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Algal endosymbionts in European *Hydra* strains reflect multiple origins of the zoochlorella symbiosis ☆

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ABSTRACT

Symbiotic associations are of broad significance in evolution and biodiversity. Green *Hydra* is a classic example of endosymbiosis. In its gastrodermal myoepithelial cells it harbors endosymbiotic unicellular green algae, most commonly from the genus *Chlorella*. We reconstructed the phylogeny of cultured algal endosymbionts isolated and maintained in laboratory conditions for years from green *Hydra* strains collected from four different geographical sites within Croatia, one from Germany and one from Israel. Nuclear (18S rDNA, ITS region) and chloroplast markers (16S, *rbc*L) for maximum likelihood phylogenetic analyses were used. We focused on investigating the positions of these algal endosymbiotic strains within the chlorophyte lineage. Molecular analyses established that different genera and species of unicellular green algae are present as endosymbionts in green *Hydra*, showing that endosymbiotic algae growing within green *Hydra* sampled from four Croatian localities are not monophyletic. Our results indicate that the intracellular algal endosymbionts of green *Hydra* have become established several times independently in evolution.

1. Introduction

Hydra is a single freshwater polyp that inhabits shallow lakes and calm, slow-moving waters. It belongs to the hydrozoan clade Aplanulata (Collins et al., 2005, 2006) within the deep-branching eumetazoan phylum Cnidaria. It provides a useful model system for comparative research in development and evolution (Galliot and Schmid, 2002; Technau and Steele, 2011), both for investigations of early branching metazoans and for the study of plant-animal symbioses. Recent phylogenetic analyses that included most globally identified *Hydra* species demonstrated they can be divided into four groups (Kawaida et al., 2010; Martinez et al., 2010): the *viridissima* group, the *braueri* group, the *oligactis* group and the *vulgaris* group. The latter three groups are not known to enter into symbiotic relationships with photoautotrophic algae, and are referred as "brown *Hydra*" (Campbell, 1987, 1989). The *viridissima* group is estimated to have diverged from all other *Hydra* species 65–55 Ma (Martinez et al., 2010), followed by the divergence of the *braueri* group, and then the *oligactis* and *vulgaris* groups. Within the genus, *Hydra viridissima* has the smallest genome, but whether its genome size directly relates to symbiotic interactions and/or adaptive changes to environment (Johnston et al., 1996; Zacharias et al., 2004) is unknown. Four species of green *Hydra* have been described: *H. viridissima*, *H. hadleyi*, *H. plagiodesmica* and *H. sinensis*, but it is not clear whether they are really separate species (Grayson, 1971).

Green *Hydra* (*Hydra viridissima* Pallas 1766) houses endosymbiotic unicellular green algae, typically *Chlorella* species that inhabit gastrodermal myoepithelial cells. They are separated from host cell cytosol by a membrane structure named symbiosome (Douglas, 1994). Symbiotic algae from *H. viridissima* polyps can be artificially eliminated by photobleaching (Pardy, 1983). Although such aposymbiotic *H. viridissima* polyps do not occur naturally, they reveal normal morphology, easily grow under laboratory conditions as symbiotic polyps and can be reinfected with freshly isolated algae from symbiotic polyps (McNeil, 1981). It is still not resolved whether a stable symbiotic relationship can be established between artificially induced *Chlorella* strains and host polyps (Kawaida et al., 2013). Yet, it has been demonstrated that certain correlations between *Chlorella* species and the *viridissima* group within the genus *Hydra* appear to exist (Rahat and Reich, 1986; Kawaida et al., 2013).

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Various invertebrates enter into symbiotic relationships with photosynthetic unicellular algae collectively named zoochlorellae (Chlorophyta) and zooxanthelle (in most cases dinoflagellates; Paracer and Ahmadjian, 2000). In Cnidaria, symbiotic relationships with dinoflagellates (genus *Symbiodinium*) are well known within the corals (class Anthozoa; Rowan, 1998; Baker, 2003). Endosymbiotic algae in freshwater *Hydra* polyps are commonly named "zoochlorellae". *Hydra viridissima* is the only *Hydra* species hosting zoochlorellae (Habetha et al., 2003). Molecular data for strains sampled so far suggests that endosymbiotic *Chlorella* of different green hydra strains represent separate species within Trebouxiophyceae that are closely related to, but distinct from, free living *Chlorella* species (Huss et al., 1993/94; Friedl, 1997; Pröschold et al., 2011; Kawaida et al., 2013).

Regarding the number of events leading to *Hydra* endosymbionts, there are conflicting reports. Recent molecular phylogenies suggested that endosymbiotic zoochlorellae of *Hydra viridissima* are polyphyletic (Pröschold et al., 2011), although Kawaida et al. (2013) argue that a single origin of the zoochlorellae endosymbiosis followed by endosymbiont escape could also account for observed host-algal patterns cospeciation. While the *Chlorella* endosymbiont species are morphologically indistinguishable from each other, phylogenetic studies have demonstrated that the canonical *Chlorella* morphology is shared with other lineages of the Trebouxiophyceae and Chlorophyceae classes within the Chlorophyta (Huss et al., 1999; Neustupa et al., 2009; Darienko et al., 2010).

