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Two-Step Evolution of Endosymbiosis between Hydra and Algae

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Abbreviations

ML: maximum likelihood

BI: Bayesian inference

Mya: million years ago

PRRs: pattern recognition receptors

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.

Keywords

Hydra, mitochondrial genome, endosymbiotic potential, *Chlorococcum*

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 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 2,500 lux illumination. The polyps were fed three times a week.

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

No.	Strain code	Identified species name	Origin of strain	Remarks
1	105	<i>H. magnipapillata</i>	Japan	Reference genome determined
2	A1	<i>H. magnipapillata</i>	Japan	Lacking holotrichous isorhiza
3	A9	<i>H. magnipapillata</i>	Japan	Another code; sf-1
4	B4	<i>H. magnipapillata</i>	Tokyo, Japan	
5	B10	<i>H. japonica</i>	Fukuoka, Japan	
6	B11	<i>H. magnipapillata</i>	Akita, Japan	
7	B12	<i>H. magnipapillata</i>	Akita, Japan	
8	D1	<i>H. magnipapillata</i>	Japan	Another code; mini-1
9	D7	<i>H. magnipapillata</i>	Japan	Another code; maxi-1
10	E4	<i>H. magnipapillata</i>	Japan	
11	F2	<i>H. magnipapillata</i>	Japan	Another code; Reg16
12	J1	<i>H. magnipapillata</i>	Japan	
13	J2	<i>H. magnipapillata</i>	Japan	
14	J6	<i>H. magnipapillata</i>	Japan	
15	J7	<i>H. magnipapillata</i>	Japan	Green algae symbiont
16	J10	<i>H. magnipapillata</i>	Japan	Green algae symbiont
17	B6	<i>H. attenuata</i>	Basel, Switzerland	
18	K5	<i>H. attenuata</i>	Basel, Switzerland	
19	K6	<i>H. attenuata</i>	Basel, Switzerland	

20	K9	<i>H. vulgaris</i>	Basel, Switzerland
21	L2	<i>H. attenuata</i>	Basel, Switzerland
22	K7	<i>H. attenuata</i>	Basel, Switzerland
23	L4	<i>H. carnea</i>	U.S.A.
24	M2	Not yet determined	CA, U.S.A.
25	M5	<i>H. vulgaris</i>	CA, U.S.A.

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 (μ T-12, TITEC, Saitama, Japan) without beads. The suspension was centrifuged at $10,000 \times g$ 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.

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 (Voight 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' -TGGCTCATGACCAGAATATAAGGG-3' and

Hydra-mt-first-R 5' -AAGCTATCTGGAAAGTCTGCA-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- μ L PCR mixture containing 10 ng of the template, 5 μ L 10 $\times$ *LA Taq*TM buffer (TaKaRa Bio Inc., Japan) containing 2 mM MgCl₂, 0.5 μ M of the primers, 0.4 mM dNTPs, and 2.5 U *LA Taq*TM polymerase (TaKaRa Bio Inc., Japan). The amplification conditions were: initial denaturation step at 94°C for 2 min; 30 cycles of 10 sec 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 PGM system with the Ion Sequencing 200 kit (Life Technologies). The raw sequencing reads of the mitochondrial genome are deposited in the DNA Database of Japan Sequence Read Archive (DRA) (accession number DRA003539, <https://trace.ddbj.nig.ac.jp/DRAsearch/submission?acc=DRA003539>).

The raw reads from the Ion PGM system were preprocessed using the scripts in the FASTX-Toolkit (Hannon lab., 2009). In each read, the first 30 nucleotides, the nucleotides after the 250 nt position, and nucleotide tails with quality scores ≤ 20 were discarded. After trimming, reads with lengths < 50 nucleotides were also discarded. The remaining reads were mapped onto the complete mitochondrial genome sequence of *H. magnipapillata*, which belongs to *H. vulgaris* group, using BWA (Li and Durbin, 2010) implemented in the DNA Database Japan pipeline (Kaminuma et al., 2010, Nagasaki et al., 2013). Through these procedures, we obtained the mitochondrial genome sequences of the 25 strains; however, some gaps were present in the sequences.

2.4 Determining the 18S rRNA nucleotide sequence of the J10 alga

A phylogenetic analysis of the endosymbiotic algae was carried out. Genomic DNA of the algae in strain J10 was extracted using a genomic DNA extraction kit (DNeasy Plant Mini Kit, Qiagen) after compacting and purifying the algae as described in the previous section.

