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A Novel Symbiotic Ciliate (Ciliophora: Peritrichia) in the Hindgut of a Stag Beetle (Coleoptera: Lucanidae)

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Bell-shaped ciliates of the subclass Peritrichia, such as *Vorticella*, *Carchesium* and *Epistylis*, are commonly found in freshwater and other aquatic environments, either solitary or colonial. Peritrichs attach to a substratum via a contractile or non-motile stalk, and collect food particles by water current using ciliary rows around the edge of the bell, called the peristome. Some peritrichs are epibiotic and ectocommensalistic associates of aquatic insects and other animals, settling on the surface of their specific hosts. Only a few peritrichs are known to establish a more internal association with their hosts, locating within the preoral cavity or esophagus of water beetles and presumably subsisting on food materials chewed and ingested by the insects. To date, no endoparasitic or endocommensalistic peritrichs have been reported from insects. Host insects reported to date have all been aquatic, and given the aquatic lifestyle of peritrichs, terrestrial hosts have been considered unlikely. In the present study, we report a dense population of bizarre microbes within the gut of a terrestrial insect, and histological, ultrastructural and molecular phylogenetic analyses identified it as a peritrich ciliate. The highly-developed hindgut of the stag beetle *Aegus currani* contained oval colonial peritrichs connected by branched stalks resembling grape clusters. Each zooid exhibited a reduced peristome without disc, a vestibulum with active ciliary movement inside, and an elongated macronucleus. These features are morphologically reminiscent of but distinct in some respects from those in *Operculariella parasitica*, known from the esophagus of dysticid diving beetles. Taxonomic, ecological and functional aspects of this gut-dwelling peritrich warrant future study.

Key words: *Aegus currani*, gut symbiosis, *Operculariella parasitica*, parasite, commensalist

INTRODUCTION

Members of the subclass Peritrichia (Ciliophora: Oligohymenophorea) comprise a large group of morphologically peculiar ciliates. Well-known representatives of the Peritrichia include *Vorticella*, *Carchesium*, and *Epistylis*. These and other peritrich ciliates are commonly found in aquatic environments, either solitary or colonial. Peritrichs attach to a substratum via a contractile or non-motile stalk, and possess a bell-shaped structure with ciliary rows along the edge of the bell (the peristome or collar), the movement of which drives food particles into a cellular opening (known as the vestibule, infundibulum, or funnel) that leads to the cytopharynx where food particles are endocytosed to form food vacuoles (Fenchel, 1987; Lynn, 2008; Foissner and Hawksworth, 2009).

In European freshwater environments, some extensive surveys of peritrich ciliates have been conducted (Stammer, 1948; Nenninger, 1948; Lust, 1950; Matthes, 1950; Biegel, 1954), and revealed that (i) approximately 30% of sessile peritrichs settle on diverse abiotic, plant and animal substrata; (ii) another 30% of the sessile peritrichs exhibit some specificity, for example, settling only on animals, for which the host species span various classes and orders; and (iii) the remaining 40% of sessile peritrichs are highly specific, each species settling on only a few or a single animal host species (Nenninger, 1948; Lust, 1950; Steffan, 1967). While most of the highly host-specific peritrich ciliates are epibionts/ectocommensals that attach themselves to the outer surfaces of host animals, previous studies reported two notable epibiotic peritrich taxa that are ecologically closer to endoparasites/endocommensals: *Orboperculariа ominosa* and *O. gnathophila*, which inhabits the preoral cavity of whirligig beetles (Coleoptera: Gyrinidae) (Lust, 1950; Matthes and Scheubel, 1971); and *Opercularіella parasitica*, which inhabits the esophagus of diving beetles (Coleoptera: Dytiscidae) (Stammer, 1948a). These peritrich ciliates exhibit several peculiar morphological traits, including reduced size of the peristome. It has been argued that the smaller peristome is related to their special ecological niche in which they subsist on food materials chewed and ingested