Algal endosymbionts isolated from green *Hydra* are notoriously difficult to maintain in stable culture (McAuley and Smith, 1982; Rahat, 1992; Huss et al., 1993/94; Friedl, 1997; Habetha et al., 2003) although free-living relatives are easily cultured under routine conditions. However, Kovačević and colleagues recently reported the stable maintenance of endosymbiont cultures isolated from green *Hydra* (Kovačević et al., 2010).

Here we investigate the phylogeny of algal endosymbionts maintained in stable laboratory cultures isolated from green *Hydra* strains, collected from six different geographical sites. All strains of endosymbiotic algae were isolated from green *Hydra*; four from different localities in Croatia (Botanical Garden, Jarun Lake, Maksimir park and Turopolje), one from Israel and one from Germany. We used nuclear (18S rDNA, the ITS region) and chloroplast markers (16S, *rbc*L) for phylogenetic analysis of 547 different sequences spanning chlorophyte diversity. We focussed on the question of whether native symbionts of Croatian *H. viridissima* strains descend from two or more symbiotic events (Huss et al., 1993/94; Pröschold et al., 2011) or whether symbiosis with *Chlorella* occurred only once in the distant past followed by subsequent cospeciation and a secondary origin of free-living algal strains from escaped endosymbionts, as suggested by Kawaida and colleagues (2013).

Table 1
List of the analysed strains of alga *Chlorella* sp. in the experiment.

Algal endosymbiont code name	Description	Original location of green hydra
BV	Isolated symbiotic alga from green hydra host (isolated in this study for the first time)	Botanical Garden, Croatia
T	Isolated symbiotic alga from green hydra host (isolated in this study for the first time)	Turopolje, Croatia
M9	Isolated symbiotic alga from green hydra host (isolated in this study for the first time)	Israel
HV	Isolated symbiotic alga from green hydra host (isolated in this study for the first time)	Germany
CZ80	Isolated symbiotic alga from green hydra host (growing in cultures for 5 years)	Botanical Garden, Croatia
CZ120	Isolated symbiotic alga from green hydra host (growing in cultures for 5 years)	Jarun Lake, Croatia
CV80	Free-living *Chlorella vulgaris* (SAG 211/11b)	Germany
A100	Free-living *Parachlorella kessleri* (LARG/1)	Croatia

Table 2
Primers used for PCR amplification and sequencing.

DNA region	Length (bp)	Primer name and sequence	Reference
16S rDNA	814	294-313F: 5′-TGG GGA ATT TTC CGC AAT GG-3′ 1131-51R: 5′-TGT AGC ACG TGT GTC GCC CAG-3′	Katana et al. (2001)
18S rRNA	1750	18SF: 5′-AAC CTG GTT GAT CCT GCC AGT-3′ 18SR: 5′-CTT GAT CCT TCT GCA GGT TCA CCT AC-3′	Medlin et al. (1988)
rbcL	1075	rbcL-F1: 5′-CCA CAA ACT GAA ACT AAA GCA-3′ rbcL-R1: 5′-CAT GTG CCA TAC GTG AAT ACC-3′	Fawley et al. (2005)
ITS	743	ITS-1: 5′-TCC GTA GGT GAA CCT GCG G-3′ ITS-4: 5′-TCC TCC GCT TAT TGA TAT GC-3′	White et al. (1990)

Table 3
PCR reaction conditions for an individual gene.

Reaction conditions	16S		18S		rbcL		ITS	
	Temp.	Duration	Temp.	Duration	Temp.	Duration	Temp.	Duration
1. Initial denaturation	98 °C	2 min	98 °C	2 min	98 °C	2 min	98 °C	2 min
2. Denaturation	98 °C	0.5 min	98 °C	0.5 min	98 °C	0.5 min	98 °C	0.5 min
3. Annealing	54 °C	0.5 min	55 °C	0.5 min	58 °C	0.5 min	60 °C	0.5 min
4. Elongation	72 °C	0.5 min	72 °C	1 min	72 °C	0.5 min	72 °C	0.5 min
Repeat from 2. to 4.	25×		35×		35×		35×	
5. Final elongation	72 °C	10 min	72 °C	10 min	72 °C	10 min	72 °C	10 min

Table 4
Species name of the NCBI sequences for each gene used in phylogenetic analysis.

