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Isolation and Cultivation of Endosymbiotic Algae from Green Hydra and Phylogenetic Analysis of 18S rDNA Sequences*

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Symbiotic associations are of wide significance in evolution and biodiversity. The green hydra is a typical example of endosymbiosis. In its gastrodermal myoepithelial cells it harbors the individuals of a unicellular green algae. Endosymbiotic algae from green hydra have been successfully isolated and permanently maintained in a stable clean lab culture for the first time. We reconstructed the phylogeny of isolated endosymbiotic algae using the 18S rRNA gene to clarify its current status and to validate the traditional inclusion of these endosymbiotic algae within the *Chlorella* genus. Molecular analyses established that different genera and species of unicellular green algae could be present as symbionts in green hydra, depending on the natural habitat of a particular strain of green hydra.

Key words: Endosymbiotic alga, green hydra, molecular phylogeny, symbiosis, taxonomy.

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The green hydra (*Hydra virdissima* Pallas, 1766) is a typical example of endosymbiosis. In its gastrodermal myoepithelial cells it harbors the individuals of a unicellular green alga, *Chlorella* (HABETHA *et al.* 2003). Each alga is embedded in an individual vacuolar membrane (O'BRIEN 1982) called a symbiosome. It is believed that all endosymbiotic algae in one host represent clones (DOUGLAS 1994). Inside the gastrodermal myoepithelial cells of green hydra, algae are regularly stacked to form columns, one alga above the other, in the basal part of the cell, towards the mesoglea (POOL & MUSCATINE 1980).

Thus far it has been accepted that endosymbiotic algae from green hydra are impossible to grow in long-term clean culture (HABETHA & BOSCH 2005; MCAULEY & SMITH 1982). *Chlorella* is a large and complex polyphyletic genus whose systematics and taxonomy are extremely complicated and without consensus (HUSS *et al.* 1993). Algae of the *Chlorella* genus are morphologically indistinguishable. The futility of the systematization of free-living and symbiotic species into the same categories demands revision of the taxonomical status of this genus. Presently, the 16S rRNA and 18S rRNA genes are used almost exclusively in the phylogeny of symbiotic organisms (NAKAHARA *et al.* 2004) as suitable, highly conserved and reliable molecular markers that evolve slowly and are functionally preserved (EARDLY & BERKUM 2004). In *Chlorella* research, the 18S rRNA gene marker has become essential (HUSS & SOGIN 1990; HUSS *et al.* 1999; KRIENTIZ *et al.* 2004).

The main goal of this study was to successfully isolate the endosymbiotic algae from green hydra and to sustain them in permanent and stable lab culture. Sequencing of the 18S rRNA gene from endosymbiotic algae and its comparison with homologous algal genes from GenBank gave a clear and definitive insight into the phylogenetic and taxonomic status of the symbiosis.

Material and Methods

Collection and culture

Hydras were collected from two localities, Jarun Lake (strain S1J-J1) and the greenhouse of the Botanical Gardens of the Faculty of Science, University of Zagreb (strain S1B-S1) from the surface of

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submersed plants. The collected animals were maintained in laboratory cultures in glass dishes that were 11 cm in diameter, 5.5 cm high and had a volume of 350 ml in aerated aquarium water. Green hydras were kept in diffused light (photoperiod 10 hrs light, 14 hrs dark; intensity 15 μmol/m^{2}s), at room temperature of 21^{o}C. They were fed once a week with the nauplia of *Artemia salina*. After each feeding they were transferred into clean aquarium water. Undamaged hydras of the same size and at the same developmental stage without buds were rinsed before the beginning of the experiment. Hydras were not fed during the experiment.

Isolated endosymbiotic algae from green hydra were maintained on sterile deep stock agar (HORVATIĆ *et al.* 2001; PRATT 1941). The composition of the medium was: 2 g agar, 100 mg KNO_3, 1 ml $MgSO_4x7H_2O$, 1 ml K_2HPO_4, 011 ml $FeCl_3$ and 100 ml dH_2O. Algae were grown in tubes that were 16 cm in length, 15 mm in diameter in a clime room in a sterile environment at 24^{o}C under a constant light intensity of 80 μmol/m^{2}s (fluorescent lamp Osram L36W/20, Cool/White/2850 lm Osram, Berlin, Germany). Each tube contained 5 ml of agar, inclined at 15^o. Algae were transferred with a platinum needle and plated into new tubes every 14 days in a sterile environment in a laminar. The content of each tube was transferred into 5 new tubes. The length of the "zigzag" smear in each tube was 10 cm. Tubes were sealed by cotton wool and transparent foil and afterwards placed in the clime room. Through this optimization and standardization of the method, a constant amount of cultures suitable for the experiment was obtained.

