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Photobiont Flexibility in *Paramecium bursaria*: Double and Triple Photobiont Co-Habitation

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ABSTRACT

The green ciliate, *Paramecium bursaria*, has evolved a mutualistic relationship with endosymbiotic green algae (photobionts). Under culture conditions, photobionts are usually unified (to be single species) within each *P. bursaria* strain. In most cases, the algal partners are restricted to either *Chlorella variabilis* or *Micractinium reisseri* (Chlorellaceae, Trebouxiophyceae). Both species are characterized by particular physiology and atypical group I intron insertions, although they are morphologically indistinguishable from each other or from other *Chlorella*-related species. Both algae are exclusive species that are viable only within *P. bursaria* cells, and therefore their symbiotic relationship can be considered persistent. In a few cases, the other algal species have been reported as *P. bursaria* photobionts. Namely, *P. bursaria* have occasionally replaced their photobiont partner. This paper introduces some *P. bursaria* strains that maintain more than one species of algae for a long period. This situation prompts speculations about flexibility of host-photobiont relationships, how *P. bursaria* replaced these photobionts, and the infection theory of the group I introns.

Keywords: *Paramecium bursaria*; Photobiont; Symbiosis

1. Introduction

The green ciliate *Paramecium bursaria* is one of the most studied protists due to its observable endosymbiosis. Their symbiotic relationship is able to start over, *i.e.*, artificially algae-removed *P. bursaria* can absorb again and fix the algae as new photobionts [1]. Despite the re-symbiosis ability of *P. bursaria*, there are unusual characteristics in terms of the small diversity of their photobionts. Although almost 50 strains of photobionts (partly directly gained sequences from *P. bursaria* extracts) have been genetically identified, most belong to either *Chlorella variabilis* or *Micractinium reisseri* (Chlorellaceae, Trebouxiophyceae) [2-11]. Neither species has ever been collected as a free-living species from natural water sources. This is possibly due to following reasons. Both species are essentially nutritionally fastidious [e.g., 12,13]. Additionally, they are very sensitive to the *Paramecium bursaria Chlorella* virus (PBCV), which is abundant in natural water sources [14-16]. Escaped photobionts from *P. bursaria* cell would be attacked by PBCV immediately. Both species appear to be highly dependent on their host refuge. *Chlorella variabilis* and *M. reisseri* are therefore thought to have adapted to exclusively dwell in *P. bursaria*. In a few cases, *P. bursaria* is associated with other species of *Chlorella* or *Scenedesmus* (Chlorophyceae) [3,9,11]. Namely, *P. bursaria* has replaced its photobionts on several occasions.

Lichens and corals, representatives of symbiotic associations with algal symbionts, experience a period without symbionts in their life cycle and must acquire fresh algae as symbionts to complete their life cycle. There is no such symbiont-less period for *P. bursaria*; the algae are retained through cell division as well as sexual reproduction [17]. Consequently, the symbiotic relationship with *P. bursaria* appears to be permanent. However, diversity in the photobionts, as mentioned above, does exist. Thus, it is not understood how *P. bursaria* gain such algal diversity.

Group I intron evidence shows that *C. variabilis* and *M. reisseri* have co-habited in a *P. bursaria* cell. Group I introns are a distinct RNA group that function as enzymes, splicing themselves out of precursor RNA transcripts and ligating exons. A distinctive character of group I introns is their mobility. Phylogenetic analyses have indicated that introns at homologous gene sites are related (position family), even among distantly related host organisms. This phenomenon is linked to intron spreading mechanisms. Namely, when an intron at a locus of a gene infects a different organism, the new intron will be inserted into the same locus of the gene in which

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it was originally located (For general characters on group I introns, see Cech [18]; Haugen *et al*. [19] and references therein). Group I introns are classified into subgroups IA through IE based on their structural diversity and phylogeny, and nuclear encoding introns belong to subgroup IC or IE [20]. *Chlorella variabilis* is particularly intron-rich, containing eight group I introns (four IC and four IE) in the nuclear rDNA [21], and *M. reisseri* also has two IE introns [22] (**Figure 1**). Due to the intron spreading mechanisms, IE introns have strong insertion bias to limited positions. In green algae, nearly all IE introns are located at S516 (S = SSU rRNA; the numbering reflects their homologous position in the *Escherichia coli* rRNA gene) [20,23]. However, four (S943, L1688, L2184, and L2449; L = LSU rRNA) out of six introns of *P. bursaria* photobionts occupied novel insertion sites. Structural and phylogenetic analyses of these IE introns led to the following extremely bizarre findings: 1) these IE introns are monophyletic and independent of those from other green algae; 2) one intron (L2449) of *M. reisseri* has an archaic state and the other introns are assumed to originate from this intron; 3) the completion of the hereditary line includes two transfer events beyond the species barrier (**Figure 1**). Explaining these intron transmissions requires a special situation in which two algal species have frequently been in contact with each other [22]. It is just as conceivable that there was a long period during which *C. variabilis* and *M. reisseri* lived sympatrically and simultaneously in the *P. bursaria* cell, where cell-cell contact within a small space may accelerate the lateral transfer of group I introns [24,25].