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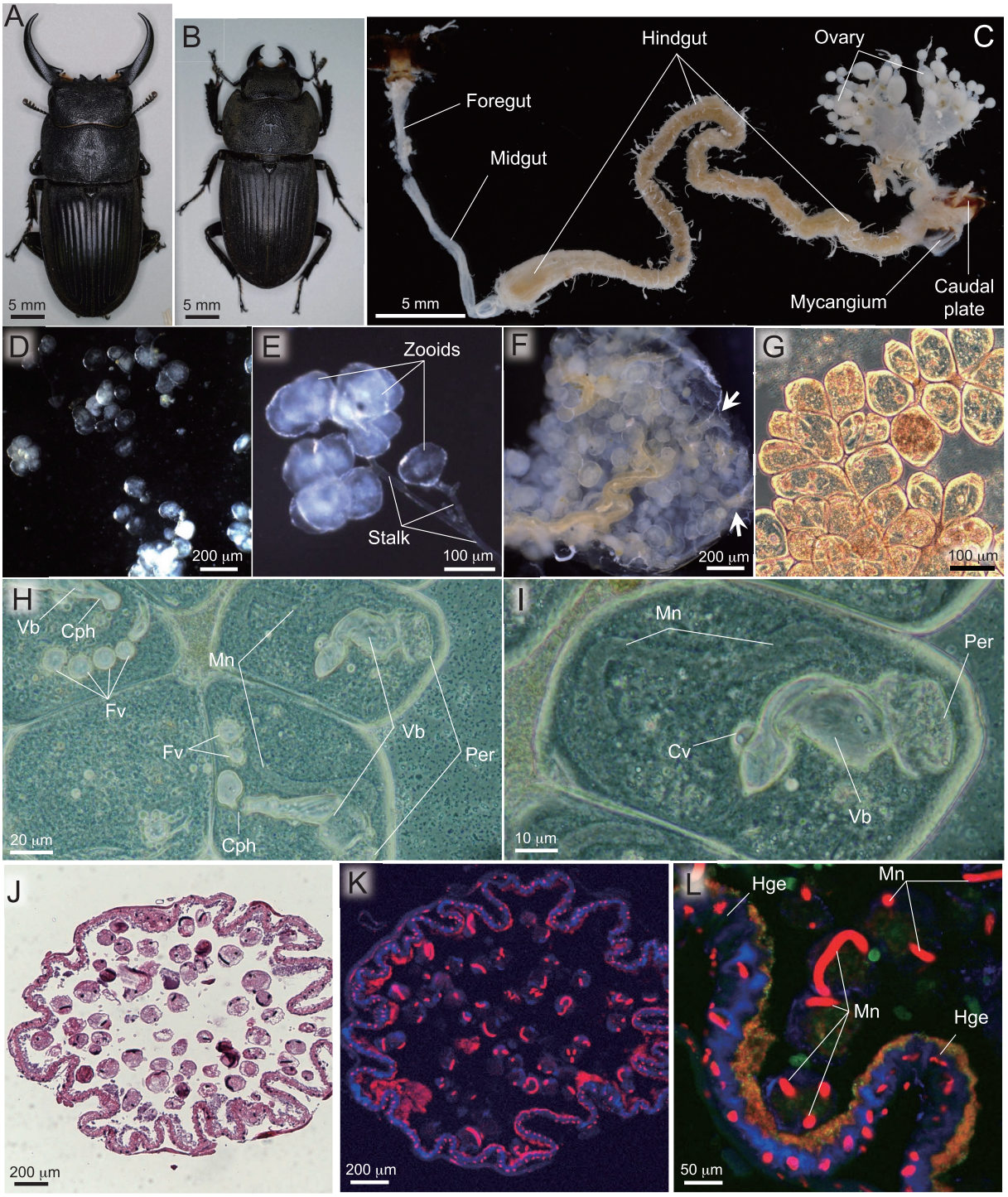

Fig. 1. The stag beetle *Aegus currani* and the novel peritrich ciliate found in the hindgut of the insect. **(A)** An adult male. **(B)** An adult female. **(C)** An alimentary tract dissected from an adult female. **(D)** Oval ciliate cells spilt out from a dissected hindgut. **(E)** A fragmented ciliate colony, in which oval ciliate cells are interconnected by a branched stalk. **(F)** A piece of dissected hindgut whose inner cavity is full of the ciliate colonies. Arrows show the stalk bases attaching to the inner wall of the hindgut. **(G)** A phase-contrast microscopic image of the ciliate colony. **(H, I)** Light microscopic images of the ciliate cells, in which ciliary movements in the vestibule and the peristrome, and also activities of food vacuoles and contractile vacuoles are highlighted. As for formation process of the food vacuoles, see Supplementary Movie S1 corresponding to **(H)**. As for activity of the contractile vacuole, see Supplementary Movie S2 corresponding to **(I)**. **(J)** A light microscopic image of a cross section of the ciliate-harboring hindgut, stained with hematoxylin and eosin. **(K)** A confocal fluorescence image of a cross section of the ciliate-harboring hindgut, in which DNA and tubulin are visualized in red and blue, respectively. For tracking serial optical sections, see Supplementary Movie S3. **(L)** An enlarged confocal fluorescence image of the ciliate cells and the hindgut epithelium, in which DNA, tubulin and bacterial 16S rRNA are visualized in red, blue and green, respectively. Abbreviations in **(H, I, L)**: Cph, cytopharynx; Cv, contractile vacuole; Fv, food vacuole; Hge, hindgut epithelium; Mn, macronucleus; Per, peristrome; Vb, vestibule.