Taxon sampling

In this study we analyzed 41 different 18S rRNA sequences that represent most of the described and known *Chlorella*. The following sequences were taken for analysis and comparison: (1) own samples of algae isolated after 10, 33 and 43 generations of cultivation (CZ10 18SF, CZ33 18SF and CZ43 18SF), (2) and other species and genera of non-endosymbiotic as well as some other described symbiotic unicellular green algae significant for the reconstruction of phylogenetic relationships of endosymbiotic green algae isolated from hydra (*Chlorella*, *Parachlorella*, *Scenedesmus*, *Desmodesmus*, *Mychonastes*, *Enallax*, *Tetradesmus*) as well as two outgroup taxa (*Ulothrix* and *Gloetilopsis*) (DOUGLAS & HUSS 1984; FAWLEY *et al.* 2004; HOSHINA *et al.* 2005). All the samples are presented in Table 1, along with abbreviations and GenBank accession numbers as well as all the sequences retrieved from GenBank with their accession numbers.

DNA extraction, gene amplification and sequencing

Total genomic DNA was extracted from 15 mg of fresh algae cells that were cultivated for 10, 33 and 43 generations, e.g. CZ10 18SF (S1B-S1 hydra strain), CZ33 18SF and CZ43 18SF (S1J-J1 hydra strain) using the Qiagen DNeasy® Tissue Kit following the manufacturer's directions.

PCR products were amplified according to standard procedures with the HotMasterMix (Eppendorf, Germany) on a Mastercycler Personal Thermocycler (Eppendorf, Germany). Reactions were performed in 50 μl volumes containing: 25 μl HotMasterMix, 22 μl mQ H_2O, 1 μl of DNA and 1 μl of each primer. For the amplification of a portion of the 18S rRNA gene, we used the primers 18SF and 18SR according to MEDLIN *et al.* (1998). The initial denaturation step at 94°C was applied for 2 minutes. The amplifications were followed by: 35 cycles of 1.5 min at 94°C, 2.5 min at 55°C, and 3.5 min at 65°C, and a final extension for 7 min at 65°C modified from PRÖSCHOLD *et al.* (2001). The PCR products were electrophoresed on a 1% agarose gel, soaked in ethidium bromide for 15 minutes, and visualized by ultraviolet light.

PCR products were purified with the Qiagen PCR Quick Purification kit (Qiagen, Germany). For sequencing purposes we used the services of Macrogene Inc. (Seoul, South Korea) performed on an Applied Biosystems 3730xl DNA Analyzer.

Phylogenetic analyses

In order to improve accuracy, both the heavy and light strands from each sample were sequenced. Sequence chromatograms were viewed and edited manually using Chromas Lite 2.0 (Technelysium Pty., Queensland, Australia). Forward and reverse sequences were checked for base ambiguity in BioEdit 7.0.5.2 (HALL 1999) before consensus sequences were compiled and aligned with ClustalX (THOMPSON *et al.* 1997) using the default parameters. The alignments were trimmed at the ends before consensus sequences were compiled using BioEdit to avoid the inclusion of missing data.

Maximum parsimony analyses were performed using PAUP* 4.0b10 (SWOFFORD 2001). Parsimony analysis included a heuristic search using random sequence addition with 100 replicates and tree-bisection-reconnection (TBR) branch swapping. Support for individual clades was evaluated using nonparametric bootstrapping (FELSENSTEIN 1985) obtained from 1000 bootstrap replicates

Table 1

Species names, abbreviations and GenBank Accession Numbers of all the sequences used in phylogenetic analyses

