

Maintenance of algal endosymbionts in *Paramecium bursaria*: a simple model based on population dynamics

Sosuke Iwai,* Kenji Fujiwara and Takuro Tamura

Department of Biology, Faculty of Education, Hirosaki University, Hirosaki 036-8560, Japan

Summary

Algal endosymbiosis is widely distributed in eukaryotes including many protists and metazoans, and plays important roles in aquatic ecosystems, combining phagotrophy and phototrophy. To maintain a stable symbiotic relationship, endosymbiont population size in the host must be properly regulated and maintained at a constant level; however, the mechanisms underlying the maintenance of algal endosymbionts are still largely unknown. Here we investigate the population dynamics of the unicellular ciliate *Paramecium bursaria* and its *Chlorella*-like algal endosymbiont under various experimental conditions in a simple culture system. Our results suggest that endosymbiont population size in *P. bursaria* was not regulated by active processes such as cell division coupling between the two organisms, or partitioning of the endosymbionts at host cell division. Regardless, endosymbiont population size was eventually adjusted to a nearly constant level once cells were grown with light and nutrients. To explain this apparent regulation of population size, we propose a simple mechanism based on the different growth properties (specifically the nutrient requirements) of the two organisms, and based from this develop a mathematical model to describe the population dynamics of host and endosymbiont. The proposed mechanism and model may provide a basis for understanding the maintenance of algal endosymbionts.

Introduction

Acquired phototrophy by algal endosymbiosis is widely distributed in eukaryotes including many protists and metazoans (Stoecker *et al.*, 2009; Johnson, 2011). Host organisms that contain algal endosymbionts within their cells can

generally benefit from enhanced growth or survival through access to photosynthetic products released by the algae. From a nutritional point of view, acquired phototrophy is usually mixotrophy that combines phagotrophy and phototrophy, and plays important roles in aquatic ecosystems (Stoecker *et al.*, 2009; Esteban *et al.*, 2010). In freshwater ecosystems, most common endosymbionts are *Chlorella*-like unicellular green algae (Reisser, 1986; 1992). The species hosting *Chlorella*-like algae include protists such as ciliates, amoebae, and heliozoa, and invertebrates such as *Hydra* (Reisser, 1992; Pröschold *et al.*, 2011). *Paramecium bursaria* is one such ciliate that harbours *Chlorella*-like algae. Like other species hosting algal endosymbionts, *P. bursaria* also benefits from enhanced growth under food-limiting conditions and survival under starved conditions (Karakashian, 1963).

In general, to maintain a stable symbiotic relationship, the number of endosymbionts in the host must be properly regulated and maintained at a constant level. For example, *P. bursaria* normally contains several hundreds of the symbiotic algae in its cytoplasm and is persistently and facultatively associated with these endosymbionts (Siegel, 1960). To explain such regulation and maintenance for a wide range of algal endosymbionts, three possible mechanisms have been suggested (Muscatine and Pool, 1979; Davy *et al.*, 2012): (i) expulsion or (ii) digestion of excess endosymbionts and (iii) regulation of endosymbiont cell growth or division. In *P. bursaria* cells, the symbiotic algae are individually enclosed in a perialgal vacuole usually attaching to the host cell cortex and normally escaping digestion via lysosome fusion (Karakashian and Karakashian, 1973; Karakashian and Rudzinska, 1981; Kodama and Fujishima, 2009), except under stress conditions such as long-term culture in darkness (Gu *et al.*, 2002). In addition, expulsion is not frequently observed under culture conditions in which *P. bursaria* grows well, and it may not be central to regulating the endosymbionts in *P. bursaria*.