Family	Species name	16S rRNA	18S rRNA	rbcL	ITS region
Trebouxiophyceae	*Parachlorella beijerinckii*		AY323841		FM205845
	'*Chlorella*' *ellipsoidea*	X12742	X63520	EU038287	EU038292
	C. kessleri	D11346	X56105	AB260912	FR865655
	C. lobophora		X63504		
	C. luteoviridis	AJ242767	AB006045		FR865658
	C. minutissima		AB006046	KC810312	
	C. mirabilis	X65100	X74000		
	'*C.*' *saccharophila*	FJ176391	AB058310	AM260446	FM946010
	C. sorokiniana	EF030600	X73993	JQ415922	KC416207
	C. sphaerica		AJ416105		
	C. vulgaris	AJ242754	AB080308	JQ415915	AY591512
	C. zofingiensis/*M. zofingiensis*		X74004	HQ902940	HQ902929
	C. sp.	EF030603	AB713411	KC810316	FM205844
	Asterochloris sp.	GU191846		JN573807	AM906012
	Auxenochlorella protothecoides	AY553213		EU038285	FN298931
	Auxenochlorella sp.	FJ890890		AM260439	FN298932
	Chloroidium ellipsoideum				FR865666
	C. saccharophilum				FR865677
	Choricystis minor			DQ219819	FN870434
	Closteriopsis acicularis	Y17632		EF113433	HM066009
	Coccomyxa glaronensis	AM292034			
	Coccomyxa sp.			HQ335208	FN298928
	Dictyochloropsis reticulata			EF113435	FJ792803
	Elliptochloris bilobata			FJ217379	
	E. subsphaerica			FJ217382	FJ648518
	Helicosporidium sp.	AF538865			AF317895
	Heterochlorella luteoviridis			HE984580	
	Heveochlorella hainangensis	EF595525			JX290372
	H. roystonensis	JN003600			
	Kalinella bambusicola			HE984581	
	Koliella longiseta				AJ431677
	K. sempervirens	AF278747			AJ431673
	K. spiculiformis	AF278746			AJ431670
	Leptosira terrestris			AM260448	
	Microthamnion kuetzingianum			EF589152	
	Myrmecia biatorellae			AF499685	
	Nannochloris sp.				JQ922411
	Neochloris aquatica			EF113456	AY577764
	Phyllosiphon arisari	FJ829885			
	Picochlorum (Nannochloris) eucaryotum	X76084		EF113454	
	Prototheca blaschkeae	FR848895			
	P. wickehamii	X74309			
	P. zopfi var. hydrocarbonea	FR848894			FR848898
	Raphidonema longiseta	AF278749			
	Stichococcus bacillaris	AF278751		AM260442	AJ431678
	Trebouxia asymmetrica				AJ249565
	T. impressa				AJ318780
	T. magna			AJ969630	
	Viridiella fridericiana				FM958481
	Watanabea reniformis				FM958480
Chlorophyceae	*Desmodesmus communis*		X73994	KC810306	AY461372
	D. subspicatus			KC810307	AY461367
	Scenedesmus abundans		X73995	KC810308	
	S. acuminatus		AB037088		AJ249511
	S. costatus		AB037090		AB762692
	S. costato-granulatus		X91265		AM228911
	S. obliquus	AF394206	AJ249515	EF113469	AJ249506
	S. obtusus		AB037091		AJ170858
	S. pectinatus var. pectinatus				FR865735
	S. raciborskii		AB037094		
	S. regularis		AB037095		FR865732
	S. rubescens		X74002	KC810310	
	S. vacuolatus		X56104		AB762693
	S. sp.		AF513373	KC810311	
	Ankistrodesmus stipitatus			EF113406	
	Bracteacoccus aerius			JQ259861	JQ281838
	Bulbochaete hiloensis			EF113415	AY962677
	Carteria cerasiformis	AB688625		D89768	
	Chaetopeltis orbicularis				FR865748
	Chlamydomonas debaryana			D86838	FR865600
	C. humicola	AF374186			DQ377088
	C. moewusii	X15850		EF113422	FR865601
	C. reinhardtii	J01395		AB511845	U66954
	Chlorococcum sp.		AB713407	AB713414	

(*continued on next page*)

Table 4 (*continued*)

Family	Species name	16S rRNA	18S rRNA	rbcL	ITS region
	Dunaliella salina			DQ173088	DQ116743
	Enallax acutiformis		AB037089		
	Haematococcus pluvialis	HQ317401		FJ438476	GQ463618
	Hydrodictyon reticulatum			EF078305	AY577738
	Monoraphidium sp.			KC810300	
	Mychonastes homosphaera		X73996		GQ477054
	Pediastrum duplex			EF113461	AY577745
	Pleodorina japonica	AB688626			
	Protosiphon botryoides			JN880463	
	Stigeoclonium hevleticum				HQ646382
	Trademus wisconsinensis		AB037097		
	Volvox carteri f. nagariensis			AB076099	
Ulvophyceae	*Gloeotilopsis planctonica*		Z28970	AF499681	
	Ulothrix zonata		Z47999		HE860526
	Ulva rigida			EF110009	AJ234319
Prasinophyceae (outgroup)	*Monomastix minuta*	AB491652			FN562446
	Prasinococcus capsulatus	AB491658	AB058384	L34834	HE610141
	Pterosperma cristatum	AB491636	AJ010407	U30281	
	Pseudoscourfieldia marina	AB491627		U30279	
	Scherffelia dubia				HE610128
	Tetraselmis striata		X708002		HE610129
	T. subcordiformis	JN561782	JN022609		