Species name	Abbreviation	GenBank Accession Number
Chlorella ellipsoidea	CEllipsoidea	X63520
C. kessleri	CKessleri	X56105
C. lobophora	CLobophora	X63504
C. luteoviridis	CLuteoviridis	AB006045
C. minutissima	CMinutissima	AB006046
C. mirabilis	CMirabilis	X74000
C. saccharophila	CSaccharophila	X63505
C. sorokiniana	CSorokiniana	X73993
C. sphaerica	CSphaerica	AJ416105
C. vulgaris	CVulgaris	AB080308 , AB162910
C. zofingiensis	CZoofingiensis	X74004
C. sp. *C.* sp. Hvt (Hydra symbiont) *C.* sp. Ssh (Hydra symbiont)	ChlorellaSp ChlorellaSp ChlorellaSp	X73992 X72706 X72707
Desmodesmus communis	DCommunis	X73994
D. subspicatus	DSubspicatus	AJ249514
Enallax acutiformis	EnallaxAcutiformis	AB037089
Gloetilopsis planctonica	GloetilopsisPlanctonica	Z28970
Mychonastes homosphaera	MHomosphaera	X73996
Parachlorella beijerinckii	ParacBeijerinckii	AY323841
Scenedesmus abundans	SAbundans	X73995
S. acuminatus	SAcuminatus	AB037088
S. costatus	SCostatus	AB037090
S. costato-granulatus	SCostatoGranulatus	X91265
S. littoralis	SLittoralis	AB055801
S. obliquus	SObliquus	AJ249515
S. obtusus	SObtusus	AB037091
S. pectinatus var. *pectinatus*	SPectinatusVarPectinatus	AB037092
S. producto-capitatus	SProductoCapitatus	X91266
S. raciborskii	SRaciborskii	AB037094
S. regularis	SRegularis	AB037095
S. rubescens	SRubescens	X74002
S. vacuolatus	SVacuolatus	X56104
S. sp.	ScenedesmusSp	AF513373
Tetradesmus wisconsinensis	TWiconsinensis	AB037097
Ulothrix zonata	UlothrixZonata	Z47999
Coccoid green alga	CoccoidGreenAlga	AY195982
Coccoid scenedesmid	CoccoidScenedesmid	AY197638
Endosymbiotic alga from green hydra/ *Chlorella zagrebiensis*	**CZ10 18SF**	**EU250280**
Endosymbiotic alga from green hydra/ *Chlorella zagrebiensis*	**CZ33 18SF**	**EU250281**
Endosymbiotic alga from green hydra/ *Chlorella zagrebiensis*	**CZ43 18SF**	**EU250282**

found by heuristic or exact search in PAUP* using the same options as the individual searches.

Maximum likelihood analyses were performed using PAUP* 4.0b10. Modeltest 3.7 (POSADA & CRANDALL 1998) was used to select the best-fit model of nucleotide substitution for the data sets. The model of substitution was evaluated via Akaike Information Criterion (AIC). The TrN+I+G model of nucleotide substitution was chosen for estimation of ML. The ML tree was evaluated with 1000 bootstrap replicates.

Bayesian analysis was performed using Mr. Bayes 3.1.1. (RONQUIST & HUELSENBECK 2003). Mr. Bayes uses a Markov Chain Monte Carlo method to approximate the posterior probability distribution of trees, which is the probability of a

tree conditioned on the observations (data). Priors were set according to the suggested model. No initial values were assigned to the model parameters, and empirical nucleotide frequencies were used. Four Markov chains were run for 1 000 000 generations and trees were sampled every 100 generations to yield a posterior probability distribution of 10 000 trees. After eliminating the first 1000 trees as "burn-in", we constructed a 50% majority-rule consensus tree, with nodal values representing the probability (posterior probability) that the recovered clades exist, given the aligned sequence data.

Results and Discussion

Isolation of endosymbiotic algae from green hydra

In this study endosymbiotic algae from green hydra were for the first time successfully isolated and permanently cultivated in stable lab conditions for years. Phylogenetic analysis of isolated endosymbiotic algae from green hydra based on the 18S rRNA molecular marker was performed. The results showed that in the examined algae, far more nucleotide diversity exists in 18S rRNA than previously thought (JOHNSON *et al.* 2007).

For the isolation of endosymbiotic alga from green hydra, we transferred 5 individuals of green hydra from culture dishes under sterile conditions in a laminar to the surface of deep stock agar in tubes. After 3 days all the hydras died and in their place only «green spots» remained. These «green spots» were the basis for future growth and isolation of endosymbiotic algae from green hydra. Every 3 days we transferred the material from the «green spots» to sterile medium in new tubes with a platinum needle. After 11-15 days, e.g. after 4-5 transfers (each transfer was considered as 1 generation of endosymbiotic alga from green hydra), we obtained a sufficient amount of unpolluted endosymbiotic algae isolated from green hydra, which could be transferred and maintained under the conditions described earlier. The isolates used for further analysis were as follows: CZ10 18SF, CZ33 18SF and CZ43 18SF (e.g. 10, 33. and 43. generation respectively).

We have maintained the algae culture continuously for more than 3 years, and momentarily the 76^{th} generation of algae has been cultivated in a stable and clean culture.