Several models have been proposed for the regulation of cell growth or the division of algal endosymbionts by hosts (for review, see Davy *et al.*, 2012). Host-regulated supply of inorganic nutrients (Muscatine and Pool, 1979; Muscatine and Neckelmann, 1981), or more directly, the 'division factor' that is essential for the division of

Received 5 June, 2015; revised 18 November, 2015; accepted 18 November, 2015. *For correspondence. E-mail iwai-so@hirosaki-u.ac.jp; Tel. (+81) 172393365; Fax (+81) 172321478.

endosymbionts (McAuley, 1985) may be preferable in that it allows for density-dependent divisions of the endosymbionts (McAuley, 1985; Taylor *et al.*, 1989; McAuley and Darrah, 1990); however, neither underlying molecular mechanisms nor the factor itself are identified. Likewise, evidence is lacking for the host-regulated supply of a 'growth inhibitor' (Muscatine and Pool, 1979; McAuley, 1982), and for the host-regulated release of photosynthetic products by endosymbionts (Douglas and Smith, 1984). The coupling of cell cycles between the host and the endosymbiont may be an important factor in regulating endosymbiont population size, as it simply leads to coordinated growth of the two organisms and thereby the maintenance of endosymbiont number in the host. In some invertebrates, the relationships of cell cycle processes between the two organisms seem to be positive, whereas the degree of the coupling is variable depending on cells, species or environments (McAuley, 1982; 1985; Fitt, 2000; Dimond *et al.*, 2013). It has been suggested that in *P. bursaria* the cell cycle of the endosymbiont depends on that of the host (Kadono *et al.*, 2004); however, close coupling of cell cycles between *Chlorella*-like algae and ciliates is likely to require complicated systems because of the differences in their respective cell division patterns. Thus, mechanisms by which endosymbionts are maintained are still poorly understood.

Paramecium bursaria is a unicellular ciliate that reproduces asexually by binary fission only and may provide a simple experimental model to elucidate mechanisms for maintaining algal endosymbionts. Although some pioneering studies have been carried out (Weis, 1969; Reisser, 1986; Kadono *et al.*, 2004), the mechanism for regulating the *P. bursaria*–algae symbiosis is still unclear. In this study, we aimed to provide a basis for understanding the mechanisms underlying the maintenance of algal endosymbionts by investigating the population dynamics of the host *P. bursaria* and its endosymbiotic algae under various experimental conditions, using a simple culture system. Our results suggest that endosymbiont population size in *P. bursaria* was regulated not by active processes such as cell division coupling, but rather by some indirect means. Consequently, we propose a simple mechanism based only on the different growth properties of the two organisms. On the basis of the proposed mechanism, we develop a mathematical model to describe the population dynamics of the host and the endosymbiont.

Results and discussion

Population dynamics of P. bursaria and its algal endosymbiont under different experimental conditions

A simple culture system is a powerful tool to study the population dynamics of microorganisms. The conventional culture systems for *Paramecium* spp. are compli-

cated and consist of bacteria and types of plant infusions (e.g. Siegel, 1960; Karakashian, 1963; Weis, 1969; Kadono *et al.*, 2004; but see Omura *et al.*, 2004). We therefore developed a simple and reliable culture system for *P. bursaria* using the yeast *Saccharomyces cerevisiae* as the sole food source. The system comprises living yeast cells suspended in a solution containing minimal salts and an antibiotic and is free of bacteria or any other microorganism. The growth rate of *P. bursaria* cultured in this system was comparable with those reported for other systems (Fig. 1A, upper panel, and Table 1; Siegel, 1960; Karakashian, 1963; Weis, 1969; Kadono *et al.*, 2004; Omura *et al.*, 2004), confirming that *S. cerevisiae* cells were suitable prey for *P. bursaria*. Neither visible cellular aggregation nor biofilm was formed in the culture at least during growth measurements, probably because of the absence of bacteria. In *P. bursaria* cells, *S. cerevisiae* cells were completely digested and did not remain during the stationary phase, unlike other yeast species (Görtz, 1982). An additional supply of yeast cells to *P. bursaria* cells that had already consumed the initially supplied food and arrested their growth led to further growth (up to $>10^4$ cells mL^{-1}), indicating that the growth arrest was caused by the depletion of nutrients rather than the accumulation of growth-inhibitory products. Our system is a simple batch culture in which the nutrients are all in particulate form and will be mostly consumed by *P. bursaria* and is particularly suited to mark the transition from the log to the stationary phase. Because the usual logistic equation could not be satisfactorily fitted to the growth curves, semilog growth plots fitted with two separate regression lines, consisting of an early linear function for the log phase and a later constant function for the stationary phase, were used to reveal growth transitions.