2. Materials and methods

2.1. Hydra and Chlorella strains

Different green *Hydra* strains were collected from 4 localities in Croatia: Jarun Lake, Maksimir Park, Turopolje and the greenhouse of the Botanical Garden from the surface of submerged plants. Strain M9 was from Israel and strain HV from Kiel, Germany. Symbiotic algae were successfully isolated from green hydras from these regions for the first time (Table 1). *Parachlorella kessleri* (Fott & Nováková) Krienitz, E.H. Hegewald, Hepperle, V. Huss, T. Rohr & M. Wolf; strain LARG/1 from collection of Botanical Garden (Faculty of Science, University of Zagreb) and *Chlorella vulgaris* Beyerinck [Beijerinck]; strain SAG 211-11b from algal collection in Göttingen, Germany were used as referent algal species.

2.2. Isolation of endosymbiotic algae from green hydra

Green hydras were cultured in aquarium water in the laboratory in 2L tanks and fed twice a week with the larvae of *Artemia salina*. The cultures were kept under diffused light (15 μmol/m^2; 10L/14D) at a temperature of 21 °C. Isolated endosymbiotic algae from green hydras were maintained on sterile deep stock agar and grown in tubes in a clime room in a sterile environment at 24 °C under a constant light intensity of the 80 μmol/m^2. We have maintained the algal cultures continuously for more than 5 years and for more than 1 year for the different Croatian strains (growing algal cultures are shown in supplementary data S1).

2.3. DNA isolation, PCR amplification, cloning and sequencing

Different strains of symbiotic algae isolated from green hydras are designated by abbreviations that come with a numerical value representing the number of generations of algae (the time elapsed since its initial isolation, during which any transfer to a fresh tube meant the next generation). Total DNA was extracted from 15 mg of fresh algae cells using Qiagen DNeasy® Tissue Kit following the manufacturer's directions. DNA was isolated from the symbiotic algae that were previously grown two to four months in the stable and sterile culture for strains BV12, HV15, M9-19, M9-20, T23 and T24 and for more than 5 years for strains CZ80 and CZ120. In order to exclude any possible contamination of algal cultures we also selectively obtain DNA of the algae from Botanical garden and Turopolje hydra strains as described by Kawaida et al. (2013). We homogenized and centrifuged polyps in the culture solution in order to separate algae from tissues of the host polyp. DNA was then extracted from the collected algae using the DNA extraction kit.

Four different phylogenetic markers were selected: chloroplast SSU (16S rDNA) gene, nuclear small ribosomal RNA (18S rRNA) gene, chloroplast DNA *ribulose-biphosphate carboxylase large subunit* (*rbc*L) gene and 18S–26S ribosomal internal transcribed spacer (ITS) region, including the 5.8S rDNA. PCR reactions were performed in a final volume of 50 μL using VELOCITY™ DNA Polymerase (BIOLINE) and 5× Hi-Fi Reaction Buffer for 16S, 18S, *rbc*L and ITS genes respectively. Primers used for PCR amplification and sequencing are listed in Table 2 and PCR cycling conditions in Table 3. The PCR products were electrophoresed on a 0.6% agarose gel with addition of ethidium bromide, and visualized by GelDoc-It™ Imaging System (UVP, Upland CA, USA). Elution of PCR blunt-ended amplicons from gel was done with Promega-Wizard SV Gel and PCR Clean-Up System in 50 μL volume, followed by setting up ligation of PCR clones with pJET 1.2/blunt Cloning Vector by protocol from manufacturer (Thermo SCIENTIFIC). Transformation was done with XL1-Blue Ultracompetent Cells (*E. coli*) on LB + Ampicillin plates placed on incubation overnight (37 °C). Positive colonies have been picked and inoculated with 5 ml of LB-medium (supplemented with Amp-ampicillin) (placed on shaker overnight at 37 °C). Plasmid isolation was done using Thermo Scientific GeneJET Plasmid Miniprep Kit and 50 μL of plasmid DNA was purified. For the end, purified plasmid DNA was restricted with BglII (NEW ENGLAND BioLabs) and NEBuffer 3 (100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). Afterwards restriction gel was done to select which positive colonies will be sent for sequencing. Sequencing was carried out through Eurofins MGW Operon (Ebesberg, Germany) and Macrogen (Amsterdam, Netherlands).

2.4. Phylogenetic analysis

In total, 37 valid sequences of symbiotic algae isolated from four Croatian green *Hydra* strains, then two strains from Europe and two