by host insects (Steffan, 1967). There have been no reports to date on true endoparasitic/endocommensalistic peritrichs associated with insects.

Stag beetles (Coleoptera: Lucanidae) comprise a large group of morphologically spectacular insects that includes some 1500 described species in the world (Fujita, 2010). Stag beetle larvae generally feed on decaying wood, although it is the fungal bodies decomposing the wood, not the wood materials themselves, which are nutritionally important for sustaining larval growth (Tanahashi et al., 2009; Tanahashi and Kubota, 2013). All stag beetles examined thus far possess a membranous pouch specialized for harboring symbiotic yeasts, called the mycangium, in association with the ovipositor of adult females (Tanahashi et al., 2010; Tanahashi and Fremlin, 2013; Tanahashi and Howes, 2016). Yeast symbionts in the mycangia of *Lucanus*, *Dorcus* and *Platycerus* species are closely related to the xylose-fermenting yeast *Scheffersomyces stipitis* (formerly called *Pichia stipitis*) (Tanahashi et al., 2010; Hawes, 2013; Tanahashi and Fremlin, 2013), which is suggestive of involvement of mycangial yeasts in the xylophagous nutritional physiology of their lucanid hosts.

Here we report the discovery of a novel microbial associate in the Lucanidae. During a global survey of fungus–lucanid mycangial symbiotic associations, we encountered a stag beetle species harboring bizarre microorganisms in its highly-developed hindgut, which we identify here as peritrich ciliates. The intra-intestinal association of a peritrich with a non-aquatic insect is to our knowledge unprecedented.

MATERIALS AND METHODS

Insects

An adult male (Fig. 1A) and four adult females (Fig. 1B) of *Aegus currani*, originating from General Nakar, Quezon, Luzon Island, Philippines (Table 1), were purchased at an insect shop (Mushi-sha, Tokyo, Japan; http://www.mushi-sha.com). *A. currani* is endemic to Luzon island, Philippine, and the insects were field-collected and imported to Japan. Stag beetles of the genus *Aegus* are distributed across southwestern Asia and include over 200 described species, of which *A. currani* is the largest in size; adults males sometimes exceed 70 mm in body length (Fujita, 2010).

Light and electron microscopy

The insects were dissected in a phosphate-buffered saline (PBS; 8 g NaCl, 2.9 g $NaHPO_4{\cdot}12H_2O$, 0.2 g KCl and 0.2 g KH_2PO_4 in 1 L distilled water) in Petri dishes using fine forceps and scissors under a dissection microscope (S8APO, Leica Microsystems) connected to a digital camera. The dissected alimentary tracts were observed under a light microscope (Axiophot, Carl Zeiss Microscopy) with differential interference and epifluorescence optics, and subjected to histological preparations. For tissue sectioning and staining, the samples were fixed in 4% paraformaldehyde in PBS, dehydrated and cleared through a xylene-ethanol series, embedded in paraffin, processes into serial tissue sections (10–15 μm thick), mounted on glass slides, dewaxed through a xylene-ethanol-water series, stained with hematoxylin and eosin, dehydrated, and cleared through a water-ethanol-xylene series, mounted in balsam with coverslips, and observed under a light microscope. For confocal fluorescence imaging, the samples were fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid), stained with Sytox Orange for visualizing DNA, with a mouse anti-β-tubulin antibody (primary antibody) and a rabbit anti-mouse IgG antibody (secondary antibody) labeled with Alexa Fluor 488 or 567 for visualizing β-tubulin, and with an oligonucleotide probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) targeting eubacterial 16S rRNA for visualizing bacteria, and observed under a laser confocal microscope (Pascal 5, Carl Zeiss Microscopy). For inspecting fine structure, the samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight, and subsequently with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4°C for 60 min. After dehydration through a water-ethanol series, the samples were embedded in Spurr resin, processed into ultrathin sections (80 nm thick) using an ultramicrotome (Ultracut-N, Leichert-Nissei), mounted on copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (model H-7000, Hitachi).