Using this culture system, we monitored the population dynamics of the host *P. bursaria* and the endosymbiotic algae simultaneously under a normal light/dark cycle (12 h/12 h). Total algal cell density was estimated by lysing the host cells from aliquots of the culture using a detergent. In parallel, algal cells external to host cells were detected and estimated to be usually less than two cells per *P. bursaria* cell, which was considerably less than those within the host cells. In the log phase, the host and the endosymbiont multiplied at similar growth rates (Fig. 1A, upper panel, and Table 1), as previously described (Weis, 1969). Our study, however, demonstrated that the number of endosymbionts per host was not always constant during the growth process (Fig. 1A, lower panel). Initially, the number of endosymbionts showed a decrease, which was ascribed to the fact that the hosts started their growth with a shorter lag than the endosymbionts (Fig. 1C). Upon entry into the stationary phase, even after the growth of the hosts was arrested, the growth of the endosymbionts continued for about one

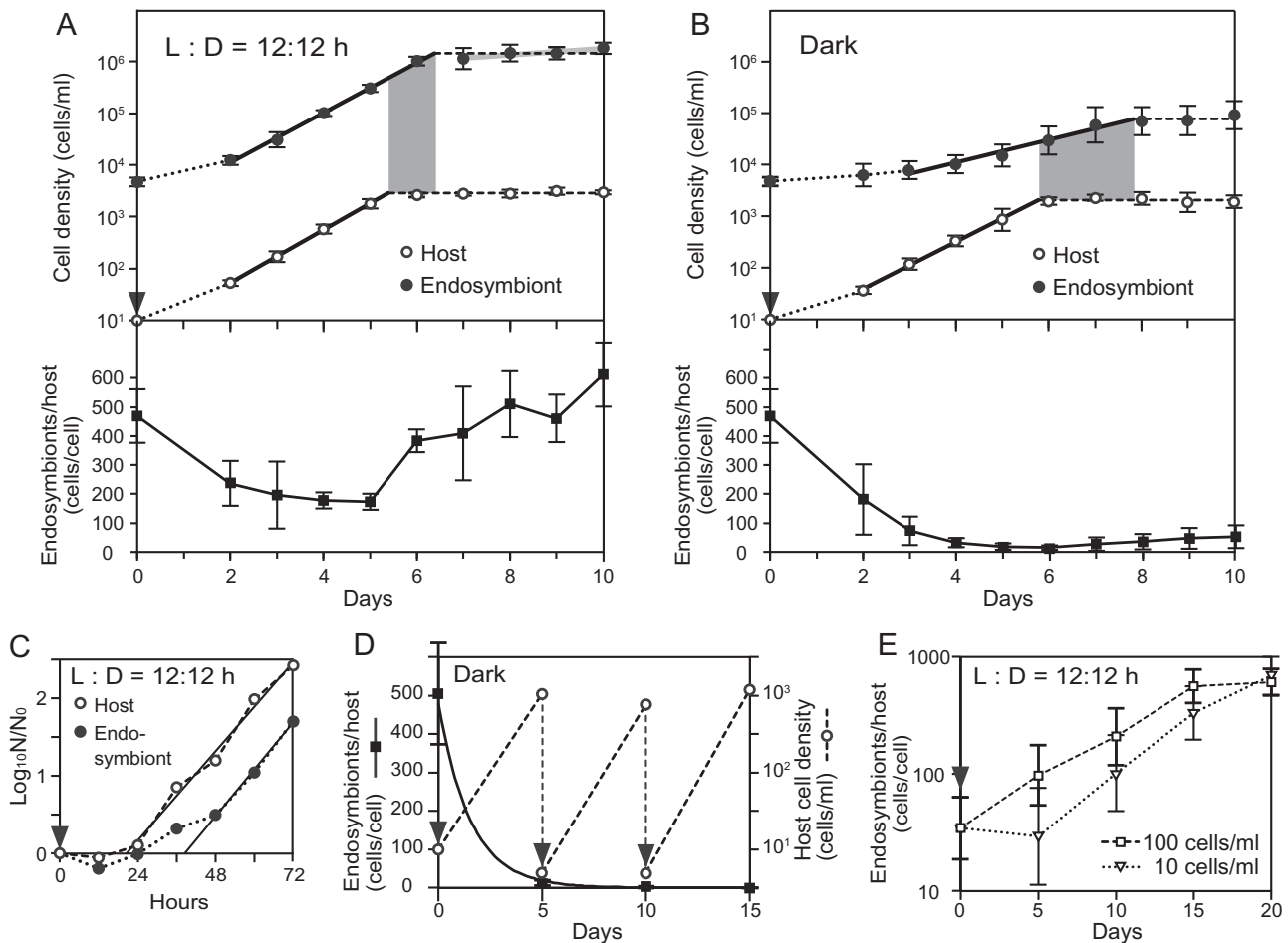


Fig. 1. Population dynamics of *P. bursaria* and endosymbiotic algae. Arrowheads indicate each supply of yeast cells. (A and B, upper panels) Growth of *P. bursaria* and endosymbiotic algae under a 12 h/12 h light/dark cycle (A) and in constant darkness (B) presented as semilog plots and mean $\pm$ standard deviation for three independent measurements. Linear (solid lines) and constant (dashed lines) functions were fitted to the plots (see Table 1 for detail). Grey areas indicate differences in the log-stationary transitions between host and endosymbiont. (Lower panel) Mean number of endosymbionts per host under light/dark cycle (A) and in constant darkness (B) calculated from the respective upper panels. (C) Lag phase and early-log growth phase of *P. bursaria* and endosymbiotic algae under light/dark cycle, presented as semilog plots of N/N_0 , where N and N_0 denote cell densities and initial cell densities respectively. (D) Loss of endosymbiotic algae in constant darkness. *Paramecium bursaria* cells were diluted and fed with yeast cells at 5 day intervals. (E) Recovery of the reduced endosymbiotic algae in *P. bursaria* with initial cell densities of 100 or 10 cells ml⁻¹ under light/dark cycle, presented as semilog plots. For (D) and (E), the numbers of endosymbionts per host were directly counted and are presented as mean $\pm$ standard deviation. ($N > 10$ cells).