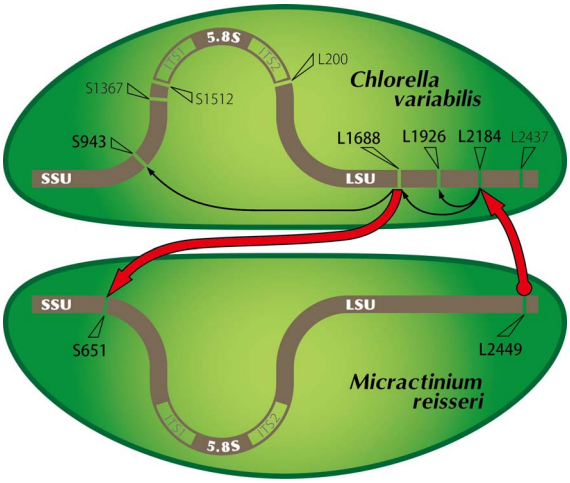

Figure 1. Group I introns intervening in rDNAs of *Chlorella variabilis* and *Micractinium reisseri*. Subgroup IE introns are in bold. Numbering reflects their homologous position in the *Escherichia coli* rRNA gene: S = SSU rRNA, L = LSU rRNA. The transmission contexts of IE introns are indicated by thick (inter specific) and narrow (intra specific) arrows. For details of intron transmission, see Hoshina and Imamura [22].

The present study will introduce some *P. bursaria* strains that maintain more than one photobiont species in a long period of culture. These strains encourage the above intron transfer theory, and possibly indicate the way to photobiont switch of *P. bursaria*.

2. Materials and Methods

2.1. *Paramecium bursaria* Culture

Particular kind of *Paramecium bursaria* strains were maintained in lettuce juice medium [26] under LED illumination (12 h L:12 h D) at 15˚C (**Table 1**). These strains were once collected by Dr. T. Kosaka (Hiroshima University) in 1992 in the United States and have been maintained in a laboratory at the University. The stock cultures were kindly donated by Prof. H. Hosoya (Hiroshima University) to RH in December 2008 and have been cultured for more than four years; therefore symbiotic conditions should be regarded as stable.

2.2. Algal Isolation

Individuals of *P. bursaria* were carefully picked from the surface of the culture medium (to avoid picking up the coccoids on the bottom of the flask, though there were not many), the cells were disrupted and suspended in pure water then spread onto an oligotrophic agar plate (1/5-concentration Gamborg's B-5 Basal Medium with Minimal Organics, Sigma Aldrich, St Louis). Observed colonies were picked and transferred to 1/5 Gamborg liquid medium. These were maintained under LED illumination (12 h L:12 h D) at 15˚C.

2.3. Microscopy

Cells of *P. bursaria* and their symbiotic algae were observed under light microscopy CX31 (Olympus, Tokyo) and photos were taken with an HDCE-31 digital camera (AS ONE, Osaka).

2.4. DNA Extraction, Amplification, and Sequencing

Paramecium bursaria strains with photobionts in each cell were directly used to extract both host and photobiont DNAs. A photobiont strain AG-35_ZF1 isolated from *P. bursaria* AG-35 (**Table 1**) was also used in DNA extraction. DNA extractions were performed using the DNeasy plant mini kit (Qiagen, Düsseldorf).

Whole *P. bursaria* DNA extracts were used to amplify both host SSU rDNA and photobiont SSU rDNA. Host targeting PCR was performed with the primer pair SR-1 (universal [27])/Paramecium800R (host specific [6]), and algae targeting PCR was also performed with CHspeR-maeF (trebouxiophyte specific [6])/INT-5R (trebouxio-

Table 1. *Paramecium bursaria* strains used in this study and their sequence data.