Using the extracted DNA as a template, part of the 18S rRNA gene was amplified. We used primers reported previously for this amplification (Kawaida et al., 2013). The primer sequences were 5' -AACCTGGTTGATCCTGCCAGT-3' and 5' -TTGATCCTTCTGCAGGTTACCTACG-3'. The PCR products were sequenced with an

ABI 3130 Avant Genetic Analyzer (Applied Biosystems). A BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for the sequencing reactions.

2.5 Phylogenetic analysis

The sequences were aligned using the Clustal omega algorithm (Sievers et al., 2011). The sequence alignment was further optimized by manual inspection using Alignment Explorer in MEGA 6 (Tamura et al., 2013). We used the maximum likelihood (ML) method with RAxML v.8.1.0 (Stamatakis, 2006) and the Bayesian inference (BI) method using MrBayes5D (Ronquist and Huelsenbeck, 2003; Tanabe, 2012) to infer the phylogenetic relationships. The best-fit substitution models at each nucleotide partition were estimated using Akaike's information criteria for the ML analyses and the Bayesian information criterion for the BI analyses implemented in jModelTest (Nylin, 2010). For BI, the settings were: number of Markov chain Monte Carlo generations, 50 million; sampling frequency, 1,000; and burn-in, 1,001. The support for internal branches was evaluated using bootstrap percentages from 1,000 nonparametric replicates for the ML method and using Bayesian posterior probabilities for the BI. The divergence time between *H. oligactis* group and *H. vulgaris* group (72–132 million years ago, *Mya*) that was estimated by Schwenk and Bosch (2015) was used to calibrate the estimated times of divergence of the clusters. The analysis was conducted using the RelTime method (Tamura et al., 2012).

3 Results

3.1 More than half of the non-symbiotic strains of *H. vulgaris* group have the potential to harbor the *Chlorococcum*

We investigated the evolution of endosymbiosis between *H. vulgaris* group strains and *Chlorococcum* by first examining the endosymbiotic potential of non-symbiotic strains. The alga in strain J7 (hereafter J7-alga) belongs to the genus *Chlorococcum* (Kawaida et al., 2013). However, the alga in strain J10 (J10-alga) has not been taxonomically identified to date. We performed an analysis of the phylogenetic relationships between the algae in the J7 and the J10 strains. The ML tree based on the 18S rRNA gene clearly showed that the J10-alga was genetically closely related to the J7-alga (Fig. 2); therefore, we used only the J7-alga in this experiment.

If our method for introducing algae is reliable, then it should be possible to re-establish the endosymbiotic relationship between the *Chlorococcum* and the *Chlorococcum*-eliminated, aposymbiotic J7 polyps. We eliminated the *Chlorococcum* from symbiotic J7 polyps by culturing the animals under dark conditions for several weeks (McAuley, 1981). Purified *Chlorococcum* cells were then injected into the gastric cavities of *Chlorococcum*-eliminated J7 polyps. We found that *Chlorococcum* was fully incorporated into endodermal epithelial cells of the *Hydra* and transmitted into offspring within 2 weeks (Fig. 3). Therefore, the endosymbiotic

relationship was clearly re-established between the artificially introduced *Chlorococcum* and aposymbiotic J7. This finding validated the rationale of the reintroduction experiment.

We performed artificial introduction of algae into 23 strains (excluding J7 and J10). For 12 strains (105, A1, B4, B6, B10, B11, D1, D7, J6, L2, K7, and K9), all treated individuals established stable endosymbiosis, and no qualitative differences in the incorporation of the *Chlorococcum* was present among individuals. In the 11 other strains, algal cells were completely absent in all five treated individuals (Fig. S2).

We confirmed endosymbiotic potential by axial grafts of half polyps of the endosymbiotic J7 strain with half polyps of the non-symbiotic strains B12, K5, K7, and L4. We found that *Chlorococcum* spread from the symbiotic J7 into non-symbiotic K7, which also harbored the *Chlorococcum* in the artificial introduction (Fig. 4A). However, transfer of *Chlorococcum* did not occur in grafts involving strains that did not establish endosymbiosis after artificial introduction of algae (B12, K5, L4) (Fig. 4B–D). Our artificial introduction and grafting experiments were therefore consistent with each other.