Molecular phylogenetic analysis of endosymbiotic algae isolated from green hydra

The molecular phylogenetic analysis of endosymbiotic algae from green hydra via Bayesian (Fig. 1), maximum likelihood (Fig. 2) and maximum parsimony (Fig. 3) analyses revealed highly similar taxa positions on trees and overall topology.

After successful DNA extraction, PCR and sequencing, we aligned sequences. A portion of the 18S rRNA gene of the analyzed specimens was submitted to GenBank under the accession numbers EU250280, EU250281 and EU250282. Some analyzed sequences possessed identical haplotypes for the 18S gene. After removing multiple identical sequences we found 41 unique haplotypes consisting of 812 base pairs.

It was clearly visible on all inferred phylogenetic trees that the examined taxa formed 2 distinctive groups. The first one comprised algae of the genus *Chlorella* and the second comprised algae of several genera and species of the class Chlorophyceae. Our samples of endosymbiotic algae from green hydra were nested on 2 opposite sides within the second group, emphasizing that they were not closely related to the genus *Chlorella* (Trebouxiophyceae). Moreover, the results showed that these algae were not closely related to each other. On this basis we could undoubtedly conclude that endosymbiotic algae from green hydra have a polyphyletic origin, as in the whole *Chlorella* group.

The samples CZ33 18SF and CZ43 18SF were shown to be haplotypes most closely related to *Desmodesmus subspicatus* (Chlorophyceae) (Chodat) Hegewald et Schmidt. The specimen CZ10 18SF was shown to be most closely related to *Mychonastes homosphaera* (Chlorophyceae) (Skuja) Kalina et Punčochářová. This unexpected biodiversity of endosymbionts probably depended on the natural habitat of a particular hydra strain.

Molecular phylogenetic analyses of endosymbiotic algae from green hydra resulted in the discovery of two unexpected and thus far undescribed endosymbiotic species in green hydra. This result confirms the notion that symbionts from green hydra are the consequence of at least 2 symbiotic events (HUSS *et al.* 1993). It was already noticed that hydra can also carry bacterial symbionts, so the types of symbionts depend on the hosts' lines (HOSHINA *et al.* 2005). A combination of different algal partners and selection of host populations could contribute to better adaptation of symbionts (KLÜTER 2006).

We have suggested a general unsystematic common name for all endosymbiotic algae isolated from green hydra with the characteristic of permanent stable lab growth to be the *Chlorella zagrebiensis* group Kovac. & Jelen. (2007) (Kovacevic & Jelencic). This group of polyphyletic origin was introduced as a tool for understanding the position of a particular endosymbiont in the phylogenetic tree of *Chlorella* and related algae. We proposed