Strain name	Syngen	Mating type	Collection date	Collection site	DNA accession				
					Host	Large band	Medium band	Small band	Isolated alga
AG-35	1	Ⅲ	Aug. 1992	Aquatic Garden, Washington	AB699097	AB699101	AB699105	AB699111	AB699112
BP-11	1	Ⅳ	Jun. 1992	Catonsville, Maryland	AB699098	AB699102	AB699106/107	—	—
DC-3	1	Ⅰ	Aug. 1992	Delaware River, New York	AB699099	AB699103	AB699108	—	—
OLG-3*	2	?	Dec. 1992	(Orlando, Florida)	AB699100	AB699104	AB699109/110	—	—

*Offspring of original strain.

phyte specific [5]) for the photobiont. The PCR product of the host DNA was purified via Quantum Prep PCR Kleen Spin Columns (Bio-Rad, CA) and directly sequenced. The amplified fragments of photobiont DNA were confirmed by agarose gel electrophoresis, collected by excising the fluorescent band, purified using the Qiaex II Gel Extraction Kit (Qiagen), and directly sequenced.

SSU rDNA to internal transcribed spacer 2 of the isolated alga, AG-35_ZF1, was amplified with the primer pairs SR-1/SR-9, SR-6/SR12k, and INT4F/HLR3R (primers are described in Hoshina *et al*. [6]). These were purified via Quantum Prep PCR Kleen Spin Columns, and directly sequenced.

2.5. DNA Sequence Comparisons

Paramecium bursaria and its photobiont DNA sequences were deposited to Standard Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) to find identical or close sequence data.

3. Results

The *P. bursaria* strains here were obviously different from the ordinary ones. Under the microscope, green coccoids of different sizes (ca. 5 μm or 1.5 μm) were clearly seen in *P. bursaria* (strains AG-35, BP-11, and OLG-3) (**Figure 2**). Note that ordinary *P. bursaria* retains a cloned single-species of alga (ca. 5 μm). The number of small balls was much larger than the large ones except DC-3 (small balls were remarkably few). The small coccoids were inserted in between the trichocysts (**Figure 2(c)**).

Next, we conducted algae-targeting PCR (18). Three *P. bursaria* strains (BP-11, DC-3, and OLG-3) produced two length-polymorphic bands, whereas strain AG-35 produced three bands (**Figure 3**). The length polymorphisms were due to the variations in intron insertions. BLAST search indicated the sequences were identical with *C. variabilis* (large bands: AB699101-104), *Chorisystis minor* (medium bands: AB699105-110), and some *Chlorella* and *Micractinium* species (small band: AB699111) (**Table 1**). The amplified region (exon) was fairly conservative among *Chlorella*-related species and we were unable to identify the small band to the species level.

On the oligotrophic agar plates with the *P. bursaria* extract, only small coccoids were obtained from BP-11, DC-3, and OLG-3 extracts. Because *C. variabilis* requires organic nitrogen sources to grow [13], this oligotrophic condition might have prevented its growth. Both sizes of green coccoids were present on the plate from the AG-35 extract. We picked up several green colonies from this plate and transferred each to liquid medium. One of the algal strains, an approximately 5 μm coccoid, was named AG-35_ZF1 and we sequenced its SSU-ITS1-5.8S-ITS2 rDNA. The sequence (AB699112) of the isolated alga, AG-35_ZF1, was very close to those of *C. vulgaris*. An NCBI BLAST Search indicated the closest taxon, *C. vulgaris* CCAP 211/80 (FM205853, covering ITS1-5.8S-ITS2 rDNA), where only two transitions and one indel were found within the ITS1 region. It is likely that this photobiont is *C. vulgaris*.

SSU rDNAs for *P. bursaria* (host) were determined. Of these, three (AG-35, BP-11, and DC3: AB699097-099) were identical and matched some previous sequences we refer to as genotype D [28], whereas OLG-3 (AB699100) differed from the others and matched what we refer to as genotype B.

4. Discussion

Paramecium bursaria usually gains energy by feeding as well as by the photosynthates of photobionts. In general, *P. bursaria* collected from nature may contain more than one green alga. Photobiont and feed are difficult to determine. These algae will be unifiedin the cell of *P. bursaria* during several days of culture conditions. In most cases, the remaining algae (namely, natural photobionts) are either *C. variabilis* or *M. reisseri*. However, the algae

