

An atypical *Chlorella* symbiont from *Paramecium bursaria*

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Summary

A useful approach to rapid identification of *Chlorella* strains in a single cell of *Paramecium bursaria* was developed. A “nested” PCR-based method with primers flanking intron insertion sites in the 18S rRNA gene of *Chlorella* ensured fast screening of large *P. bursaria* collections for the presence of the two basic types of *Chlorella* endosymbionts without isolating them in pure culture. The method applied to several *Paramecium* isolates showed that the *P. bursaria* clone T-24-5 from Tajikistan presumably bears an atypical *Chlorella* symbiont possessing an atypical intron in the 18S rRNA gene. This sequence was cloned and sequenced. BLAST analyses of the intron sequence cloned showed the presence of 136-bp stretch highly similar to the *Chlorella* virus intron fragment in major capsid protein Vp54 gene. The similarity value indicated a common origin of the sequences and suggested that the horizontal transfer of this DNA fragment had happened between the *Chlorella* and the virus attacking it.

Key words: *Paramecium bursaria*, *Chlorella*, PBCV, 18S rRNA gene, group I intron

Abbreviations: PBCV – *Paramecium bursaria* *Chlorella* virus

Introduction

Unicellular green algae, also called zoochlorellae, are part of the triple symbiotic system “*Paramecium bursaria* – *Chlorella* sp. – PBCV virus (*Chlorovirus*, *Phycodnaviridae*)” (Migunova et al., 1999). One cell of *P. bursaria* contains several

hundred green algae in the cytoplasm, as forage or endosymbionts. Each algal cell is enclosed in an individual perialgal vacuole, protecting it both from the ciliate’s digestive enzymes and from contacts with the virus. Apart from virus protection, *P. bursaria* supplies its endosymbionts with some vitamins and nitrogen-containing organic substances. The

Table 1. *Paramecium bursaria* clones used in this work.

Clone	Origin	Collection
G12	Russia, St. Petersburg	St. Petersburg University, K.P. Vorobyev
E1	Russia, St. Petersburg	St. Petersburg University, K.P. Vorobyev
PA-2-2	Russia, Altay	Biological Research Institute of St. Petersburg University, M.S. Rautian
26-1	Russia, Novgorod	Biological Research Institute of St. Petersburg University, M.S. Rautian
T-24-5	Tajikistan, Dushanbe	Biological Research Institute of St. Petersburg University, M.S. Rautian
Bal	Ukraine, Balaklava	St Petersburg University, Migunova
Cs-2	Japan	Biological Research Institute of St. Petersburg University, M.S. Rautian
Ard-5	USA	Biological Research Institute of St. Petersburg University, M.S. Rautian
Ard-16	USA	Biological Research Institute of St. Petersburg University, M.S. Rautian

algae, in turn, produce for their host photosynthetic products – oxygen and sugars (maltose and glucose), so the symbiosis is practically obligate. Zoochlorella strains isolated from ciliates were divided into two types: “Southern” and “Northern” according to preferred temperature conditions and specific viruses hosted by them. Viruses of “Southern” type infect only “Southern” type of symbiotic *Chlorella*, whereas “Northern” type of symbiotic *Chlorella* is resistant to these viruses, and vice versa (Linz et al., 1999; Migunova et al., 1999, 2000; Van Etten, 2003, Kvitko et al., 2001, 2004).

All symbiotic *Chlorella* types (northern and southern ecotypes) and intron-bearing free-living forms are highly diverse regarding the number of introns and their location in the 18S rRNA gene (Hoshina et al., 2004, 2005; Gaponova et al., 2006; Vorobyev et al., 2006, 2007) (Fig. 1). Thus, introns in the 18S rRNA gene of *Chlorella* are good candidates for a taxonomic marker (in the “presence-absence” style); they are also a promising area for evolutionary investigations.