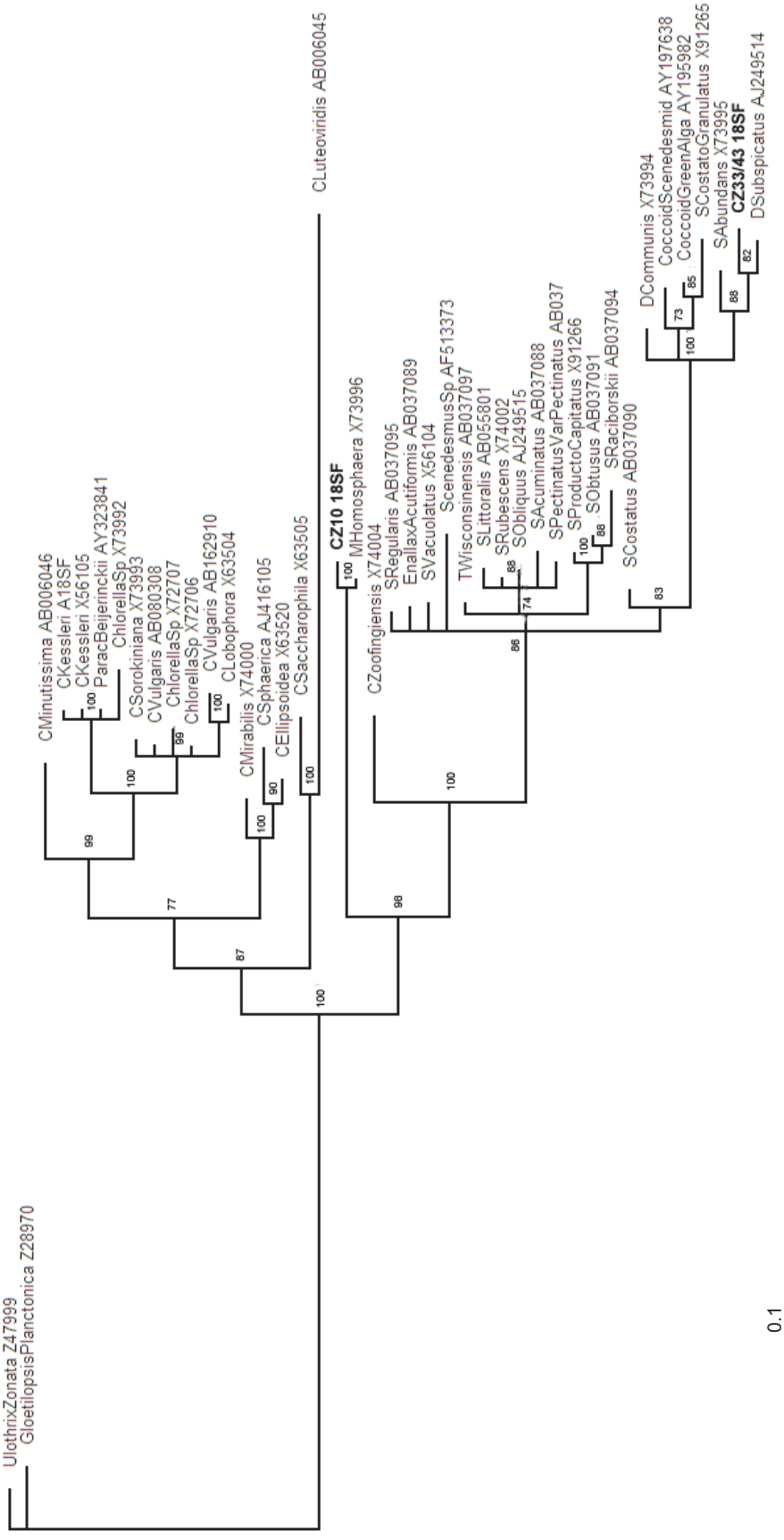

Fig. 1. Bayesian tree based on 18S rRNA gene sequences of endosymbiotic algae from green hydra, algae from the genus *Chlorella* and related genera. Endosymbiotic algae from green hydra isolated in our experiments are noted with abbreviations and bolded. Numbers on branches correspond to posterior probability values.

