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Two-step evolution of endosymbiosis between hydra and algae

Masakazu Ishikawa [a,b], Hiroshi Shimizu [c], Masafumi Nozawa [a,b], Kazuho Ikeo [a,b], Takashi Gojobori [c,*]

[a] Department of Genetics, SOKENDAI, Yata, Mishima, Shizuoka 411-8540, Japan
[b] Center for Information Biology, National Institute of Genetics, Yata, Mishima, Shizuoka 411-8540, Japan
[c] King Abdullah University of Science and Technology (KAUST), Computational Bioscience Research Center, Thuwal 23955-6900, Saudi Arabia

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ABSTRACT

In the *Hydra vulgaris* group, only 2 of the 25 strains in the collection of the National Institute of Genetics in Japan currently show endosymbiosis with green algae. However, whether the other non-symbiotic strains also have the potential to harbor algae remains unknown. The endosymbiotic potential of non-symbiotic strains that can harbor algae may have been acquired before or during divergence of the strains. With the aim of understanding the evolutionary process of endosymbiosis in the *H. vulgaris* group, we examined the endosymbiotic potential of non-symbiotic strains of the *H. vulgaris* group by artificially introducing endosymbiotic algae. We found that 12 of the 23 non-symbiotic strains were able to harbor the algae until reaching the grand-offspring through the asexual reproduction by budding. Moreover, a phylogenetic analysis of mitochondrial genome sequences showed that all the strains with endosymbiotic potential grouped into a single cluster (cluster γ). This cluster contained two strains (J7 and J10) that currently harbor algae; however, these strains were not the closest relatives. These results suggest that evolution of endosymbiosis occurred in two steps; first, endosymbiotic potential was gained once in the ancestor of the cluster γ lineage; second, strains J7 and J10 obtained algae independently after the divergence of the strains. By demonstrating the evolution of the endosymbiotic potential in non-symbiotic *H. vulgaris* group strains, we have clearly distinguished two evolutionary steps. The step-by-step evolutionary process provides significant insight into the evolution of endosymbiosis in cnidarians.

1. Introduction

Hydra is a cnidarian that inhabits freshwater and has no medusa stage. Phylogenetic studies of the genus *Hydra* have shown that the species can be classified into four monophyletic groups, namely, *H. vulgaris* group, *H. oligactis* group, *H. braueri* group, and *H. viridissima* group (Kawaida et al., 2010; Martínez et al., 2010; Schwentner and Bosch, 2015). One of the primary characteristics of the *H. viridissima* group is endosymbiosis with the alga *Chlorella* (Dunn, 1987; McAuley, 1986). The National Institute of Genetics (Mishima, Japan) maintains the world's largest collection of hydra strains, with samples collected worldwide. This collection contains six *H. viridissima* group strains, which all harbor green algae. A previous study suggested that endosymbiosis occurred before the divergence of the *H. viridissima* group strains and that the hydra and algae continued to co-speciate (Kawaida et al., 2013).

In addition to the *H. viridissima* group, the *H. vulgaris* group also harbors green algae (Rahat and Reich, 1985). Among the 25 *H. vulgaris* group strains in the collection, only two strains (J7 and J10) currently harbor algae; the algal genus residing in strain J7 has been identified as *Chlorococcum* (Kawaida et al., 2013). However, it is unknown whether the other non-symbiotic *H. vulgaris* group strains have the potential to harbor algae. A previous study showed that some *H. vulgaris* group strains were capable of forming a stable symbiosis with algae (Rahat and Reich, 1986; Rahat and Sugiyama, 1993). This observation suggests that evolution of endosymbiosis in the *H. vulgaris* group may have occurred in two steps: first, the endosymbiotic potential was obtained before or during radiation of the *H. vulgaris* group strains; and second, a symbiont was acquired only in the strains that are currently endosymbiotic. The objective of this study was to elucidate the evolutionary timing of endosymbiosis in the *H. vulgaris* group. First, we examined whether non-symbiotic strains had the potential for endosymbiosis by artificial introduction of endosymbiotic algae. Second, we sequenced the mitochondrial genomes of the 25 strains of the *H. vulgaris* group in the collection from the National Institute of Genetics and conducted a phylogenetic

Abbreviations: ML, maximum likelihood; BI, Bayesian inference; Mya, million years ago; PRRs, pattern recognition receptors.

* Corresponding author.
E-mail address: takashi.gojobori@kaust.edu.sa (T. Gojobori).

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analysis to estimate their evolutionary timing. On the basis of these experiments and data analysis, we propose a possible pathway for the evolution of endosymbiosis between *Hydra* and green algae.

2. Materials and methods

2.1. Hydra strains

Twenty-five strains of the *H. vulgaris* group maintained at the National Institute of Genetics were used in the present study (Table 1). Polyps were kept in a plastic dish filled with modified 'M' solution (Sugiyama and Fujisawa, 1977) at 18 °C under 12 h dark/light conditions with 2500 lux illumination. The polyps were fed three times a week.

2.2. Introducing endosymbiotic Chlorococcum into non-symbiotic strains

To examine whether the non-symbiotic strains have endosymbiotic potential, the endosymbiotic *Chlorococcum* was introduced into each of the strains. First, *Hydra* polyps with endosymbiotic *Chlorococcum* were disrupted in the *Hydra* culture solution using a crusher (μT-12, TITEC, Saitama, Japan) without beads. The suspension was centrifuged at 10,000*g* for 5 min and washed several times with the culture medium. This process resulted in a compact pellet of purified *Chlorococcum* at the bottom of the tube. The purified *Chlorococcum* were re-suspended in the *Hydra* culture solution and introduced into the gastric cavity of individual *Hydra* through the mouth opening using a microglass capillary. The increase in the number of *Chlorococcum* in the treated *Hydra* cells was periodically checked using a fluorescence microscope (Axiophot, Zeiss); the *Chlorococcum* appeared as red dots inside the *Hydra* cells (Fig. 1). The polyps were allowed to bud asexually for 2 weeks, and their offspring also budded, which resulted in grand-offspring polyps in the 2-week period. If *Chlorococcum* fully proliferated in the grand-offspring polyps (Fig. S1), we considered this host *Hydra* strain to have potential for endosymbiosis. Five individuals from each non-symbiotic strain were examined in this experiment.

