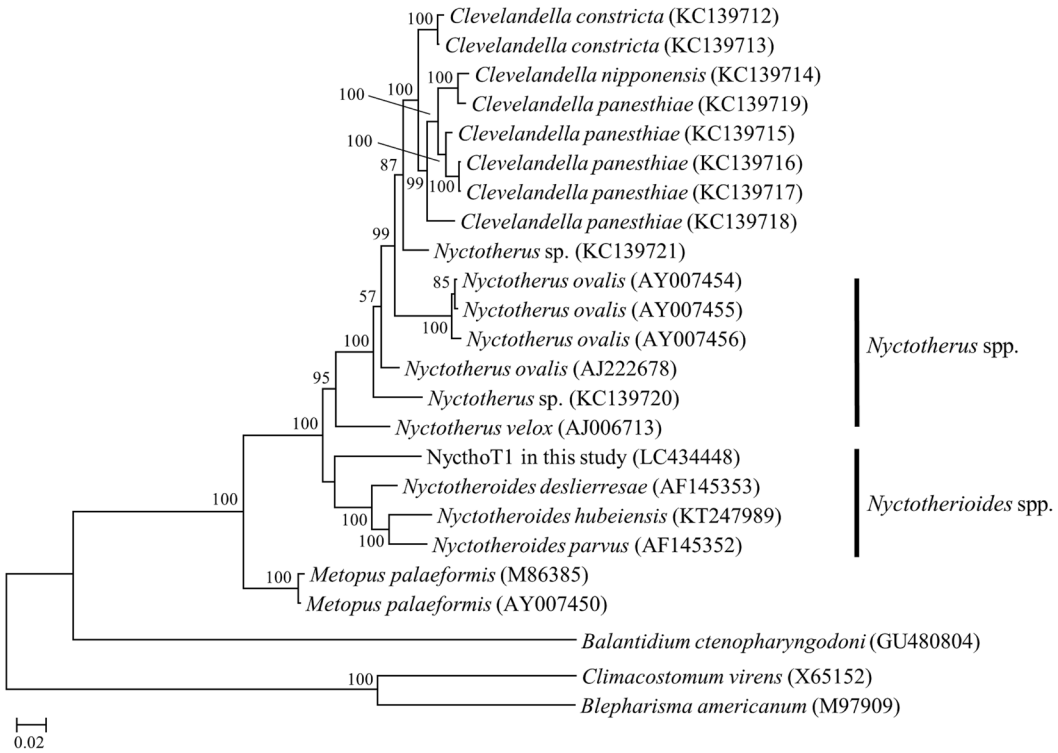

Fig. 3. Phylogenetic relationships of order Clevelandellida ciliates using Bayesian analysis of 18S rRNA sequences. Bayesian analysis was run using four simultaneous chains (nchain=4), 2,000,000 generations, and an initial burn-in of 2,500, at which point the average standard deviation of split frequencies had convergence. The general time-reversible model with a proportion of invariant bases and four categories of among-site rate variation were used, and trees were sampled every 100 generations. Significant bootstrap support (>50%) from Bayesian analysis is shown above the node. The scale bar represents the distance in substitutions per nucleotide. GenBank accession numbers are shown in parentheses.

Some spherical or hemispherical and electron-dense structures were identified, but they differed from the characteristic structures of cilia. We hypothesized that this structure was a sensor-like organ because the hemispherical form was exposed to the outside to sense environmental changes.

In summary, the morphological details of *N. teleacus* (NictoT1) were determined from cultured cells, and this organism was classified in the genus *Nyctotherus* based on the morphological analysis.

Additional genetic studies using more nyctotherid spp. from reptiles, including tortoises, are needed to determine the genus of nyctotherid spp. Hence, we are continuously trying to culture isolates of independent species of *Nyctotherid* from reptiles in order to support this study's genetic classification of *N. teleacus*.

ACKNOWLEDGMENTS. This study was partially supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research [(C) 24590511 and (B) 16H05819].

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