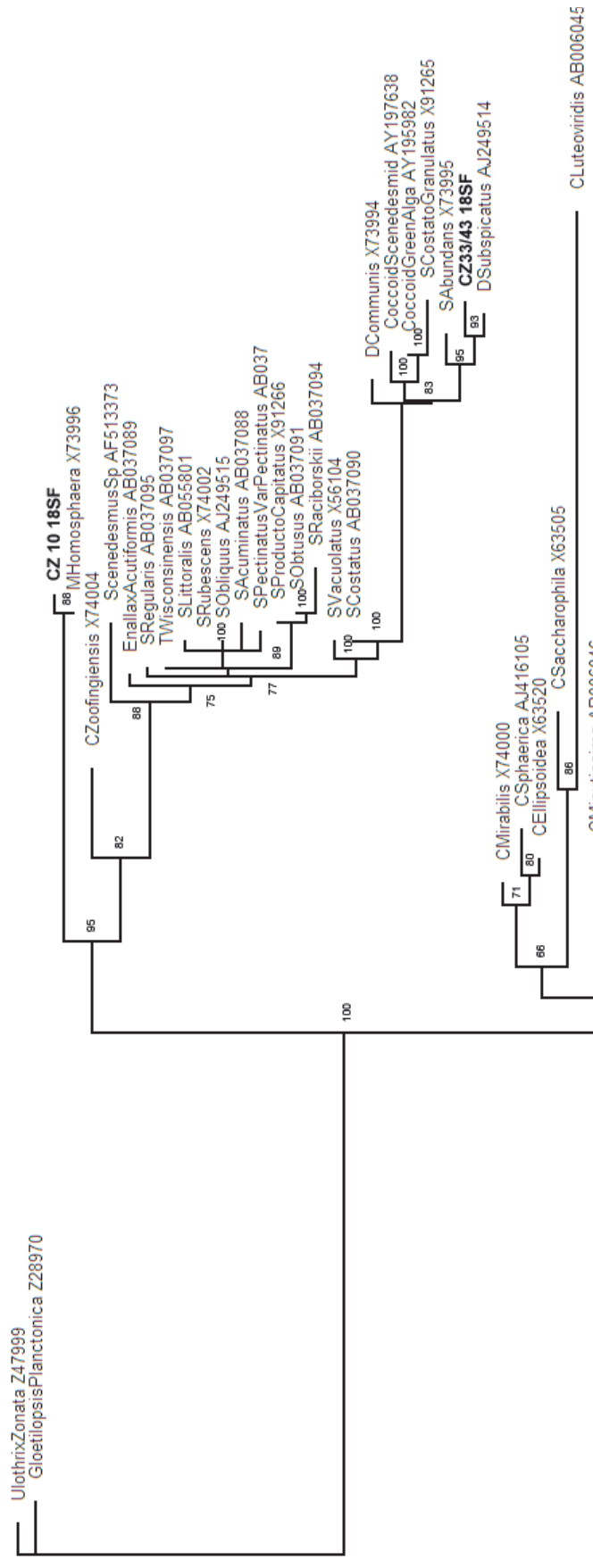

Fig. 2. Maximum likelihood tree based on 18S rRNA gene sequences of endosymbiotic algae from green hydra, algae from the genus *Chlorella* and related genera. Endosymbiotic algae from green hydra isolated in our experiments are noted with abbreviations and bolded. Numbers on branches correspond to bootstrap values.