We also examined endosymbiotic potential by axially grafting a half symbiotic J7 polyp to a half non-symbiotic polyp using the previously described axial transplantation procedure (Kawaida et al., 2013). At 2 weeks after grafting, the transfer of *Chlorococcum* from the J7 tissue into the non-symbiotic tissue was observed using a fluorescence microscope. Three axial transplanted polyps were prepared for each non-symbiotic strain tested.

Table 1
List of the analyzed strains of *Hydra vulgaris* group.

No.	Strain code	Identified species name	Origin of strain	Remarks
1	105	*H. magnipapillata*	Japan	Reference genome determined
2	A1	*H. magnipapillata*	Japan	Lacking holotrichous isorhiza
3	A9	*H. magnipapillata*	Japan	Another code; sf-1
4	B4	*H. magnipapillata*	Tokyo, Japan	
5	B10	*H. japonica*	Fukuoka, Japan	
6	B11	*H. magnipapillata*	Akita, Japan	
7	B12	*H. magnipapillata*	Akita, Japan	
8	D1	*H. magnipapillata*	Japan	Another code; mini-1
9	D7	*H. magnipapillata*	Japan	Another code; maxi-1
10	E4	*H. magnipapillata*	Japan	
11	F2	*H. magnipapillata*	Japan	Another code; Reg16
12	J1	*H. magnipapillata*	Japan	
13	J2	*H. magnipapillata*	Japan	
14	J6	*H. magnipapillata*	Japan	
15	J7	*H. magnipapillata*	Japan	Green algae symbiont
16	J10	*H. magnipapillata*	Japan	Green algae symbiont
17	B6	*H. attenuata*	Basel, Switzerland	
18	K5	*H. attenuata*	Basel, Switzerland	
19	K6	*H. attenuata*	Basel, Switzerland	
20	K9	*H. vulgaris*	Basel, Switzerland	
21	L2	*H. attenuata*	Basel, Switzerland	
22	K7	*H. attenuata*	Basel, Switzerland	
23	L4	*H. carnea*	U.S.A.	
24	M2	Not yet determined	CA, U.S.A.	
25	M5	*H. vulgaris*	CA, U.S.A.	

2.3. Determining the mitochondrial genome sequences of the H. vulgaris group strains

A phylogenetic analysis of mitochondrial sequences in the *Hydra* strains was performed. Genomic DNA was extracted from a single polyp of each of the *H. vulgaris* group strains using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. PCR primers were designed to cover as much as possible of the mitochondrial genome. Because the *Hydra* mitochondrial genome comprises two mitochondrial chromosomes (Voigt et al., 2008), we designed primers for the ends of each of mitochondrial chromosome based on the mitochondrial genome sequence of *H. magnipapillata* (Accession No. NC_011221). For chromosome 1, the primers used were *Hydra*-mt-first-F 5′-TGGCTCATGACCAGAA TATAAGGG-3′ and *Hydra*-mt-first-R 5′-AAGCTATCTGGAAAGTC TGCA-3′. For chromosome 2, the primers were *Hydra*-mt-second-F 5′-TGGCTCATGACCAGAATATAAGGG-3′ and *Hydra*-mt-second-R 5′-AAGCTATCTGGAAAGTCTGCA-3′. Amplifications were conducted in a 50-μL PCR mixture containing 10 ng of the template, 5 μL 10 × *LA Taq*™ buffer (TaKaRa Bio Inc., Japan) containing 2 mM $MgCl_2$, 0.5 μM of the primers, 0.4 mM dNTPs, and 2.5 U *LA Taq*™ polymerase (TaKaRa Bio Inc., Japan). The amplification conditions were: initial denaturation step at 94 °C for 2 min; 30 cycles of 10 s at 98 °C; 7 min annealing at 64 °C; and a final elongation step of 10 min at 72 °C. Amplified fragments of the expected lengths were extracted after gel electrophoresis and purified using a MiniElute Gel Extraction Kit (Qiagen). Using the products obtained as the starting materials, DNA libraries were constructed with NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (NEB, Ipswich, MA). The constructed libraries tagged with multiplex bar codes were enriched and loaded onto the Ion 316 chips, and the multiplex sequencing was conducted by the Ion

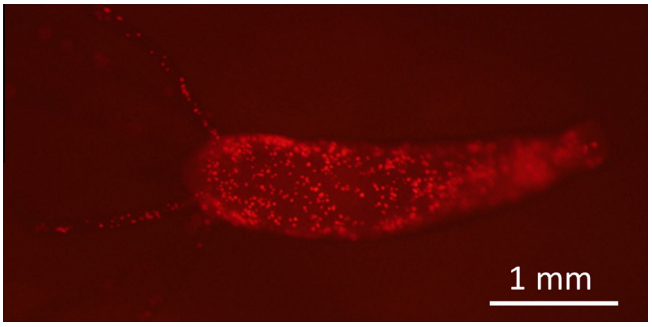

Fig. 1. A *Hydra* polyp harboring endosymbiotic algae. This photograph was taken under a fluorescence microscope. The small bright red particles inside the *Hydra* body are the endosymbiotic algae. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)