Molecular phylogenetic analysis

The dissected hindgut samples were crushed in liquid nitrogen, suspended in a lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton-X, 20 mg/ml lysozyme) and subjected to DNA extraction using QIAmp DNA Mini Kit (Qiagen). A 1.5 kb region of eukaryotic 18S rRNA gene was amplified by PCR from the DNA samples using the primers NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and FS2 (5′-TAG GNA TTC CTC GTT GAA GA-3′) under a temperature profile of 95°C for 5 min followed by 28 cycles consisting of 95°C for 1 min, 53°C for 1 min and 72°C for 1.5 min. The PCR products were subcloned into pT7Blue plasmid vector (Novagen). Competent cells of *Escherichia coli* DH5α (TaKaRa) were transformed with the plasmids, and white colonies (8–16 colonies per plate) were subjected to PCR amplification using pT7Blue-specific primers U-19 and R-20 (Novagen). The PCR products were sequenced using a DNA sequencer (3130xl Genetic Analyser, Applied Biosystems) with the sequencing primers U-19, R-20 and NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′) (White et al., 1990). These short reads (~600 bases) were then assembled into contigs using the software ContigExpress (Invitrogen). The contig sequences were inspected by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to exclude non-ciliate contigs.

The ciliate 18S rRNA gene sequences were subjected to multiple alignment using the program ClustalW (Larkin et al., 2007). The alignments were inspected and corrected manually, from which ambiguously aligned sites were removed. Phylogenetic analyses were conducted by the maximum-likelihood (ML) method using the program Mega 6 (Tamura et al., 2013). We selected the Tamura + G + I model on the basis of the Akaike criterion. Bootstrap tests were performed with 1000 replications.

Table 1. Samples of *A.currani* examined in this study.

Sample ID	Sex[1]	Collection date[2]	Collection locality	Purchased date[3]	Body length[4]	Ciliate density[5]	Accession number[6]
#817	Female	2012 Fall	General	17 Jan 2013	32.55	+++	LC212810
#819	Male	2012 Fall	Nakar,	17 Jan 2013	39.30	+++	LC212811
#826	Female	2012 Fall	Quezon,	7 Feb 2013	34.35	+++	–
#839	Female	2012 Fall	Luzon Island,	11 May 2013	32.53	+++	–
#951	Female	2013 Fall	Philippines	12 Jan 2014	35.65	+	–

[1]All adults.
[2]Exact date not available.
[3]At Mushi-sha, Tokyo, Japan (http://www.mushi-sha.com).
[4]In millimeters including mandibles.
[5]+++, very dense; +, sparse.
[6]18S rRNA gene (1495 bp).

RESULTS AND DISCUSSION

In order to examine the yeast-harboring mycangium, an adult female of *A. currani* was dissected. The mycangium is an invaginated pouch-like organ made of inter-segmental membrane located at female's dorsal abdominal tip (Fig. 1C). During the dissection, we noticed that, when the hindgut was torn in the saline, numerous cyst-like particles spilt out (Fig. 1D). The particles were connected to each other by branching filaments, forming clusters resembling bunches of grapes (Fig. 1E–G). Careful dissection of the hindgut revealed that the particles filled the hindgut cavity as huge clusters, being attached to the inner wall of the hindgut via the filaments (Fig. 1F).

Light microscopic observations showed that the particles exhibited active ciliary movements inside, but not outside (Fig. 1H and I; Supplementary Movies S1 and S2), suggesting the possibility that the particles are ciliate cells of a particular type. Given their characteristic traits, we conjectured that the particles might represent a ciliate of the subclass Peritrichia. Members of the Peritrichia embrace *Vorticella*, *Carchesium*, *Epistylis* and other bell-shaped sessile filter feeders commonly found in aquatic environments, which generally lack surface cilia except for ciliary rows around the cytostome, namely the peristome and the vestibule, for feeding (Fenchel, 1987; Lynn, 2008; Foissner and Hawksworth, 2009). Each particle was regarded as a peritrich cell or a zooid (Fig. 1D and E). The particle cluster looked like a peritrich colony consisting of zooids and branching stalks (Fig. 1E). The colonies were often so large and intermingled in the hindgut cavity that it was difficult to count the number of zooids in a colony (Fig. 1F and G). Distinct from free-living colonial peritrichs, such as *Carchesium* and *Epistylis*, whose zooids are bell-shaped with a ciliated peristome extending outward for efficiently collecting food particles, the zooids in the hindgut of *A. currani* were egg- or bud-shaped, their peristome was reduced and encased internally, and consequently their cytostome opening was very small, which probably reflects adaptation to the nutrition-rich intrahost environment (Fig. 1G-I). Morphometry of the zooids yielded ciliate cell dimensions of 127 ± 22 μm in length and 94 ± 22 μm in width (mean ± SD; n = 20).