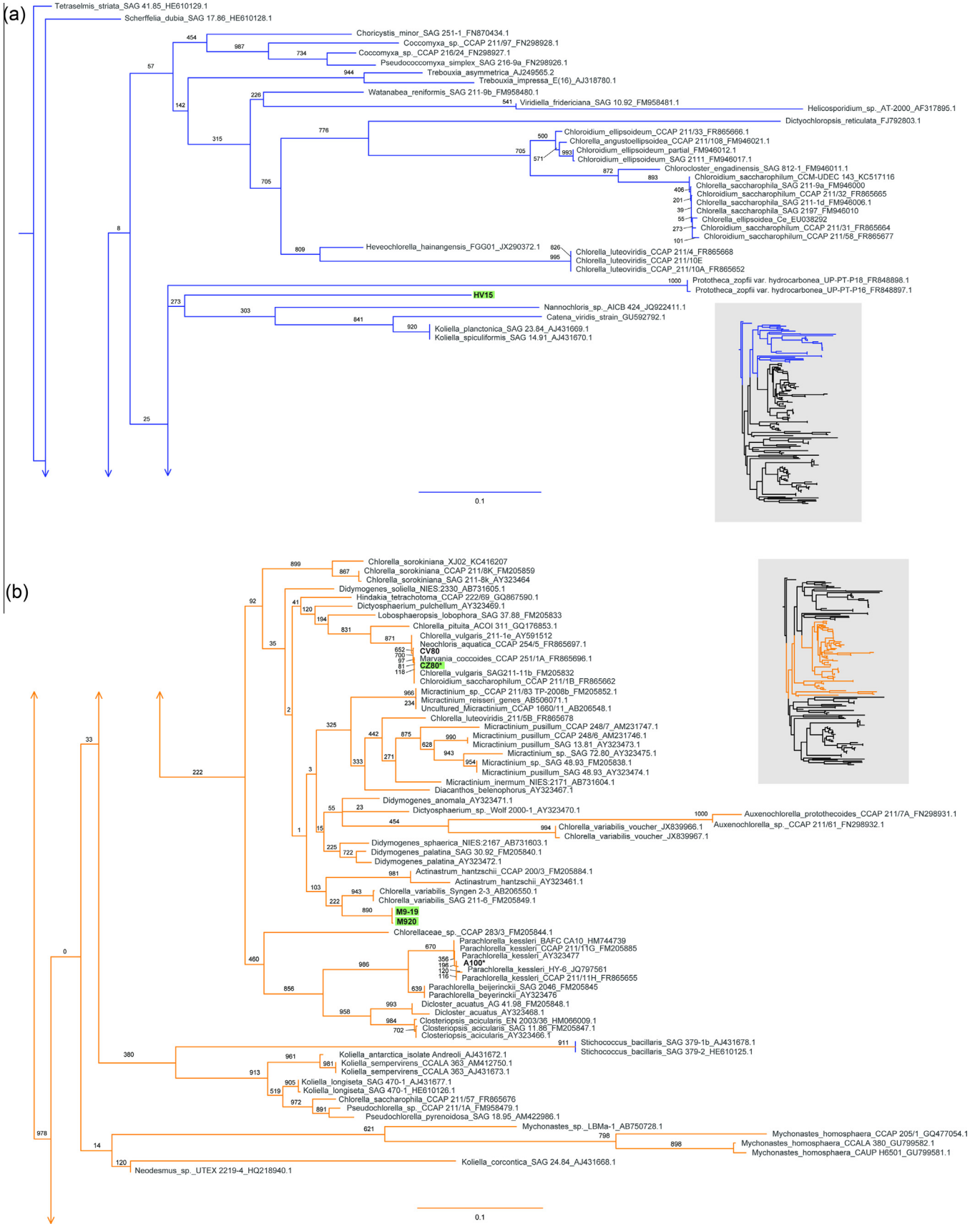

Fig. 1. (a–c) Molecular phylogenetic tree of 171 taxa of green lineage Chlorophyta inferred by maximum likelihood method based on ITS region sequences comparisons. The numerals near the nodes indicate 1000 time bootstrap values. Sequences gained in this study are in abbreviations and bold. Bold abbreviations with green background are algal endosymbionts isolated from green *Hydra* in this study. Bold abbreviations with green background and asterisks are algal endosymbionts isolated from Croatian strains of green *Hydra*. Strain and accession numbers are given after the species name. The species *Tetraselmis striata* from Prasinophyceae was used as outgroup. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

