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Protistology

Genomic dactyloscopy of *Chlorella* sp., symbionts of *Paramecium bursaria*

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Summary

The exon nucleotide sequences of the last part of the 18S rRNA gene in the northern and the southern ecotypes of zoochlorella strains (in the interval from 1315 to 1766 sites in accordance with sequence X56105 of *Chlorella kessleri* strain SAG-211-11g) were compared with the exon sequence of this gene in different *Chlorella* strains. These two ecotypes were shown to be the closest neighbours of *Ch. vulgaris, Ch. sorokiniana, Ch. lobophora.* All strains of northern and southern ecotypes were different from free-living *Chlorella vulgaris* in having introns in the first part of the 18S rRNA gene. The southern zoochlorellae had at least one more intron in middle part of the gene. We assume that in the course of evolution the ancestors of the northern zoochlorellae (*Ch. vulgaris*) got an intron (of the size about 330 nucleotides in the interval of 106-1315 b.p. of the 18S RNA gene), and southern zoochlorellae have got several introns, one similar to that of the northern ecotype in size and position and the others in the part of the gene, 1276-1766 (the size 647 nucleotides) and 495 b.p (according to the data of Hoshira et al., 2004, 2005) at the end of the gene.

Key words: *Chlorella*, PCR, 18S rRNA, phylogeny, introns, ecotypes of zoochlorella

Introduction

Unicellular green algae, zoochlorellae, are a part of the symbiotic system "*Paramecium bursaria* - *Chlorella* sp. - PBCV virus (*Chlorovirus, Phycodnaviridae*)". The cytoplasm of a *Paramecium bursaria* individual may contain several hundreds of zoochlorella cells. 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.

According to their sensitivity to specific viruses, zoochlorella strains isolated from ciliates were divided into two types: NC64A and Pbi (Van Etten, 2003). We refer to them, respectively, as the "southern" and the "northern" ecotype (Kvitko et al., 1996, 2001, 2004; Migunova et al., 1996, 1999, 2000). It is not clear whether these two zoochlorella ecotypes belong to the same species or to different species. In cell morphology, the northern and southern *Chlorella* sp. are close to *Chlorella vulgaris* (Reisser et al., 1988), whereas in cell wall structure they are close to the *Ch. vulgaris/Ch. sorokiniana* group. Various strains of the same ecotype are identical in numerous physiological-biochemical parameters (Kessler et al., 1991) and other characteristics, such as sugars secretions, size of protein markers, 8 isoenzyme patterns (Linz et al., 1999) and surface antigens (Migunova et al., 1992), but strains of separate ecotypes are different. Genomic dactyloscopy by UPPCR-patterns (Migunova, 2002) allows one to divide zoochlorella strains into two different types (the northern and the southern ecotype) or, probably, even into two different species. The aim of our investigation was to reveal differences and similarities between zoochlorella ecotypes at the genomic level.

Material and Methods

Algal strains and culture conditions

We used virus-sensitive and virus-resistant *Chlorella* sp. strains isolated from *P. bursaria* as well as free-living *Ch. vulgaris* from the CALU collection of the Biological Research Institute (St. Petersburg State University) (Table 1). All strains were grown in standard BBL medium diluted to 1:4 (1.25 g of gelatin, 0.75 g of meat extract and 15 g of agar for 1 l of water), in tubes with agar slants at 15-28°C. Illumination was 2000-4000 lux.

For strain cultivation mineral media BBM was used: $NaNO_3$ - 250 mg/ml, KH_2PO_4 - 175 mg/ml, K_2HPO_4 - 75 mg/ml, $CaCl_2 \cdot H_2O$ - 25 mg/ml, $MgSO_4$ - 75 mg/ml, NaCl - 25 mg/ml, microelements - 1 ml); BBM + AP (10 % aminopeptid); BPS: BBM+peptone - 1 g/l, sucrose - 5 g/l. For preparation of solid nutrient medium BPS, 15 g/l of agar was taken. Flasks with a volume of 250-300 ml filled with 200 ml of liquid media BBM, enriched with AP (10 %), or Petri dishes with solid BPS nutrient medium were used for cultivation. They were kept at 15-28°C and illuminated by luminescent lamps (2000-4000 lux).

DNA manipulation

DNA isolation from *Chlorella* strains was performed with "Nucleon PhytoPure" plant and fungal DNA extraction kits (Amersham) according to the producer's instructions. 18S rRNA fragments were amplified using MyCycler (BioRad) thermocycler and DyNAzyme II DNA Polymerase (Finnzymes). The primers used are listed in Table 2. All PCRs were performed with the following temperature profile: an initial denaturation at 95°C for 3 min, 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), extension (1 min at 72°C) and final extension at 72°C for 3 min. Amplified DNA was examined by electrophoresis in 1% agarose gel.