The total DNA from a *Paramecium* culture was already used for symbiotic *Chlorella* detection by Tanaka with colleagues (2002), but to simplify identification of *Chlorella* species in *P. bursaria* we have developed a “nested” PCR-based technique allowing identification of *Chlorella* ecotypes in a single cell of this ciliate. Such a method could be very useful for screening large collections of *Paramecium* (and other protists) for *Chlorella* symbionts, since it does not require the time-consuming isolation of zoochlorella in pure culture. Initially, we intended to report only the details of the method developed. However, when the method was applied to a *Paramecium* collection an unusual algae was detected. The 18S rRNA gene of this putative *Chlorella* symbi-

ont had an atypical intron containing 136-bp stretch highly similar to *Chlorella* virus intron fragment in major capsid protein Vp54 gene. The last finding turned out to be the main object of the article.

Material and Methods

The ciliates (Table 1) were cultivated at room temperature on lettuce medium inoculated with *Enterobacter aerogenes* at 15–22° and illuminated by luminescent lamps (2000–4000 lux) (Sonneborn 1970). Pure *Chlorella* DNA representing the southern (*Chlorella* sp. strain NC64A (USA, Nebraska University)) and the northern (*Chlorella* sp. strain SAG 241-80 (Germany, SAG collection)) ecotype were used as control in each experiment.

Primer design. As the quantity of DNA extracted from a single cell of *Paramecium* is very low, the “nested” PCR was used for amplification of the target sequences. Three primer pairs have been designed (Tab. 2, Fig. 1). Primers 3F/4R were used in the first round of the nested PCR. Primers SEV_EX_F/SEV_EX_R and YUG_EX_F/YUG_EX_R flanking introns in the “Northern” and the “Southern” *Chlorella* ecotypes were used in the second round of the nested PCR, allowing ecotypes identification depending on fragment sizes (longer fragments with intron and shorter intronless ones). According to the results of the “in-silica” test, all the primers designed should fit perfectly for amplification of the target sequence in almost all known *Chlorella* – both free-living ones and *Paramecium* inhabitants.

DNA manipulation. From one to five cells of *P. bursaria* containing symbiotic *Chlorella* were taken with the pipet tip from the liquid culture medium,

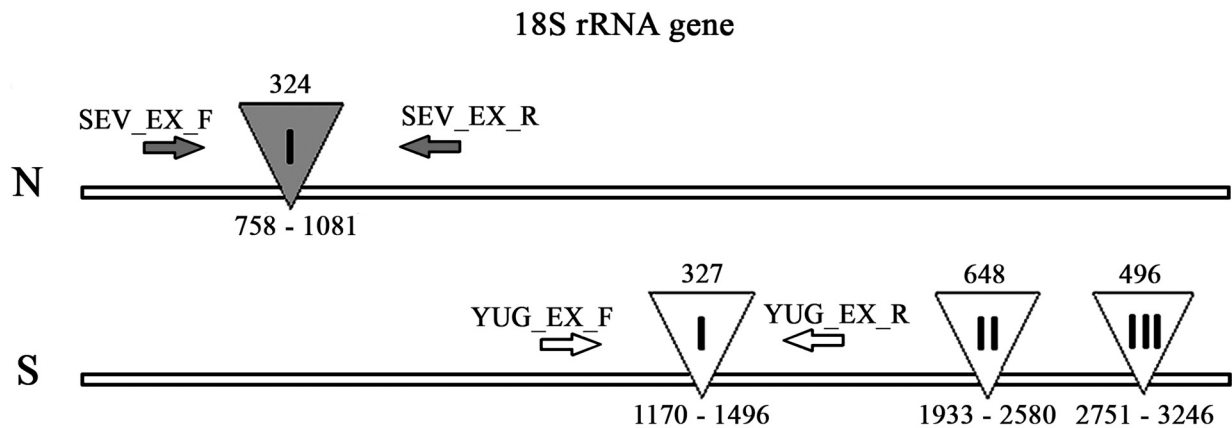


Fig. 1. Localization and size (bp) of introns in two basic types of symbiotic *Chlorella*. N is the northern ecotype symbiotic chlorella with a single intron, and S is the southern ecotype of chlorella with three introns. Arrows show 18S rDNA primers used for detection of *Chlorella* spp. Intron insertion sites are given according to *C. kessleri* strain SAG-211-11g (X56105).