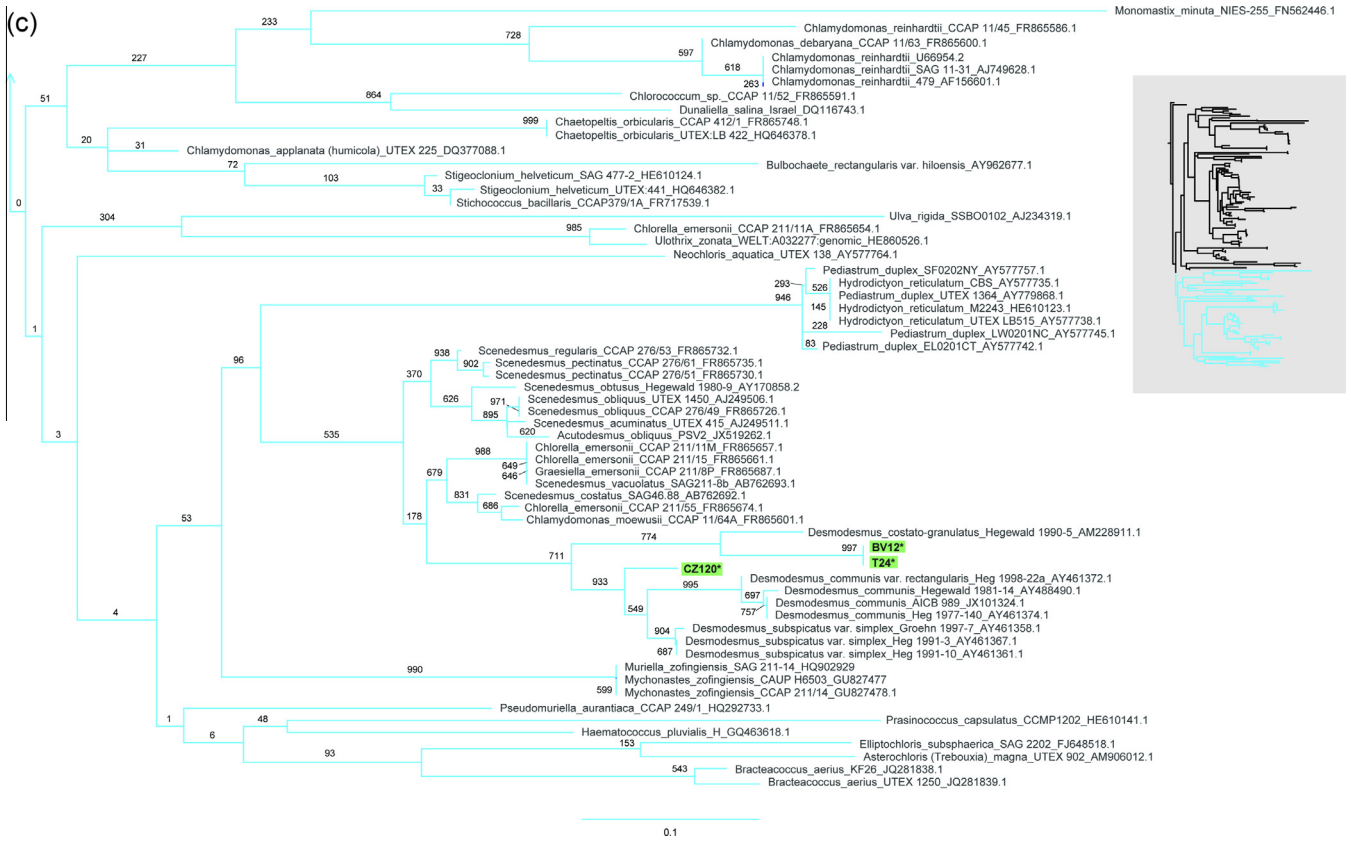

Fig. 1 (*continued*)

reference strains based on 4 different genes (16S rDNA, 18S rDNA, *rbc*L genes and ITS region) were used in maximum likelihood analysis. 510 published sequences from NBCI of green algae from three classes of the lineage Chlorophyta were also used in this analysis: Trebouxiophyceae (in total 50 taxa/species), Chlorophyceae (in total 36 taxa/species) and Ulvophyceae (in total 3 taxa/species), respectively for each gene (Table 4). Prasinophyceae were used as outgroup (in total 7 taxa/species) (Table 4). Sequences were checked by tblastn program *Chlorella vulgaris* Beyerinck [Beijerinck] strain SAG 211-11b as the referent sequence. Extraction by significant alignment was subsequently performed. Alignment was done using MAFFT with the following parameters: maxiterate 1000 and local pair; bootstrap with 1000 samples. Phylogenetic analysis was performed using PhyML, with parameters as follows: Substitution model: HKY85 (Hasegawa et al., 1985); Ts/Tv ratio: estimated; Number of substitution rate categories: 4; Gamma distribution parameter: estimated; Nucleotide equilibrium frequencies: ML; Optimized tree topology: yes; Tree topology search: SPRs (subtree pruning and regrafting); Optimize branch lengths: yes; Optimize substitution model parameters: yes. FigTree v1.4.0 was used to visualize and root phylogenetic trees.

3. Results

3.1. Cultivation of endosymbionts from green hydra

We were able to isolate clean cultures of algal endosymbionts from green hydras from two Croatian localities (strain T from Turopolje and strain BV from Botanical garden) and algal endosymbionts from two *Hydra* strains from Israel (strain M9) and Kiel (Germany; strain HV). They were maintained in the laboratory in stable, sterile and continuous culture since the original isolation in 2012.