Aliquots of the DNA amplified were digested with *Hae*III and *Msp*I restriction endonucleases. The restriction fragments were separated in 4% agarose gels, stained with ethidium bromide and photographed with the Kodak EDAS290 system. 100 b.p. DNA ladder was used as a molecular weight marker. For sequencing, the fragments amplified were cloned in pTZ57R/T plasmid, "Ins T/A clone TM PCR Products Cloning Kit" (Fermentas) was used. The fragments cloned were sequenced with ABI-310 DNA sequencer according to the producer's instructions. RFLP tree was constructed with the help of MEGA version 3.0 (Saitou and Nei, 1987; Felsenstein, 1985).

Nucleotide sequences determination

The sequences obtained were submitted to the Gene Bank (accession numbers AY876290-AY876301). Intron locations were determined by aligning sequences obtained with the 18S rRNA gene of *Chlorella kessleri* strain SAG 211-11g (accession X56105) by using ClustalX program (Thompson et al., 1997). For 18S rRNA tree construction all introns were excised from sequences. The tree including some *Chlorella* representatives was constructed by simple estimation (number of differences) and the neighbor-joining method using MEGA version 3.0 (Saitou and Nei, 1987; Felsenstein, 1985). Bootstrapping with 1000 samplings was used to evaluate clusterization.

Results

According to the results of PCR amplification with 3F/4R and 6F/HR primers, the *Chlorella* strains investigated can be divided into three groups by the size of the fragments amplified (Fig.1 and 3). Comparison between the free-living *Chlorella vulgaris* strain, the northern ecotype and the southern ecotype has shown that the latter group had an insertion (more than 300 b.p.) between positions 106 and 1296 ns (Fig.1).

To study genetic relatedness of the strains, the restriction analysis of 18S rRNA gene fragments amplified with primers 3F/4R was performed. *Msp*I and *Hae*III restriction fragments turned out to be strictly specific for the three *Chlorella* groups studied. Moreover,

Table 1. Strain description.

Strain abbreviation	Year of isolation, provenance, author and characteristics of the strain
Strains sensitive to NC64A type viruses (Van Etten, 2003), or southern ecotype (Kvitko et al., 1996, 2004)	
NC64A	1963; the USA; M. Karakashjan and L. Muskatin. Received from J.L. Van-Etten. The type strain for the southern ecotype.
Ac-21SCβ21	1988; subclone of str. NC64A (Migunove et al., 1002). Selected as resistant (able to growth on solid media with addition of streptomicin, canavanin, β-alanin).
211-6	1934; the USA; Loeffer strain. Received from Goetingen in 1990, SAG-211-6
N-1-A	1986, the USA, R. Meints (state Nebraska), received from J.L.Van-Etten
Strains sensitive to Pbi type viruses (Van Etten, 2003), or the northern ecotype (Kvitko et al., 1996, 2004)	
OCH	1985; Karelia, Loukhsky area, Lake Cherlivoe; E. Kraeva
OCH cr4; OCH cr 6	1999; subclones of str. OCH (Canavanin-resistant mutants); M.J. Prokosheva and A.V. Migunova.
OS-1 and OS-6	1999; Karelia, Loukhsky area, island Sredny; M.J. Prokosheva and A.V. Migunova
241-80	1974; Germany, Goettingen, a pond in the Botanical garden; W. Koch
Pbi	1974, Germany, Goettingen, the same pond in the Botanical garden; W. Reisser. The type strain for the northern ecotype
Strains of free-living *Chlorella vulgaris* insensitive to viruses of both types	
CALU-183	1892; M. Beijerinck. The type strain of *Chlorella vulgaris*. Received from Cambridge Collection in 1964. SAG211-11b=UTEX259=CCAP211-11b=CALU183
CALU-157	1962. from loamy ground of r. Kuban', Russia. Isolated and identified (as *Chlorella vulgaris*) by B.V. Gromov.

*Hae*III enzyme allowed the determination of genotype differences in free-living *Chlorella vulgaris* strains and in the northern ecotype strains (Fig. 2).

However, the level of homology inside zoochlorella groups was very high. The difference in restriction patterns between the ecotypes might be determined by insertions of introns.