washed thoroughly in sterile water and placed in a PCR-tube. Thirty μ l of milliQ water and glass beads ($d=0,35$ mm, 1/3 of total milliQ water volume) were added. The mix was homogenized (two times for 60 seconds) in FastPrep 24 system (MP Biomedicals) with maximal intensity. The suspension was incubated at 95°C for 5 min, shortly vortexed and centrifuged at 16000g for 2 min. Seven microliters of the supernatant was used as a DNA-matrix in the 50 μ l PCR-mix with 3F / 4R primers pair with standard thermal cycle (3 min at 95°C, then 30 cycles – 30 s at 94°C, 30 s at 56°C and 1 min at 72°C and additional extension for 5 min at 72°C). All 18S rRNA fragments were amplified in MyCycler (BioRad) thermocycler with Taq polymerase and buffer supplied (Helicon, Moscow, Russia). One μ l of the PCR product resulting from the first round was used as the DNA matrix in the second PCR round with two primer pairs flanking “northern” and “southern” intron insertion sites. This second step was carried out under the same conditions. Pure *Chlorella* DNA representing southern and northern ecotypes was used as control in each experiment.

For sequencing, the fragment of interest (T-24-5 strain) was excised from gel, purified and cloned into pTZ57R/T plasmid (Ins T/A clone TM PCR Products Cloning Kit, Fermentas). The cloned fragment (three different clones) was sequenced with M13 standard primers by using Beckman Coulter CEQ 8000 sequenator, according to the manufacturer instruction. The sequence obtained was submitted to GenBank (EU281549).

Sequence analyses. To determine intron size and its location, the sequence was aligned with intronless 18S rRNA gene of *Chlorella kessleri* strain SAG-211-11g (X56105). BLAST analyses were used for nucleotide similarity search (Altschul et al., 1990).

Results

The method developed works well with a single *P. bursaria* cell (data not shown), but for the better reproducibility in experiments we used up to five cells. The method in this modification was applied to seven different clones of *P. bursaria* of various origins

Table 2. Primers used in this work.

Primer	Sequence	Reference
3F	5'-AACTGCGAATGGCTCATTA	Kvitko et al., 2006
4R	5'-ATCAACCTGACAAGGCAACC	Kvitko et al., 2006
SEV_EX_F	5'-ATTCCAGCTCCAATAGCGT	This work
SEV_EX_R	5'-CCCGACYGTCCCTWTTAA	This work
YUG_EX_F	5'-GAAAGAYGAACYDCTGCGAAAG	This work
YUG_EX_R	5'-ACAAGGCAACCCACCAAC	This work