By gently pressing the zooids on glass slides using a coverslip, we were able to observe ciliary movements and cellular activities within the ciliate cells (Fig. 1H and I; Supplementary Movies S1 and S2). In addition to the movement of shorter cilia around the reduced peristome, very active movement of longer cilia was seen within the vestibule, which appears to contribute to food intake from the surrounding milieu. At the bottom of the vestibule was located the cytopharynx, in which food vacuoles form one after another (Fig. 1H; Supplementary Movie S1). We also observed, in association with the vestibule, a contractile vacuole, the contraction of which forces excess water into the vestibule cavity (Fig. 1I; Supplementary Movie S2).

Tissue sectioning and histological staining of the ciliate-harboring hindgut clarified the structural and cytological configuration of the host and the microbe. Hematoxylin and eosin staining visualized the zooids distributed throughout the hindgut cavity, each containing a densely-stained intracellular body (Fig. 1J). Fluorescent DNA binding dye intensely stained a slender C-shaped structure in each zooid, which sometimes exceeded 150 μm in length (Fig. 1K and L; Supplementary Movie S3). The structure is evidently the macronucleus of the ciliate. Note that this structure was also distinguishable in light microscopic images of the live ciliate cells (Fig. 1H and I). Transmission electron microscopy revealed fine structures of the macronucleus, mitochondria, vestibular cilia, food vacuoles, etc. (Fig. 2A–C).

Fluorescence in situ hybridization using an oligonucleotide probe targeting bacterial 16S rRNA detected some sig-