3.2. Molecular identification of endosymbiotic algae isolated from green hydra hosts using nuclear marker ITS region

ITS phylogenetic results in this study show that Croatian algal endosymbionts isolated from green *Hydra* belong to two different green algal lineages. In ITS analysis, 161 different taxa sequences were used, together with Croatian samples. Taxa from all three lineages of green algae (Trebouxiophyceae, Chlorophyceae, and Ulvophyceae) available from Gen Bank were included in our analysis because of getting better understanding of green algae phylogeny. *Tetraselmis striata* (SAG 41.85) from the Prasinophyceae was used as outgroup for ITS phylogenetic trees (Fig. 1a). Our results in ML tree revealed that Croatian symbiotic *Chlorella* strains isolated from Croatian hydras did not form a monophyletic clade consisting only of those strains. Rather, the phylogenies indicate polyphyly and independent origins of the symbiosis at different geographic locations (Fig. 1b and c). Croatian samples named BV12 and T24 from two different geographical locations (Table 1) branched with *Desmodesmus costato-granulatus* (Hegewald 1990-5) from Chlorophyceae, Sphaeropleales, Scenedesmaceae, genus *Desmodesmus* (Fig. 1c). Croatian sample CZ120 from a third different geographical location (Table 1) clustered with *Desmodesmus communis* var. *rectangularis* (Heg1998-22a) also from Chlorophyceae. Those endosymbionts isolated from Croatian hydras cluster within the Chlorophyceae lineage in Chlorophyta, they were not closely related to the genus *Chlorella* (Trebouxiophyceae). A fourth Croatian sample CZ80 from a different geographical location (Table 1) clustered with *Chlorella vulgaris* SAG 211-11b from Trebouxiophyceae, Chlorellales, Chlorellaceae, genus *Chlorella*. This unexpected diversity of endosymbionts might reflect the natural habitat of particular *Hydra* strains. These results confirm multiple origins of endosymbionts in green *Hydra* stemming from two highly divergent algal

classes. In the ITS tree, samples M9-19 and M9-20 from Israel formed a clade with *Chlorella variabilis* (SAG 211-6) (the *Chlorella*-endosymbiont of *P. bursaria*) and sample HV15 from Germany with *Nannochloris* sp. (AICB 424), both taxa from Trebouxiophyceae. Reference strain A100 formed a clade with *Parachlorella kessleri* (CCAP 211/11H) and reference strain CV80 with *Chlorella vulgaris* (SAG 211-11b) (Trebouxiophyceae).

To do effective search, we used three more molecular markers in phylogenetic algal analysis besides ITS: two chloroplast genes *rbc*L (large subunit of the ribulose-bisphosphate carboxylase gene) and 16S, and nuclear 18S sequences (supplementary data S2–S4).

3.3. Identity between the endosymbiotic algae isolated from green hydra hosts and cultured algae strains

16S rRNA and rbcL algal genes isolated from green hydra hosts in order to exclude possible cross contamination of cultured samples were amplified using same procedure and PCR conditions as used for cultured algae strains. Results of algal gene sequences from Botanical garden and Turopolje polyps and their cultured algal "partners" showed identity under Mega BLAST so we further on decided to use only sequences from cultured strains which was primary intention and aim of our research.

4. Discussion

Symbiosis of green algae with different protozoa and invertebrates has been studied for more than 100 years. Endosymbiotic green algae in invertebrates, like *Hydra,* have been traditionally identified as named or unnamed species of *Chlorella* Beij. or *Zoochlorella* K. Brandt or referred to as *Chlorella*-like algae or zoochlorellae. Molecular studies can provide new insides.

So far investigations on the evolution and origin of symbioses between *Chlorella*-based endosymbionts from different protozoa and invertebrates show multiple symbiont origins (Huss et al., 1993/94; Friedl, 1997; Hoshina and Imamura, 2008; Kovačević et al., 2010; Pröschold et al., 2011). These data suggest that multiple symbiont origins in *Hydra* are more likely than a single symbiont that diverged into more species. Phylogenetic results in this study also indicate multiple origins of endosymbionts in Croatian green *Hydra* hosts, and are thus congruent with reports of multiple origins for *Chlorella*-based endosymbionts in different protozoa and invertebrates.

In early work, Huss et al. (1993/94) argued that their phylogenetic tree based on 18S did not show monophyly of symbiotic *Chlorella* strains, and showed that the native green *Hydra* symbionts result from at least two recent but independent symbiotic events. Strains of symbiotic algae isolated from green hydra, named "European" Esh from UK, "Swiss" Ssh and HvT from Israeli green *Hydra* host were closely related to free-living species *Chlorella vulgaris*, *C. lobophora* and *C. sorokiniana* (Huss et al., 1993/94). However, strain Jsh isolated from Jerusalem was related to species *Chlorella protothecoides* (Huss et al., 1993/94), indicating polyphyletic origin of symbionts. The CCAP 211/7A and the symbiont called JSH (both from Israel) were closely related to the authentic *Auxenochlorella protothecoides* strain (Pröschold et al., 2011). Also, all "European" symbionts from green hydra, strains SSH, ESH and HvT belonged to *Chlorella* clade (Pröschold et al., 2011). These phylogenetic results were confirmed by phylogenetic tree based on 18S rRNA gene. There are no available ITS sequences for these strains with which we can compare isolated endosymbionts.