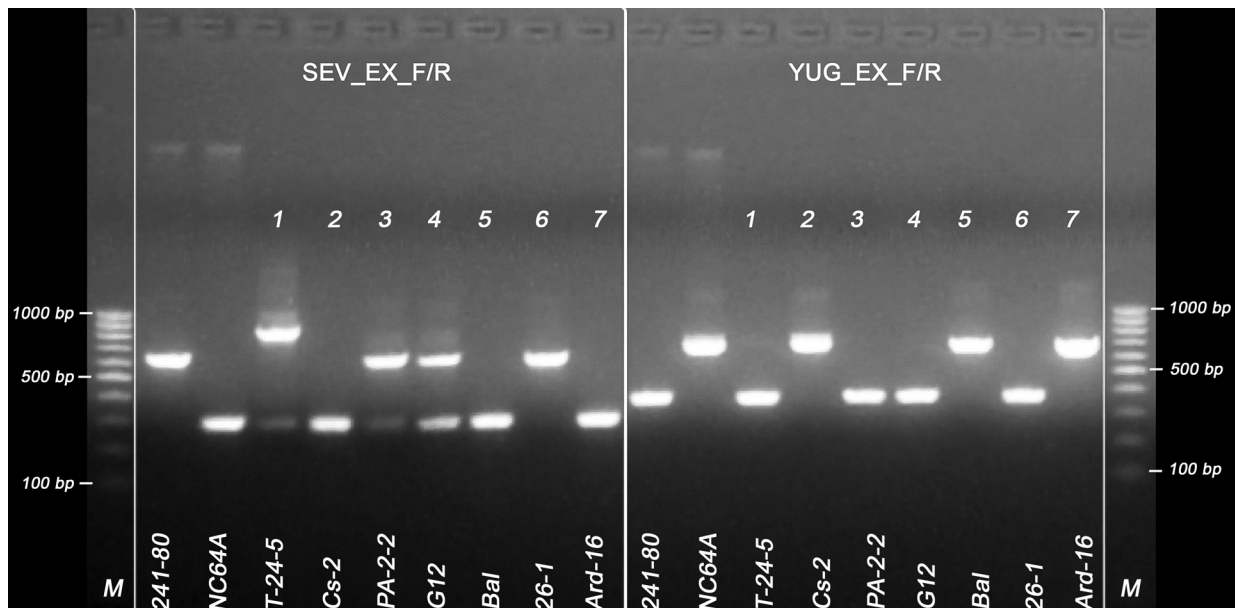


Fig. 2. 18S rRNA gene fragments corresponding to symbiotic and free-living *Chlorella* amplified in the second round of PCR from different *Paramecium* clones. 1-7 lanes – clones of *P. bursaria*, 241-80 and NC64A – controls, M – marker Ladder 100 bp.

(Table 1). As a result of “nested” PCR, one or two amplified fragments were detected (Fig. 2).

Fig. 2 represents the results of screening of seven *P. bursaria* clones for different ecotypes of symbiotic *Chlorella*. Ciliate clones are clearly distinguished by the products specific for the “Northern” (lanes # 3, 4, 6 – primers SEV_EX_F/R) or “Southern” (lanes # 2, 5, 7 – primers YUG_EX_F/R) ecotype of symbiotic *Chlorella*. The sizes of these products correspond to the sizes of intron-bearing 18S rRNA gene of the “Northern” and the “Southern” symbiotic *Chlorella* ecotypes (600 and 650 bp correspondingly). In several cases (lanes # 1, 3, 4) there were additional shorter bands specific for intronless fragments, presumably originating from nonsymbiotic *Chlorella* species inhabiting *Paramecium* cells.

In T-24-5 *P. bursaria* clone we detected a “strange” fragment of 18S rRNA gene with an unusual molecular size (~ 850 bp) amplified with SEV_EX_F/SEV_EX_R pair primers (Fig. 2, line 1). It is not the only “strangeness” of this fragment. This *P. bursaria* clone was isolated in Tajikistan, where only the “Southern” *Chlorella* ecotype has been found so far. Nevertheless, the PCR product detected presumably contains an intron with a location characteristic of the “Northern” ecotype. For a more precise analysis, this DNA fragment was purified, cloned and sequenced. Its alignment with the intronless 18S rRNA gene of *Chlorella kessleri* strain SAG-211-11g (X56105) demonstrated intron presence in the fragment analyzed.

The location of the intron detected did not differ from that in the “Northern” type, but the T-24-5 intron size was significantly larger (554 bp) and its nucleotide sequence had nothing in common with the intron of the “Northern” type. Finally, the 16 bp direct repeat flanking the intron was identified. It is of special interest that the repeat identified is an imperfect one (single nucleotide deletion/insertion). It means that the exact intron boundaries could be established only after sequencing the corresponding cDNA. In summary, our results presumably indicated detection of a new type of symbiotic *Chlorella* that differs from the “Southern” and the “Northern” ecotype. BLAST search (Altschul et al., 1990) showed that the closest identified relatives of the exon part of the cloned sequence are the well-known symbiotic *Chlorella* sp. NC64A (DQ057341) and several free-living *Chlorella* species. The intron sequence did not show any significant similarity to the known sequences when standard BLAST search parameters were applied (except three distantly similar fungal ribosomal sequences with the similarity region from 33 to 113 bp). The most interesting was the result of BLAST analysis of the intron sequence against the virus database with “soft” parameters (“somewhat similar sequences” search). In this analysis we detected the presence of a 136-bp region highly similar (expectation 10^{-16} , identities 108/140 (77%)) to the fragment of group I self-splicing intron of major capsid protein Vp54 gene of *Chlorella* virus strain CVT2 (AB006978) (Fig 3). It is especially

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