

## ORIGINAL PAPER

# DNA Analysis of Algal Endosymbionts of Ciliates Reveals the State of Algal Integration and the Surprising Specificity of the Symbiosis



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Many freshwater protists harbor unicellular green algae within their cells, but little is known of their degree of integration and specificity. Using algae-targeted PCR of whole ciliate cells collected at irregular intervals over 15 months from Lake Biwa, Japan, we explored the SSU-ITS rDNA of the endosymbiotic algae and its changes over time, obtaining sequences of algal rDNA fragments from four ciliate species. A high proportion of clonal algae was evident within the ciliate cells. The differences observed in those sequences from the SSU through to the ITS region were less than 1%. The name 'Chlorb' is proposed for these algae, with the implication that they represent a single 'species.' The sequences of the algal DNA fragments were identical for any given host species throughout the collection period, thus we conclude that these four ciliates stably retain their algae over long term. In contrast, algal DNA fragments obtained from *Didinium* sp. were variable within each sample, which indicates that this ciliate only temporarily holds its algal cells. The ITS1 sequences of Chlorb populations are close (at intraspecific level) to those of algae isolated from ciliates in Austria, which raises the possibility that Chlorb algae are universally shared as symbionts among various ciliates.

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## Introduction

Endosymbiosis is one of the major driving forces of evolution. Through modern studies on algae, it has become accepted that the endosymbiosis has arisen on many occasions, thereby producing

the current diversity of photosynthetic organisms (e.g. [Armbrust 2009](#)). We call organisms that were generated by symbiosis with cyanobacteria as 'primary plants' (green plants, red algae, glaucophytes), but we call those that absorbed such primary plants as 'secondary plants' (e.g. diatoms, brown algae, euglenoids). For these secondary symbioses, [Inouye and Okamoto \(2005\)](#) proposed a three-stage process for the development of a complete chloroplast. Endosymbiosis may be initiated when a phagotrophic protist engulfs an alga

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as prey. The protist may fail to digest the alga and thus accidentally keeps it within the cell (Stage I). The temporary symbiont then becomes a persistent symbiont when synchronization of the cell cycle is established between the host and the symbiont (Stage II). In this stage, the host retains its own (natural) symbiont, and it is unnecessary to incorporate the new alga from outside. As the next step, lateral gene transfer from the symbiont nucleus to the host nucleus occurs, which results in decreased symbiont autonomy and increased control by the host. The symbiont's organelles are then reduced one by one, but a relict symbiont nucleus is conserved for a period of time as a nucleomorph (Stage III) until control by the host is fully established (cryptophytes, chlorarachniophytes). Stage III is quite different from Stages I or II in that the symbiont is not merely a 'symbiotic alga,' but more like a chloroplast. The differences between Stages I and II, however, are perhaps not very obvious. It might be easy to differentiate them if the protist is cultivable and the number of symbionts is one or two, as in *Hatena* (Okamoto and Inouye 2005) and some dinoflagellates (e.g. Horiguchi and Pienaar 1992; Onuma and Horiguchi 2015). When the number of symbionts in a host cell is overwhelmingly high, it is hard to prove whether the symbionts are clones at all, and whether they divide synchronously. In freshwater, many protists have a large number of green endosymbionts like this. Ciliates (Alveolata) are the most common, along with certain Amoebozoa and Heliozoa (Brandt 1882). These protists usually contain hundreds of algae within a host cell, and we refer to them collectively as 'multi-algae retaining protists' (MARP).

In MARP symbioses, little is known of the degree of integration of the symbionts as indicated, e.g. by their or their descendants' retention time. If a carnivorous ciliate consumes a heliozoan that carries symbiotic algae, the heliozoan per se might be digested rapidly, leaving behind its internal algae, which may not be digested so easily. If the ciliate is observed at precisely such a time, one might erroneously judge it to be a Stage I symbiosis. Even in the case of real symbioses established in this way, it is largely uncertain how long the internalized algae are kept, and whether or not they establish clones within the host ciliate. The ciliate *Paramecium bursaria*, a textbook organism, is the only MARP species for which the degree of integration of its symbionts is becoming understood. Nearly 100 strains of this ciliate have been analyzed so far. In most cases, *P. bursaria* retains either *Chlorella variabilis* or *Micractinium reisseri* (Chlorellaceae, Trebouxiophyceae) as cloned symbionts. Which

partner *P. bursaria* possesses is strictly dependent on geography: in mid-latitudes it retains *C. variabilis* whereas in high latitudes it retains *M. reisseri*. Both algae share certain physiological features. They both require organic nitrogen compounds and are sensitive to so-called *Chlorella* viruses, which are abundant in natural freshwater. Both *C. variabilis* and *M. reisseri* have already mutated to a level that they cannot survive as free-living algae (Hoshina et al. 2010 and references therein). Because several hundred algal cells are maintained in a single *P. bursaria* cell, it cannot be proven that the cell cycles of host and symbionts are synchronized. It does seem certain that *P. bursaria* has established a persistent symbiosis, i.e. a Stage II symbiosis, with them, but might this not be a special case among the MARP symbioses? In order to check other cases, in the present study we intermittently collected ciliate species possessing green algae in Lake Biwa, the largest and oldest lake in Japan, and the DNA analyses of these symbionts has provided us with provocative evidence concerning the specificity and degree of integration of MARP symbioses, as recounted below.

## Results

### Algae-bearing Ciliates Collected in Lake Biwa

Several species of ciliates with endosymbiotic algae were observed in Lake Biwa, especially in winter and spring. In order to ascertain their degree of assimilation, as suggested by the retention time of the symbiont or their descendants, we limit our discussion here to ciliate species that were collected twice or more often, namely, *Stokesia vernalis* (Stokesiidae), *Pelagodileptus trachelioides* (Tracheliidae), *Bursellopsis spumosa* (Urotrichidae), *Cyclotrichium viride* (Didiniidae), and *Didinium* sp. (Didiniidae) (Table 1, Fig. 1). For convenience, they are all usually referred to below by their generic names. *Didinium* sp. is probably an undescribed species because its macro-nucleus is dumbbell-shaped, unlike the C-shaped nucleus of known *Didinium* species with symbiotic algae such as *D. chlorelligerum* (Foissner et al. 1999). Over a period of 15 months, *Stokesia* was collected five times, *Pelagodileptus* four times, *Bursellopsis* four times, *Cyclotrichium* twice, and *Didinium* sp. four times. All five species contained from nearly 100 to several hundred green spheres (symbiotic algal cells) within their cells, although photographs do not always show them clearly (Fig. 1).

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