3.2 Strains with endosymbiotic potential were grouped into a single phylogenetic cluster

Kawaida et al. (2010) sequenced several genes of *Hydra*, including the mitochondrial *COI* and nuclear *CnNOS1* genes, and constructed a phylogenetic tree for the genus *Hydra*. However, as the selected genes are known to evolve at slow rates, there was insufficient sequence divergence to resolve the phylogenetic relationships among the *H. vulgaris* group strains (Kawaida et al., 2010; Kayal and Lavrov, 2008). In the present study, we used mitochondrial genome sequences to investigate the phylogenetic relationships among *H. vulgaris* group strains as these generally evolve at a fast rate. Sequencing using the Ion Torrent PGM platform yielded approximately 40,000–255,000 trimmed reads in each sample, and more than 80% of these reads were successfully mapped to the reference genome (Table S1). Scaffolds of more than 12,000 bp were obtained for each strain.

We used the aligned mitochondrial genome sequences to conduct a phylogenetic analysis of the *H. vulgaris* group strains. The ML tree based on mitochondrial genomes is shown in Fig. 5. The sequence of *H. oligactis* (NC_010214) was used as the outgroup. The BI tree had an identical topology to the ML tree. The analyses identified three clusters in the *H. vulgaris* group strains.

The first cluster consisted of strains L4, M2, and M5 (cluster α) that were collected in the United States. The second cluster consisted of strains K5 and K6 (cluster β), with origins in Europe. The third cluster (cluster γ) included strains that were primarily of Japanese origin,

although B6, K7, K9, and L2 were collected in Europe. We sub-divided cluster γ into four subclusters (γ -1, γ -2, γ -3, and γ -4) with high bootstrap values. All strains that were identified to have endosymbiotic potential in the algal introduction experiment were in cluster γ (indicated by asterisks in Fig. 5). All strains in subclusters γ -1, γ -2, and γ -3 showed endosymbiosis, whereas only one strain (K7) in cluster γ -4 showed endosymbiosis. These results suggest that endosymbiotic potential evolved in the common ancestor of the γ -cluster and that this potential was lost in the γ -4 cluster lineage after the divergence from strain K7 under a parsimony principle.

3.3 Evolution of endosymbiotic potential occurred more recently in *H. vulgaris* group than *H. viridissima* group

Schwentner and Bosch (2015) estimated the divergence time of *H. viridissima* group and three other *Hydra* groups to be 254–404 Mya. Based on their data, the radiation of *H. viridissima* group strains was estimated to be 77–152 Mya. All of the *H. viridissima* group strains show endosymbiosis; therefore, the *H. viridissima* group gained endosymbiotic potential 77–404 Mya.

Our phylogenetic analysis showed that the *H. vulgaris* group strains were grouped into three clusters, and cluster α diverged first, followed by β and γ . Among the clusters, only cluster γ strains have endosymbiotic potential. This suggests that the *H. vulgaris* group gained endosymbiotic potential after divergence of clusters β and γ ; therefore, we estimated the

divergence time between clusters β and γ to be 28.1–42.0 Mya. In addition, the divergence time between γ -1 and γ -2 was estimated to be 4.7–7.5 Mya. These results suggest that *H. vulgaris* group gained endosymbiotic potential between 4.7 and 42.0 Mya, which is more recent than in the *H. viridissima* group. Moreover, our study also showed that cluster γ -4 strains other than K7 do not have endosymbiotic potential. This suggests that endosymbiotic potential was lost after the divergence of K7 and the other γ -4 strains. The divergence time between strain K7 and other γ -4 strains was estimated to be 0.34–1.7 Mya. This suggests that a clade of the γ -4 *H. vulgaris* group strains lost endosymbiotic potential more recently than 1.7 Mya. Although the timing of both events was recent, we were able to clearly distinguish these two events.