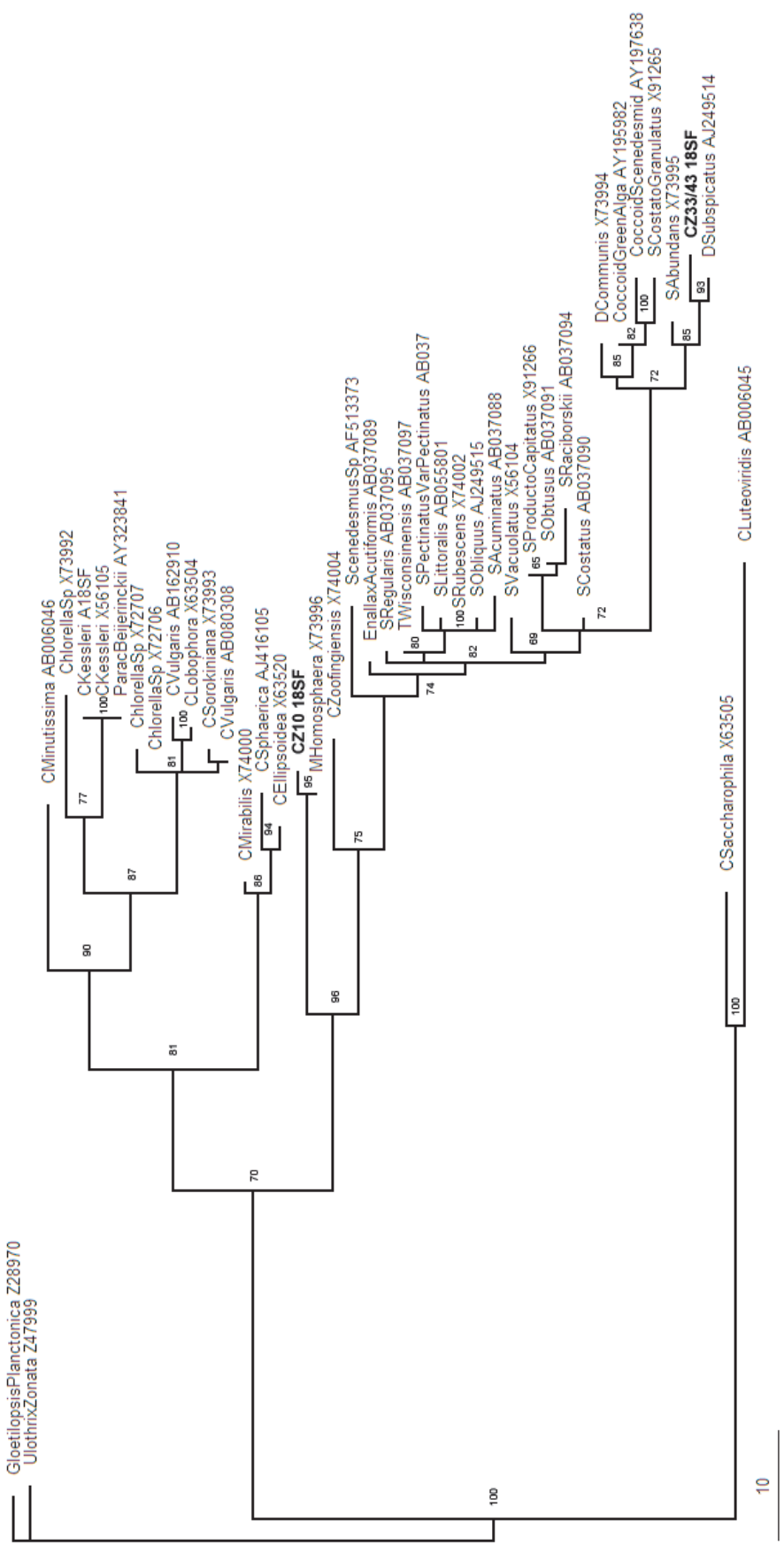

Fig. 3. Maximum parsimony tree based on 18S rRNA gene sequences of endosymbiotic algae from green hydra, algae from the genus *Chlorella* and related genera. Endosymbiotic algae from green hydra isolated in our experiments are noted with abbreviations and bolded. Numbers on branches correspond to bootstrap values.

this name for the group due to historical reasons, as well as owing to the starting point for this work in which *Chlorella* was presumed to be the endosymbiont in green hydra. Groups including green algae are known, for example, as the *Chlorella vulgaris* group on the basis of the 18S rRNA marker (KESSLER 1982).

Evolutionary aspects of hydra-alga symbiosis

The symbiosis between hydra and alga represents an important model for studying the specificity between host and symbiont in the invertebrate-alga system, as well as for understanding the biology of higher animals. Hydra is also considered a «living fossil». The growing interest for green hydra and recognition of symbiogenesis as one of the most important evolutionary concepts led to the re-questioning of host-symbiont relationships and, in addition, fueled many possibilities for scientific research and cognition of the evolution of the living world (MEINHARDT 2002; SHIMIZU & FUJISAWA 2003).

The taxonomy of the genus *Hydra* is not entirely clear therefore molecular analyses will be important for its resolution. Since green hydra has a small genome, it is considered to represent the earliest hydra from which other hydra species had evolved (ZACHARAS *et al.* 2004). We could assume that for their evolution a specific symbiotic association was necessary. A "trigger" for evolution is needed, which was found in the (algal) symbiotic partner. For example, it is assumed that the acquisition of *Symbiodinium* in dinoflagellates could stimulate further evolution and diversification of the species by the process of symbiogenesis. Via co-evolution the effect of mutual action between species is achieved by means of natural selection and this is one of the main processes influencing biodiversity (POCHON & PAWLOWSKI 2006). The same principle could be applied to hydras. It is possible that during evolution hydra changed symbionts and a symbiont may have existed that was lost or replaced by *Chlorella*. Genomes of basal metazoans could be much more dynamic than previously thought. It is also possible that even an endosymbiotic candidate for green hydra could not survive in the environment on its own. This kind of symbiotic combination could contribute to the evolution of the genus *Hydra*. After millions of years of co-evolution and preadaptations in hydra-alga symbiosis, the following evolutionary scenario seems possible: a primary parasitic relationship became obligatory mutualistic and today could be facultatively mutualistic, although most likely regularly mutualistic during the lives of the individuals that form the symbiosis. It is assumed today that lateral gene transfer is possible between the symbionts in green hydra symbiosis (HABETHA & BOSCH 2005). These reflections open up different possibilities for further investigations into hydra-alga symbiosis that could explain the phylogenetic status of hydras themselves, maintenance of the symbiosis and the course of symbiogenesis in green hydra.

The results of our experiments underline the fact that symbiogenesis in green hydra has probably not yet been terminated.

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References

DOUGLAS A. E. 1994. Symbiotic Interactions. Oxford University Press Inc., Oxford & New York.

DOUGLAS A. E., HUSS V. A. R. 1986. On the characteristics and taxonomic position of symbiotic *Chlorella.* Arch. Microbiol. **145**: 80-84.

EARDLY B., BERKUM P. V. 2004. Use of population genetic structure to define species limits in the rhizobiaceae. Symbiosis **38**: 109-122.

FAWLEY M. W., FAWLEY K. P., BUCHHEIN M. A. 2004. Molecular diversity among communities of freshwater *Microchlorophytes*. Microb. Ecol. **48**: 489-499.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39**: 783-791.

HABETHA M., ANTON-ERKSLEBEN F., NEUMANN K., BOSCH T. C. G. 2003. The *Hydra viridis/Chlorella* symbiosis. Growth and sexual differentiation in polyps without symbionts. Zoology **106**: 1-8.

HABETHA M., BOSCH T. C. G. 2005. Symbiotic *Hydra* express a plant-like peroxidase gene during oogenesis. J. Exp. Biol. **208**: 2157-2165.