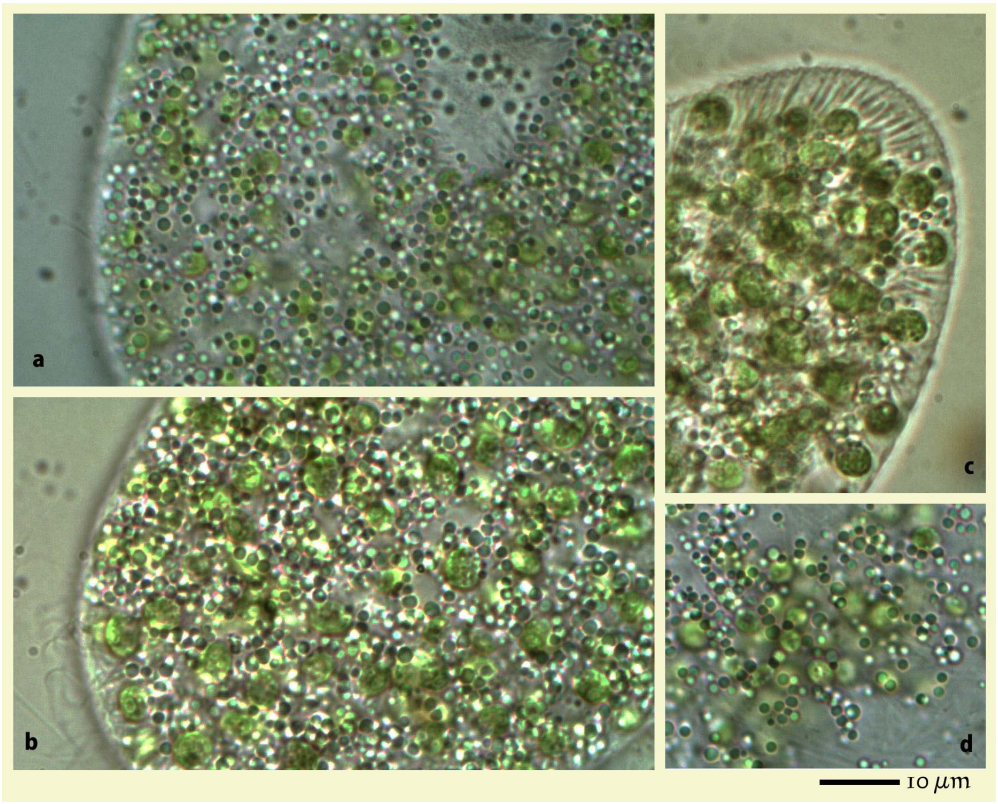

Figure 2. Microscopic images of *Paramecium bursaria* and its contents. a: AG-35; b: OLG-3; c: Head of OLG-3. Both large (*Chlorella variabilis* or *Chlorella vulgaris*) and small (*Choricystis minor*) coccids in between the trichocysts can be seen. d: Sample from AG-35 leakage.

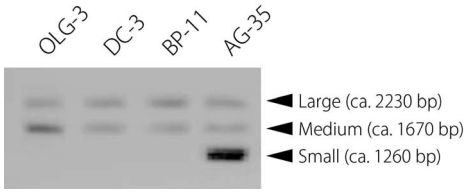

Figure 3. PCR results for four *Paramecium bursaria* strains. This PCR targeted only green algae using the specific primers CHspeRmaeF/INT-5R. Each *P. bursaria* strain produced differently sized bands attributed to intron insertions, which indicate that *Paramecium* maintains two or three kinds of green algal species. Fluorescent values of the bands are presumably influenced by cell wall disruption (*C. variabilis* is indestructible) or primer matching (four out of 20 nucleotides in the forward primer do not match *Choricystis* = medium-size band).

of *P. bursaria* strains cited here have not been unified during 20 years of culture conditions. The mother stock of OLG-3 also has shown the same feature [29]. Microscopic observations showed *C. minor* (small coccoids) were inserted in between the trichocysts. This phenomenon can be thought of as the advanced symbiosis stage rather than feed in the food vacuole [30]. Therefore, it is regarded that these *P. bursaria* strains deal both *C. variabilis* and *C. minor* as their steady photobionts. This situation, namely, stable symbiotic relationships between *P. bursaria* and multiple photobionts, will encourage the hypothetical theory for the group I intron transmitions between *C. variabilis* and *M. reisseri* (**Figure 1**).

The present study also focused on the genotype of the host *P. bursaria*. We reported that *P. bursaria* is separated into A through D genotypes based on SSU rDNA [28]. Genotype D seems to be the first diverged genotype, with differences of at least 12 substitutions and four indels from the others. For these circumstances, Pröschold *et al*. [9] suggested that *P. bursaria* is a complex of several species, similar to the *P. aurelia* complex. We previously found that host genotypes and these photobiont types are closely linked [6], and proposed a *P. bursaria* evolutionary scenario concerning genotype diversification and photobiont choice [31] (**Figure 4**). The strain OLG-3 is the first *P. bursaria* that retains *C. variabilis* among genotypes A to C and this characteristic negates the evolutionary scenario. Instead, the flexibility of the combination of hosts and photobionts becomes apparent. It is possible that *P. bursaria* groups exchange their photobionts in some way. In most cases, the most preferred photobiont is either *C. variabilis* or *M. reisseri*. Geographic or climatic conditions probably influence the photobiont choice of *P. bursaria*.

