The molecular analysis of ciliates for identification and phylogeny is usually conducted through PCR amplification and DNA sequencing, with DNA extracted from a large number of cells. Therefore, the analysis of ciliates is limited to only those species that can be cultured. We propose a single-cell PCR procedure to overcome the difficulty in the analysis of unculturable species. The procedure has been tested on 6 *Stichotrichia* strains and uncultured *Levicoleps biwae* cells, targeting 18S rRNA gene sequences, after carrying out microscopic observations and obtaining photographic records. This procedure enables the systematic analysis of unculturable ciliate strains in natural environments by linking the morphology and genotype of a single cell.

**Key words:** Ciliates, single-cell PCR, 18S rRNA gene, uncultured cell, light microscopic analysis
The PCR mixture consisted of 10× Ex Taq buffer, 0.25 mM dNTP mixture, 0.1 μM primers, 2.5 U of Ex Taq DNA polymerase (Takara, Tokyo, Japan), and single cell or template DNA. Amplifications were performed under the following conditions: 28 cycles of 93°C for 1 min, 50°C for 2 min, and 72°C for 3 min for the first PCR, and 1 cycle of 93°C for 1 min followed by 35 cycles of 93°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and finally 5 min at 72°C for the second PCR. Amplicons were analyzed by electrophoresis on a 1% agarose gel. For 6 Stichotrichia strains and uncultured L. biwae cells, the 18S rRNA gene was amplified using the various primer sets (Fig. 2). The success rates for the amplification of 18S rRNA gene fragments by nested PCR from intact single cells without DNA extracts are shown in Table 2. Amplicons could be obtained from more than half of the cells tested. In particular, we achieved a success rate of 87% for obtaining amplicons from L. biwae cells, which are covered with hard armors, indicating that single-cell PCR is extremely useful for ciliates.

To confirm the amplicons of 18S rRNA genes and to identify the taxa of the isolated cells, we determined DNA sequences. The 18S rRNA genes amplified by nested PCR using 4 primer sets (CS322F-EU929R, SR6-SR9, SR8-SR11 and SR10-SR12) were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), ligated using the pGEM-T Easy Vector kit (Promega Co., WI, USA), and cloned into Escherichia coli strain DH5α. The inserted fragments were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Approximately 1,450 bp of the 18S rRNA gene sequence were assembled from the 4 DNA sequences. Database searches for related SSU rRNA gene sequences were conducted using BLAST (NCBI). Almost the entire sequence of the 18S rRNA gene was obtained from the Stichotrichia and L. biwae cells. The results of the BLAST search are shown in Table 2. Morphological examination revealed that all the cells belonged to Stichotrichia and Colepsidae. Six strains of Stichotrichia, St03, St13 and St20; St04; St05; and St09 were similar to the genera Oxytricha, Orthomastixiella, Gonostomum, and Pattersonia, respectively. For the uncultured cells of L. Biwae, all the cells tested had identical sequences.

The single-cell PCR procedure without DNA extraction is suitable for use in ciliates regardless of whether or not they can be cultured. Besides L. biwae having calcified armors, various types of ciliate cells, very tiny cells of the Halteria sp. (ca. 25 μm in length) and several ciliate species (Histobalanantium natans viridis, Didinium sp. and Pelagodileptus sp.) were found to produce specific amplicons (data not shown, success rate approximately 60–90%). In conclusion, this procedure can be used for the systematic analysis of various types of ciliates including those that cannot be cultured, in the natural environment. Associating the morphological and molecular information of a single cell will help in revealing the ecology, taxonomy, and phylogeny of ciliates.

For dinoflagellates, it was reported that the 18S rDNA gene of a single cell could be amplified after scanning electron microscopy with osmium vapor fixation using single-cell PCR. We have not succeeded in amplifying the 18S rDNA gene of ciliates after obtaining scanning electron microscopic photographs showing clear morphological features with osmium vapor fixation. This issue should be resolved in future studies.
The reported 18S rRNA gene sequences, i.e., St03, St04, St05, St09, St13, (F) St20 and (G) L. biwae, have been deposited under DDBJ accession numbers AB449360, AB449361, AB449362, AB449363, AB449364, and AB449365, respectively.

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References

6) Griffin, J.L. 1960. An improved mass culture method for the large,