Zoochlorella strains of southern ecotype, in their turn, had an insertion (more than 500 b.p.) between positions 1315 till 1750 compared to the free-living Chlorella vulgaris and zoochlorella strains of north ecotype (Fig. 3). The insertions detected are presumably introns, that can be found occasionally in 18S rRNA genes of different Chlorella species (Huss et al., 1999).

Presence or absence of introns in these two positions can be used to distinguish between free-living *Chlorella vulgaris* (no introns), zoochlorella of the northern ecotype (one intron) and zoochlorella of the southern ecotype (two introns). However, presence of introns in other positions in the end of the 18S rRNA gene of the strains in question cannot be ruled out. To do so, nucleotide sequences of the 18S rRNA gene of

Table 2. Primers used in the work.

Primer	Nucleotide sequence	Location*)	Reference
1F	5'-WACCTGGTTGATCCTGCCAGT-3'	**1-21**	Huss et al., 1999
3F	5'-AACTGCGAATGCCTCATTAAA-3'	**86-106**	this work
6F	5'-ATGGCCGTTCTTAGTTGGTG-3'	**1276-1295**	this work
2R	5'-GTAGGTGAACCTGCAGAAGGATC-3'	**1773-1795**	this work
4R	5'-GGTTGCCTTGTCAGGTTGAT-3'	**1296-1315**	this work
5R	5'-AAACGGCTACCACATCCAAG-3'	**399-418**	this work
HR	5'-GGAGAAGTCGTAACAAG-3'	**1750-1776**	Huss et al., 1999

*) Here and in the following text all positions are given in accordance with sequence of 18S rRNA strain SAG-211-11g (X56105).

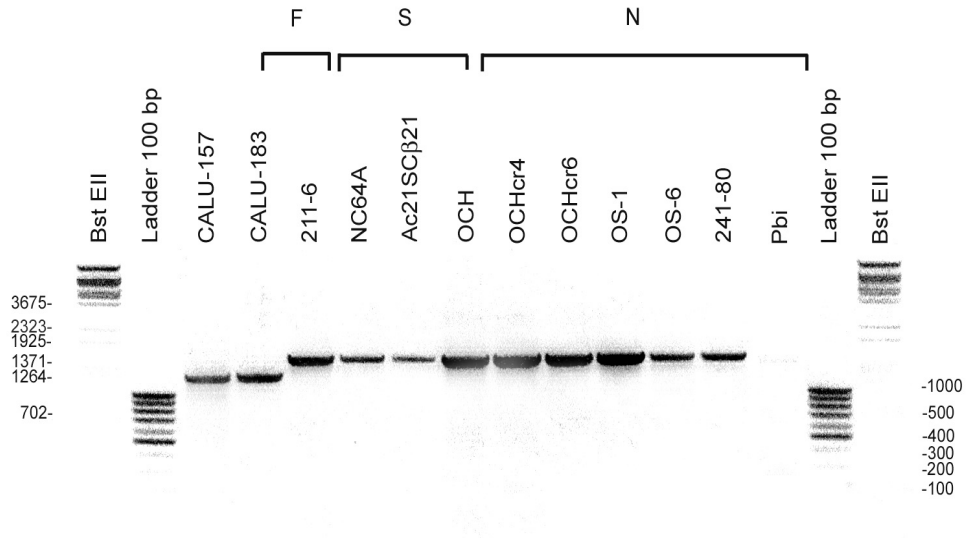

Fig. 1. Amplificates of the first part of the 18S rRNA gene (from 106 till 1296 sites) of free-living *Chlorella vulgaris* (F), southern (S) and northern (N) zoochlorella strains. Only CALU-157 and CALU-183 do not have introns in this part of the gene. Markers - λBstE-III, Ladder 100 b.p.

Chlorella strains belonging to separate species were aligned with the gene sequence of the *Ch. kessleri* strain SAG 211-11g lacking introns. Besides, the BLAST programme revealed, after intron sequences of the southern zoochlorellae (strains NC64A, Ac21ckβ21 and N-1-A) were used as query, a zoochlorella strain So13-7k of the Japanese origin (Hoshina et al., 2004), whose 18S rRNA gene had 3 introns. Altogether, the search produced 4 types of introns, with different localization sites. Table 3 presents the list of symbiotic *Chlorella* strains containing introns that were investigated in the present work.

The above difference in the number of introns between zoochlorella strains allows us to make an assumption about the origin of the northern and the southern ecotype at the level of the 18S rRNA gene. The 18S rRNA gene of zoochlorellae appears to have originated from the homologous intron-lacking gene of free-living *Chlorella* by means of consecutive inclusion first of one intron (northern zoochlorellae) and then of other introns (southern zoochlorellae) (Fig. 4).