4 Discussion

4.1 A clade in *H. vulgaris* group gained endosymbiotic potential once during the divergence of the group

Phylogenetic analysis of the *H. vulgaris* group showed that they could be grouped into three clusters (α , β , and γ) and that cluster α diverged first, followed by clusters β and γ . Cluster γ strains, except for six strains belonging to the subcluster γ -4, had endosymbiotic potential (Fig. 5). Our data indicate that endosymbiotic potential was gained in the γ lineage during the divergence of the *H. vulgaris* group strains (Fig. 6) and that it was lost in the clade of γ -4 after

the divergence of K7 and the other γ -4 strains (Fig. 6). Moreover, the phylogenetic relationship between J7 and J10 was relatively distant (Fig. 6). Two possible scenarios can be envisaged for the evolution of symbiosis between the *H. vulgaris* group and *Chlorococcum*. First, two strains of the γ clade obtained symbionts independently. Second, symbionts were obtained by the ancestor of the γ clade strains, but were lost except in strains J7 and J10. Based on the parsimony principle, the former explanation appears more likely (Fig. 6). Therefore, we suggest that the evolution of endosymbiosis between the *H. vulgaris* group and *Chlorococcum* was a two-step process: first, the endosymbiotic potential was gained in an ancestor of the γ lineage; second, symbionts were acquired in two of the current endosymbiotic lineages.

However, we cannot completely exclude the possibility that the evolution of endosymbiotic potential occurred before the separation of the γ lineage and that the endosymbiotic

Chlorococcum changed the host specificity. Therefore, the current endosymbiotic

Chlorococcum in J7 may not show endosymbiosis with the α and β lineages. However, it has

been shown that in the *H. viridissima* group, an aposymbiotic strain can harbor a symbiont from

a different strain even though the divergence time between the strains was estimated to be

77–152 Mya (Schwentner and Bosch, 2015). If this time scale is applied to the *H. vulgaris*

group, it is unlikely that the *H. vulgaris* group would have experienced such changes in host specificity among strains.

4.2 For understanding the molecular mechanisms of the endosymbiotic potential

Various molecular mechanisms have been postulated to be involved in the gain and loss of endosymbiotic potential. Pattern recognition is one possible candidate: when a host encounters microbes in the environment, the host recognizes the microbes using pattern recognition receptors (PRRs) (Crosnier et al., 2011; Fujita et al., 2004; Jimbo et al., 2000; Meyer and Weis, 2012; Weis et al., 1998; Wood-Charlson et al., 2006). For example, lectins are a ubiquitous and diverse group of PRRs that bind glycans; in parasitic interactions, lectins play an important role in the innate immune response that leads to the destruction of the pathogens (Fujita et al., 2004). Lectin/glycan interactions are also implicated as an inter-partner signaling mechanism during the onset of symbiosis in associations between anthozoa and *Symbiodinium* (Jimbo et al., 2000). In *H. vulgaris* group strains, endosymbiotic potential was apparently gained after the divergence of clusters β and γ , and this potential was lost after the divergence of K7 and the other γ -4 strains (Fig. 6). For our analysis, we used K7 and other cluster γ -4 strains that are genetically closely related. Possibly, by comparing the genomes of these strains we will be able to identify genetic differences associated with endosymbiotic potential.

4.3 Why do not most of the *H. vulgaris* group strains with endosymbiotic potential have symbionts actually?

Our algal introduction experiment demonstrated that many non-symbiotic strains have endosymbiotic potential. Therefore, the absence of symbionts did not always indicate a lack of endosymbiotic potential. Although many strains possess endosymbiotic potential, why do only two strains naturally harbor the symbionts at present? One possible reason is that the hydras or algae were transported to the location of the partner. Many animals and plants are transported worldwide by humans and migratory birds (Mack and Lonsdale, 2001; Heck et al., 2008). A previous study on the phylogenetic relationships of several hydra species showed that many did not match their geographic origins (Martinez et al., 2010). Our phylogenetic analysis also demonstrated that strains with Japanese and European origins were both part of cluster γ , implying that hydra is often transported. Another possible explanation is that the *H. vulgaris* group has not yet adapted to this endosymbiosis. A recent study has shown that evolutionary patterns of mutualism are similar to those of parasitism (Sachs et al., 2011); in addition, Toft and Anderson (2010) suggested that bacterial mutualism evolved from parasitic lineages. *H. viridissima* group use photosynthetic products from endosymbiotic algae (Douglas and Smith, 1984) and are more tolerant of starvation (Muscatine and Lenhoff, 1963). Thus, the *H. viridissima* group has apparently established a mutualistic endosymbiotic relationship with the

algae. Here, we found that the *H. vulgaris* group had gained endosymbiotic potential much more recently than the *H. viridissima* group; therefore, it is possible that the mechanisms for stable endosymbiosis in the *H. vulgaris* group is not as well established as those in the *H. viridissima* group. In future studies, mechanisms of tolerance for endosymbiosis in the *H. vulgaris* group through analysis of the gene expression patterns and protein profiles in the endosymbiotic *H. vulgaris* group should be investigated.