HALL T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41**: 95-98.

HORVATIĆ J., VIDAKOVIĆ-CIFREK ., REGULA I. 2000. Trophic level, bioproduction and toxicity of the water of lake Sakadaš (Nature park Kopački rit, Croatia). Limnol. Report **33**: 89-94.

HOSHINA R., KATO Y., AKMAKO S., IMAMURA N. 2005. Genetic evidence of «American» and «European» type symbiotic Algae of *Paramecium bursaria* Ehrenberg. Plant Biol. **7**: 526-532.

HUSS V. A. R., SOGIN M. L. 1990. Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. J. Mol. Evol. **31**: 432-44.

HUSS V. A. R., HOLWEG C., SEIDEL B., REICH V., RAHAT M., KESSLER E. 1993. There is an ecological basis for host/symbiont specificity in *Chlorella/Hydra* symbioses. Endocyt. Cell Res. **10**: 35-46.

HUSS V. A. R., FRANK C., HARTMANN E. C., HIRMER M., KLOBOUCEK A., SEIDEL B. M., WENZLER P., KESSLER E. 1999. Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). J. Phycol. **35**: 587-598.

JOHNSON J. L., FAWLEY M. W., FAWLEY K. P. 2007. The diversity of *Scenedesmus* and *Desmdesmus* (Chlorophyceae) on Itasca State Park, Minnesota, USA. Phycologia **46**: 2214-2229.

KESSLER E. 1982. Chemotaxonomy in the *Chlorooccales*. Prog. Phycol. Res. **1**: 111-135.

KLÜTER A., LOH W., HÖGH-GULDBERG O., DOVE S. 2006. Physiological and genetic properties of two recent colour morphs of the coral *Montipora digitata*. Symbiosis **42**: 123-134.

KRIENITZ L., HEGEWALD E., HEPPELE D., HUSS V. A. R., ROHR T., WOLF M. 2004. Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia **43**: 529- 542.

MCAULEY P. J., SMITH D. C. 1982. The green hydra symbiosis. V. Stages in the intracellular recognition of algal symbiont by digestive cells. Proc. R. Soc. Lond. B. **216**: 7-23.

MEDLIN L., ELWOOD H. J., STICKEL S., SOGIN M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**: 491-499.

MEINHARDT H. 2002. The radial-symmetric hydra and the evolution of the bilateral body plan: an old body became a young brain. BioEssays **23**: 185-191.

NAKAHARA M., HANDA S., WATANABE S., DEGUCHI H. 2004. *Choricystis minor* as a new symbiont of simultaneous two-species association with *Paramecium bursaria* and implications for its phylogeny. Symbiosis **36**: 127-151.

O'BRIEN T. L. 1982. Inhibition of vacuolar membrane fusion by intracellular symbiotic algae in *Hydra viridis* (Florida strain). J. Exp. Zool. **223**: 211-218.

POCHON X., PAWLOWSKI J. 2006. Evolution of the soritids-*Symbiodinium* symbiosis. Symbiosis **42**: 477-88.

POOL R. R. Jr., MUSCATINE L. 1980. Endosymbiosis and Cell Biology, vol. 1. A synthesis of recent research. (In: Endocytobiology. W. Schwemmler, H. E. A. Schenk eds. WdeG, Berlin): 223-238.

POSADA D., CRANDALL K. A. 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics **14**: 817-818.

PRATT R. 1941. Studies on *Chlorella vulgaris*. Am. J. Bot. **28**: 492-497.

PRÖSCHOLD T., MARIN B., SCHLÖSSER U. G., MELKONIAN M. 2001. Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas Ehrenberg* and *Chloromonas Gobi*, and description of *Oogamochlamys gen. nov*. and *Lobochlamys gen. nov*. Protist **152**: 265-300.

RONQUIST F., HUELSENBECK J. P. 2003. Mr. Bayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**: 1572-1574.

SHIMIZU H., FUJISAWA T. 2003. Penducle of hydra and the hearth of higher organisms share a common ancestral origin. Genesis **36**: 182-186.

SWOFFORD D. L. 2001. PAUP* Version 4.0b10 [Computer software and manual]. Sinauer Associates. Sunderland, MA.

THOMPSON J. D., GIBSON T. J., PLEWNIAK F., JEANMOUGIN F., HIGGINS D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. **25**: 4876-4882.

ZACHARAS H., ANOKHIN B., KHALTURIN K., BOSCH T. C. G. 2004. Genome sizes and chromosomes in the basal metazoan. Hydra. Zoology **107**: 219-227.