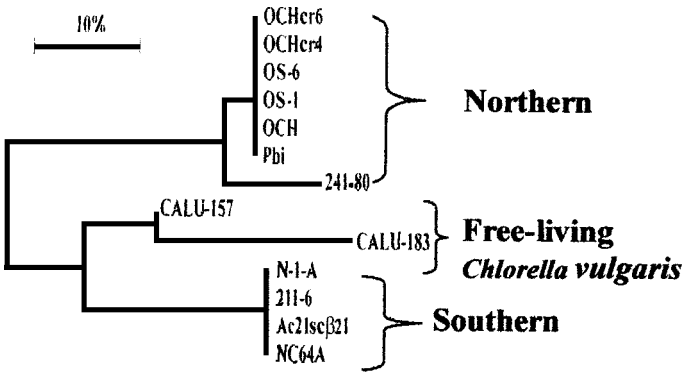

Fig. 2. Similarity tree of the southern and the northern zoochlorella ecotype and Chlorella vulgaris, inferred from restriction pattern of the first part of the 18S rRNA gene sequence (106-1296 ns, accession numbers AY876290-AY876301).

According to T. Yamada (Yamada et al., 1994), intron introduction into the 18S rRNA gene of zoochlorellae can be caused, directly or indirectly, by transfer by the third party involved in symbiosis, the virus. BLAST search demonstrated the identity of the rRNA fragment sequenced (1142 b.p.) with 18S rRNA gene fragments of six *Chlorella* isolated from *Paramecium bursaria* in Japan (accessions AB162912-AB162917, Hoshina et al., 2004). This fact, in combination with the presence of an additional intron between positions 106 and 1296, gives us reasons to suppose that the strains of the southern ecotype studied in our work are close relatives of the *Chlorella* strains from Japan.

By the program BLAST, using as inquiry intron sequences of southern zoochlorellae, strains NC64A, Ac21SCβ21 and N-1-A, we have found out that zoochlorella strains, So13-Zk (and some others) of Japanese origin (AB162 912-AB162917; Hoshina et al., 2004, 2005), containing three introns, are highly similar to 18S rRNA gene fragments of southern zoochlorellae, studied in this work.

On the basis of 18S rRNA gene sequences (the 3' exon part), a genetic tree was constructed

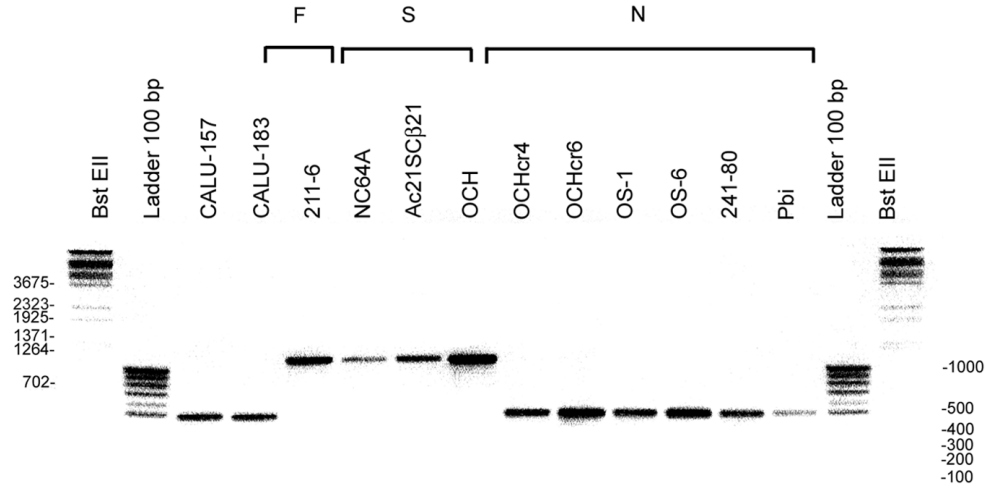

Fig. 3. Amplificates of the middle part of 18S RNA gene (from 1315 till 1766 sites). Only southern zoochlorella (group S) have the insertion. Markers - λBstE III, Ladder 100 b.p.

by the neighbor-joining method (Fig. 5). The tree topology together with the bootstrap values shows that all the strains of the northern and the southern ecotype are closest to 3 species of *Chlorella (sensu stricto): Ch. vulgaris, Ch. lobophora* and *Ch. sorokoniana*. While the northern zoochlorella strains are closer to *Ch. vulgaris* and *Ch. sorokiniana*, the southern strains are closer to *Ch. lobophora* and form, together with the Japanese strain So13-7k, a separate cluster with a high bootstrap value. These findings suggest that the northern and the southern ecotypes may belong to two different species. We will be able to tell whether this is indeed so after complete rRNA sequencing.