In conclusion, we propose two evolutionary events for development of endosymbiosis between *H. vulgaris* group strains and the algae found in the different lineages; first, the evolution of endosymbiotic potential occurred once during the divergence of the *H. vulgaris* group strains; and second, strains J7 and J10 acquired *Chlorococcum* independently. Our study implies that a step-by-step evolutionary process was required to establish the endosymbiotic relationship. To understand the establishment of stable endosymbiosis in general, further elucidation of the two-step evolutionary process of the endosymbiosis found in *H. vulgaris* group will be valuable.

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Figure legends

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.

Fig. 2. Maximum likelihood (ML) tree inferred from the nucleotide sequences of the 18S rRNA gene of *Chlorococcum*. The numbers along branches indicate the bootstrap value for ML (Left) and the Bayesian posterior probability for Bayesian inference (BI, right).

Fig. 3. Increases in symbiotic *Chlorococcum* after injection into aposymbiotic J7 polyps. Live samples on days 0 (A), 4 (B), 7 (C), and 14 (D) after the introduction of the algae were photographed under a fluorescence microscope. Bright-red particles in the polyps show the symbiotic *Chlorococcum*. The arrowheads in A indicate incorporated *Chlorococcum* in the new host polyp. Arrows in B-D indicate offspring.

Fig. 4. Axial graft of a symbiotic J7 and non-symbiotic K7 (A), B12 (B), K5 (C), and L4 (D). Half of a non-symbiotic polyp was transplanted onto half of a symbiotic J7 polyp. Live samples were photographed under a fluorescence microscope at 2 weeks after transplantation. Grafted parts are indicated by dotted lines.

Fig. 5. Maximum likelihood (ML) tree inferred from nucleotide sequences of the mitochondrial genomes of the *Hydra vulgaris* group strains. The numbers along branches indicate the bootstrap value for ML (left) and the Bayesian posterior probability for Bayesian inference (BI, right). Strains that could establish endosymbiosis with introduced algae are indicated by asterisks.

Fig. 6. A possible scenario for the origin and evolution of endosymbiosis in *Hydra*. Gray and open circles represent strains with and without endosymbiotic potential, respectively. Solid circles indicate strains that currently harbor algae. The length of the line is not correlated with the actual divergence time. Numbers at several nodes indicate divergence times estimated by the RelTime method (Tamura et al., 2012). The bold line shows divergence of strains with endosymbiotic potential. The yellow stars show the origins of endosymbiotic potential in *Hydra*, whereas the red cross indicates the loss of the potential. The dashed arrows with green circles indicate the times when endosymbiosis between hydras and algae was established.

Fig. S1. Images of the grand-offspring of a polyp in which algae were introduced. (A) A polyp of strain 105, with endosymbiotic potential, in which the algae show full proliferation. (B) A polyp of strain E4, without endosymbiotic potential, in which no algae are present.

Fig. S2. Images of strain E4 using a fluorescence microscope. (A) An individual 2 h after the introduction of algae. (B) The same individual 14 d after the introduction of algae. The arrowheads in (A) indicate the incorporated *Chlorococcum* in the new host polyp.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

- ★ Acquisition of endosymbiotic potential
- ➡ Acquisition of endosymbiotic *Chlorococcum*
- ✗ Loss of endosymbiotic potential
- The strains with endosymbiotic potential, and with harboring the algae
- The strains with endosymbiotic potential, but without harboring the algae
- The strains without endosymbiotic potential

Highlights

- Evolution of the endosymbiosis between *Hydra vulgaris* group and symbiotic *Chlorococcm* occurred in two steps: first is gain of the endosymbiotic potential, second is actual obtainment of the endosymbiotic algae.
- The endosymbiotic potential in *H. vulgaris* group acquired once during radiation of the *H. vulgaris* strains, and the potential was lost in one *H. vulgaris* group lineage.
- The *H. vulgaris* group strains which currently show endosymbiosis obtained the endosymbiotic algae independently after radiation of the *H. vulgaris* strains.