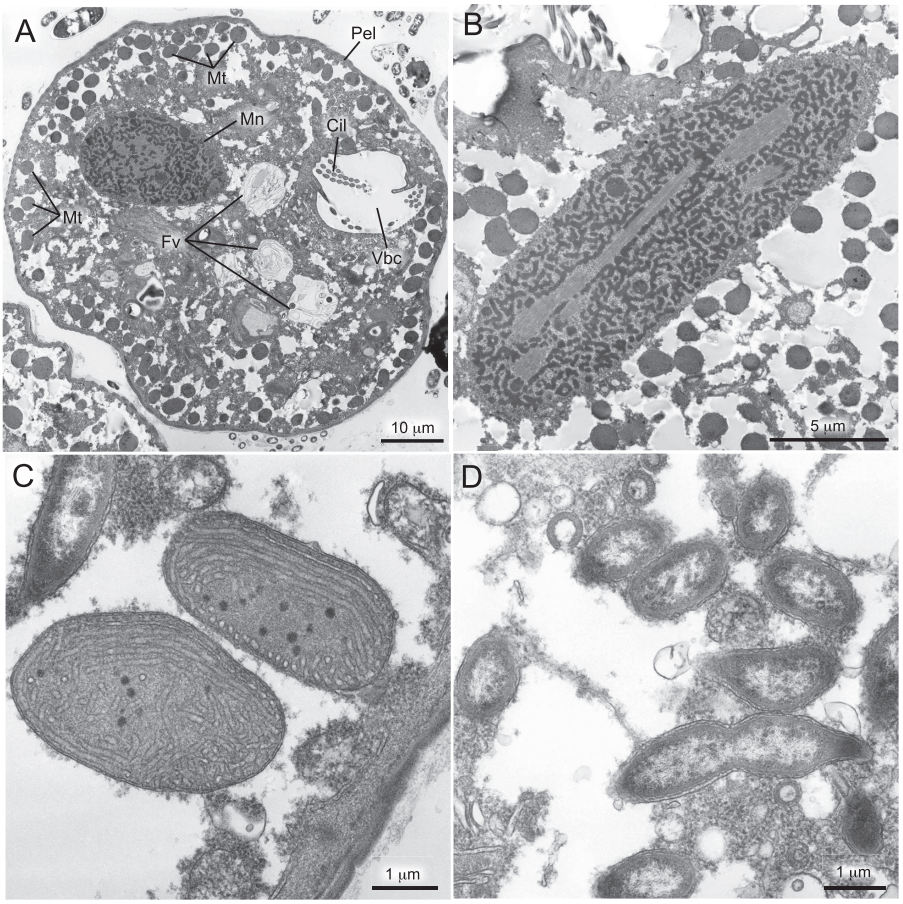

Fig. 2. Transmission electron microscopic images of the ciliate. **(A)** A ciliate cell, in which a macronucleus, a vestibule cavity and cilia inside, several food vacuoles at the center, and numerous mitochondria in the periphery are seen. **(B)** A macronucleus. **(C)** Mitochondria. (D) Bacterial cells in the cytoplasm, which are presumable endosymbionts. Abbreviations: Cil, cilium; Fv, food vacuole; Mn, macronucleus; Mt, mitochondrion; Pel, pellicle; Vbc, vestibule cavity.

nals within the ciliate cells (Fig. 1L). Transmission electron microscopy consistently detected bacterial cells within the cytoplasm (Fig. 2D), which probably represent endosymbiotic bacteria of the ciliate; their characterization will require future study. Note that endosymbiotic bacteria are commonly found in diverse ciliates and other protozoans (Heckmann and Görtz, 1992; Fujishima, 2009).

On a review of the literature, we noted that the ciliate in the hindgut of *A. currani* is morphologically and cytologically similar to *Opercullariella parasitica* known from the esophagus of dysticid diving beetles (Stammer, 1948). The cell shape and size, the reduced peristome, the basic configuration of stalks, the structure of vestibule, etc., were quite similar between the ciliate and *O. parasitica* (Table 2). On the other hand, the ciliate exhibited much larger colony size, different morphology of macronucleus, and above all, distinct ecology and host specificity in comparison with *O. parasitica* (Table 2). Molecular phylogenetic analysis based on 18S rRNA gene sequence confirmed the placement of the ciliate within the Peritrichia (Fig. 3), but because no genetic information is available for *O. parasitica*, it is currently unknown whether the ciliate is phylogenetically related to *O. parasitica*. The morphological resemblance between them seems to support their phylogenetic affinity, but the possibility that their host-associated lifestyle has led to convergent evolution cannot be excluded. Hence, while we suggest that the ciliate in the hindgut of *A. currani* may be a new member of the genus *Opercullariella*, we withhold its taxonomic treatise at this stage until more samples from other host populations are examined and its molecular phylogenetic relationship to *O. parasitica* is established.

In the present study, we inspected five adult insects of *A. currani* in total, and the peritrich ciliates were found in the hindgut of all individuals. In four insects inspected in 2013, the ciliate density was very high, whereas the infection density was low in an insect additionally inspected in 2014 (Table 1). All these insects were derived from the same locality in Philippines; however, as *A. currani* is a relatively rare lucanid species, we were able to examine only a limited number of the insects.

In conclusion, we report a peritrich ciliate symbiotically inhabiting the hindgut of the stag beetle *A. currani*. Our finding is unprecedented in that (i) this is the first peritrich ciliate living deep in a host alimentary tract and isolated from the ambient environment, and (ii) this is the first peritrich ciliate symbiotically associated with a terrestrial insect. All peritrich