Acknowledgments

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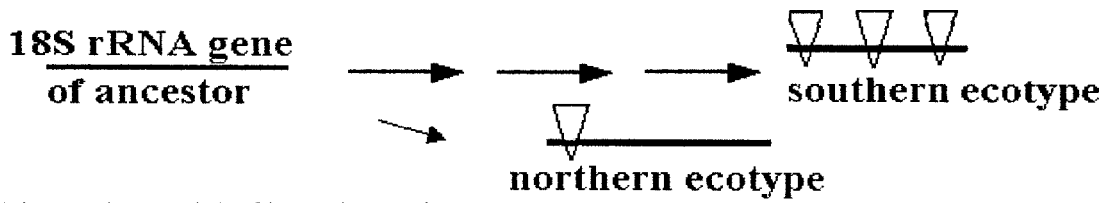

Fig. 4. The model of insertions of introns into the 18S rRNA gene of zoochlorella, ∇ - intron.

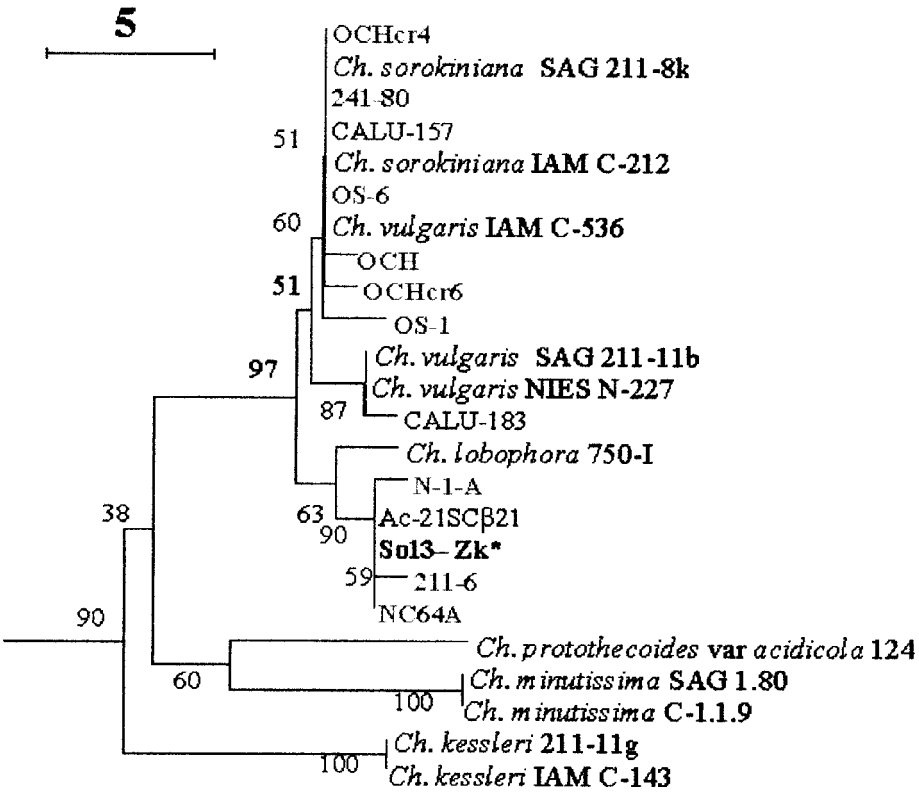

Fig. 5. Phylogenetic tree inferred from partial 18S rRNA sequence zoochlorella of southern, northern ecotypes and free-living *Chlorella*. * - zoochlorella strains So13-Zk the Japanese origin. Scale - number of substitutions per studied part of exon. Accession numbers of free-living *Chlorella*: *Ch. sorokiniana*, SAG211-8k - X62441; IAM C-212 - AB080307; *Ch. vulgaris*, CALU-157 - AY876301; IAM C-536 - AB080308; SAG 211-11b - X13688; NIES N-227 - AB162910; CALU-183 - AY876300; *Ch. lobophora*, Andeeva750-I - X63504; *Ch. protothecoides* var. *acidicola*, str. 124 - AJ439399; *Ch. minutissima*, SAG1.80 - AB006046; C-1.1.9 - X56102; *Ch. kessleri*, 211-11g - X56105; IAM C-143 - AB162911.

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