Our findings also give us an imagination about how *P. bursaria* have replaced their photobionts. Compared to other protozoa that carry coccoid green photobionts, the host-symbiont relationship is much stronger in *P. bursaria*. In many of the other protozoa, the species of photobionts depends on environment (e.g., lake, pond) rather than host species [10]. *P. bursaria* carries the symbiotic algae throughout its life cycle even during cell division and sexual reproduction [17]. *Paramecium bursaria* has lost none of its ability to take in algae as new symbionts. Consequently, algal switching can occur in two ways. The first mechanism is "new gain after symbiont loss" (**Figure 5**). Alternatively, we propose another process for algal switching: "choice after co-symbiosis" (**Figure 5**). Because *P. bursaria* maintains its re-symbiosis ability, it is possible that it routinely tries to achieve other symbionts through feeding and temporary symbiosis. As mentioned above, *C. variabilis* and *M. reisseri* are heavily dependent on *P. bursaria*. However, the photobionts are not indispensable to *P. bursaria*. In other words, *P. bursaria* is in a position to be selective about photobionts. The *P. bursaria* strains shown here (**Figure 2**) can be thought of a process to choose for a better partner. Although *C. variabilis* and *C. vulgaris* are not distinguishable under microscopic observation, *P. bursaria* AG-35 showed the triple photobiont status of *C. variabilis*, *C. vulgaris*, and small *Choricystis* (**Figure 3**). *Chlorella vulgaris* is a well-known cosmopolitan coccoid and *Choricystis minor* is the most common eukaryotic picoalgae in freshwater environments[e.g., 32,33]; therefore, *P. bursaria* can ingest them at any time. Photosynthate contributions from *C. vulgaris* or *Choricystis* in *P. bursaria* are not known, however this must have occurred in the event of symbiont switching given that *P. bursaria* possessing *C. vulgaris* have been found [3,9].

In a single host individual, multiple symbiont species performing similar-functions often have negative effects on the host's growth [34]. However, if it is regarded as a phase for determining a more optimal partner, it can be an advantage for survival in the long term.

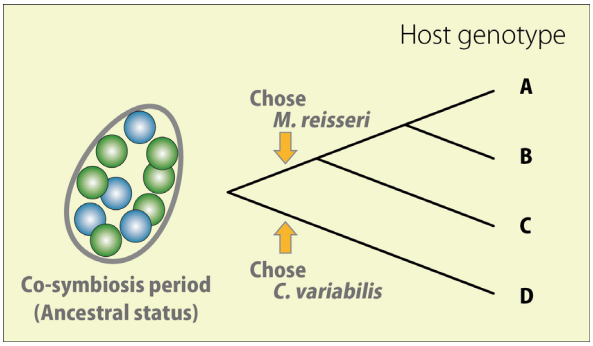

Figure 4. An evolutionary scenario for *P. bursaria* concerning genotype diversification and photobiont choice proposed by Hoshina and Imamura [31].

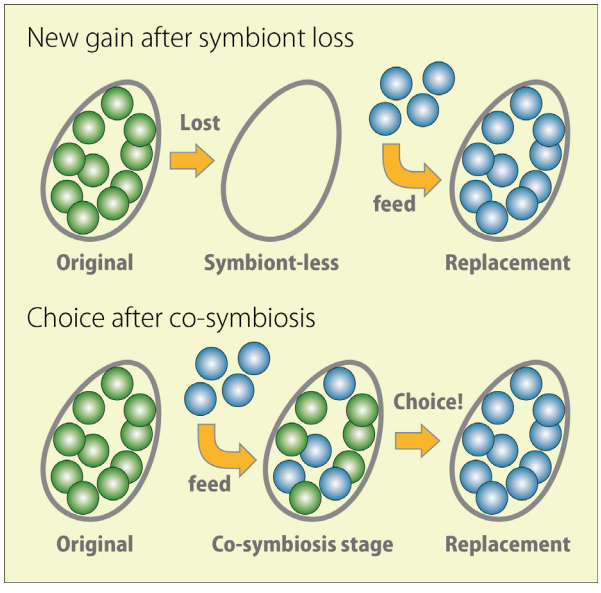

Figure 5. Two possible contexts in which *Paramecium bursaria* may switch photobionts.

5. Acknowledgements

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