In research of phylogenetic relationships between symbiotic algae isolated from green hydra, endosymbiotic algal strains CZ33 and CZ43 18SF were closely related to the species *Desmodesmus subspicatus* (Chlorophyta) Hegewald et Schmidt. The sample CZ10 18SF was associated with species *Mychonastes homosphaera* (Chlorophyta) (Skuja) Kalina et Punčochářová. These results confirm that the green hydra symbiosis is result of at least two symbiotic events (Kovačević et al., 2010). Sample CZ120 in our study formed a clade with samples Scenedesmid sp. DF-2007 CZ43 and CZ33, confirming the phylogenetic analysis by Kovačević et al. (2010).

The occurrence of closely related, but independently acquired endosymbionts was found in lichen (Friedl, 1997). Multiple symbiont origins have also been demonstrated to be more likely than a single one that diverged into the three clades in ciliate *Paramecium bursaria* (Hoshina et al., 2006). *P. bursaria* has repeatedly acquired or replaced its photosynthetic algae over its evolutionary history, and such events occurred at least four times (Hoshina and Imamura, 2008). Phylogenetic 18S analysis of algal endosymbionts in *Paramecium bursaria* showed that they belonged to two lineages in the Trebouxiophyceae; three algal symbionts belonged to one clade Chlorellaceae, but symbiont CCAP 1660/13 belonged to clade which included species of *Coccomyx*a, *Paradoxia multiseta* and an endosymbiotic alga found in Ginkgo (Hoshina and Imamura, 2008). Similar findings like ones in *P. bursaria* were identified in endosymbiotic zoochlorellae inhabiting sea anemone *Anthopleura elegantissima* using phylogenetic 18S and rbcL markers (Lewis and Muller-Parker, 2004). The green algal symbiont from *A. elegantissima* was a member of Trebouxiophyceae, and its 18S sequence formed a well-supported clade with the lichen symbiont *Coccomyxa glaronensis*, the small green endophytes of *Ginkgo biloba* and free-living taxon *Paradoxia multiseta*, but clearly being distinct from the *Chlorella* symbionts of *Hydra* (Lewis and Muller-Parker, 2004).

Our ITS tree revealed that Croatian symbiotic *Chlorella* strains isolated from Croatian hydras did not form a monophyletic clade consisting only of those strains. Rather, the phylogenies indicate polyphyly and independent origins at different geographic location. This unexpected diversity of endosymbionts might depend on the natural habitat of a particular strain of green hydra (Kovačević et al., 2010). Fawley et al. (2004) examined the molecular diversity among freshwater microchlorophytes from classes Trebouxiophyceae and Chlorophyceae (orders Sphaeropleales and Chlamydomonadales) from two different localities. It was found that the communities of planktonic, coccoid green algae varied among two sites, only two sequence types were found at both sites. Their results about diversity among communities suggest that green algae may be responding to their habitat in a fine-grained manner. Even though ecological tolerances of microalgae may be broad in culture, individual niches may be narrow under natural conditions.

Rahat (1991) argues that today's algae probably are descendants of the colonizers of various cells in *Hydra*. The symbiosis has occurred in the way that some algae inside of *Hydra* cells used better ultravacuolar sources during intracellular competition between algae of the genus *Chlorella*, or some other photosynthetic partner. *Hydra* cells that benefitted from algal survival without hampering their own viability had an evolutionary advantage. Over millions of years of coevolution, what was originally a parasitic relationship became obligatory mutualistic and today is optionally mutualistic, although probably mutualistic throughout the life of the individuals in this symbiosis (Kovačević et al., 2010). That we were able to successfully isolate symbiotic algae from green hydra hosts and obtain them in stable cultures indicates a loose mutualistic relationship between the animals and their symbionts.

A recent analysis similar to ours concluded that symbiosis with *Chlorella* occurred once in an ancestral *viridissima* group of *Hydra* and has since been perpetuated long-term with occasional

co-speciation (Kawaida et al., 2013). In contrast, our results in the present study on ITS phylogenetic tree show that *Chlorella*-like endosymbionts from green *Hydra* hosts isolated from four different Croatian localities reflect symbioses involving two classes within the Chlorophyta: Trebouxiophyceae (order Chlorellales, genus *Chlorella*) and Chlorophyceae (order Sphaeropleales, genus *Desmodesmus*). Molecular phylogenies with four different genes revealed that the situation concerning algal endosymbiont origin in green *Hydra* is more complicated. Furthermore, the M9 and HV strains from Israel and Germany belonged to the *Chlorella*-clade. It is evident that green algae are divided into three major classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae. Within the class Trebouxiophyceae, the *Chlorella* clade clearly differs from the *Parachlorella* clade, confirming previous studies (Krienitz et al., 2004; Kovačević et al., 2010; Luo et al., 2010; Pröschold et al., 2011). Species from the class Prasinophyceae, as a separate class diverging the earliest from common ancestor of green algae, were used as outgroup in phylogenetic studies. Phylogenetic tree based on the ITS region do not support a monophyletic origin of endosymbiotic algal strains isolated from Croatian green *Hydra* hosts. It thus appears that *Hydra* – algal endosymbioses have been established multiple times during the evolution of these strains. Host and symbiont factors responsible for the successful establishment of these endosymbioses have yet to be identified.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.07.014.

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