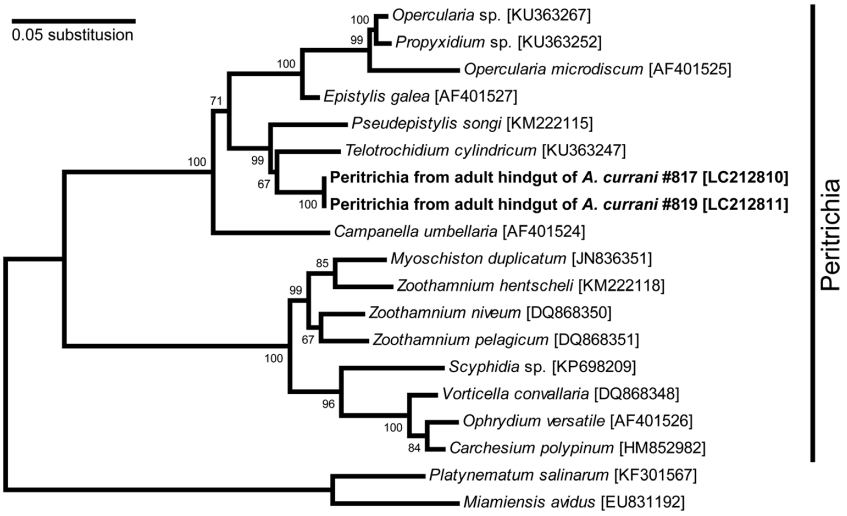

Fig. 3. Phylogenetic position of the ciliate in the subclass Peritrichia on the basis of 18S rRNA gene sequence. A maximum-likelihood phylogeny inferred from 1321 aligned nucleotide sites is shown. Bootstrap probabilities are indicated at the nodes.

Table 2. Comparison between *Opercullariella parasitica* and the ciliate in the hindgut of *Aegus currani.*

	Original description of *Opercullariella parasitica* by Stammer (1948)	Our observation of the ciliate in the hindgut of *Aegus currani* (this study)
Cell shape and size	Barrel-shaped with somewhat bulging anterior body; 100–110 μm in size	Similar in shape to *O. parasitica*; 127 ± 22 μm in length, 94 ± 22 μm in width (n = 20)
Stalk	Rigid, short and branched; the short main stalk usually dividing into two secondary branches, and at the end of further branches zooids are sitting	Similar in structure to *O. parasitica*, but much longer and complicated in connection
Colony size	Size of the colonies 2–3 times of the length of a zooid	Very large, probably dozens of zooids are interconnected by branching stalks
Peristome	Small and reduced; width only a quarter of the largest body width; small and smooth with ciliary rows; disc missing	Similar in structure to *O. parasitica*
Vestibule	Short and broad; width somewhat larger than the width of peristome; covered with many long cilia moving strongly	Similar in structure to *O. parasitica*
Contractile vacuole	Connected to and discharging into the end of the vestibule; contraction interval up to 1 h	Similar in structure to *O. parasitica*
Macronucleus	Oval in shape; twice as long as the width; 30 μm long on average	Slender and C-shaped; often over 10 times as long as the width; sometimes over 150 μm long
Host and localization	Esophagus of diving beetles (Coleoptera: Dytiscidae)	Hindgut of a stag beetle *A. currani* (Coleoptera: Lucanidae)
Molecular data	Not available	18S rRNA gene [LC212810, LC212811]

ciliates identified to date are aquatic, and all known peritrich ciliates associated with animals are epibiotic/ectocommensalistic (Nenninger, 1948; Lust, 1950; Steffan, 1967). It thus seems reasonable that all host insects of peritrich ciliates reported to date have been aquatic, such as water beetles and water bugs, enabling the ciliates to swim in, settle on, and swim away in the aquatic environment unrestricted. However, the stag beetle studied here is a terrestrial insect which lives in and feeds on decaying wood as a larva, and consumes tree sap as an adult (Fujita, 2010). This poses an enigma regarding how this ciliate is able establish and maintain its association with the hindgut of the host insect. Where does the ciliate come from? How does it infect the hindgut of the host insect? Is the ciliate acquired from the environment or vertically transmitted across host generations? Is the ciliate also present in the larval alimentary tract? If so, how does it persist through the host metamorphosis? Is the ciliate infection parasitic, commensalistic, or mutualistic for the host insect? These questions remain to be addressed in future studies on both natural populations and laboratory experimental colonies of *A. currani*.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

MT designed and conducted the research. XYM performed transmission electron microscopy. TF and MT wrote the paper.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs170012).

Supplementary Movie S1. The peritrich ciliate cells dissected from the hindgut of *A. currani*. Ciliary movements in the vestibule and the peristome, and formation of a food vacuole at the cytopharynx are highlighted. Also see Fig. 1H.

Supplementary Movie S2. The peritrich ciliate cells dissected from the hindgut of *A. currani*. Ciliary movements in the vestibule and the peristome, and contraction of the contractile vacuole are highlighted. Also see Fig. 1I.

Supplementary Movie S3. Serial optical cross-sectioning of the ciliate-harboring hindgut of *A. currani* obtained by confocal fluorescence microscopy. DNA and tubulin are visualized in red and blue, respectively. The 3D structures of C-shaped macronuclei and branching stalks are highlighted in the movie. See also Fig. 1K and L.

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