Table 1. Fit of models to the population dynamics of *P. bursaria* and endosymbiotic algae, and their log growth rates under a 12 h/12 h light/dark cycle or in constant darkness.

Condition	Organism	Log-stationary transition (day)	Log growth rate (h ⁻¹) (Doubling time (h))	R ²
Light/dark cycle (12 h/12 h)	Host	5.4	0.049 (14.1)	0.999
	Endosymbiont	6.4	0.046 (15.0)	0.995
Constant dark	Host	5.8	0.044 (15.9)	0.997
	Endosymbiont	7.8	0.021 (32.5)	0.979

The semilog plots in Fig. 1A and 1B were separated into two groups and were fitted using two separate regression lines (linear and constant functions respectively). The models in which the joint point of the two lines, i.e. the log-stationary transition, was located between the two groups and had high R^2 values were selected as optimal.

more day (Fig. 1A, grey area, and Table 1), allowing endosymbiont numbers to catch up. Moreover, even in the stationary phase, the endosymbionts grew very slowly [Fig. 1A, grey line; Akaike's information criterion (AIC) = -6.0 and -18.0 for constant and linear models respectively], eventually reaching ~1000 cells per host. As described above, algal cells were mostly within host cells in our culture system, suggesting that expulsion was not so frequent that its contribution to endosymbiont regulation was small. Collectively, despite the similar log growth rates between the host and endosymbiont, the endosymbiont population size in the host showed a dynamic behaviour, caused mainly by the time lags between the growth processes of the two organisms.

To examine the effects of algal photosynthesis on the population dynamics, *P. bursaria* cells were next cultured in constant darkness. The growth rate of the host in darkness was comparable with that under the normal light regime (Fig. 1B, upper panel), suggesting that in the presence of excess food it was not affected by endosymbiont photosynthesis. In contrast, the growth rate of the endosymbionts was markedly reduced in darkness, indicating that it depended on the symbionts' own photosynthetic activity. It should be noted that even in darkness, the endosymbiont did not cease growth but increased slightly (up to ~30-fold). Isolated algal cells also showed growth in darkness on nutrient-rich agar (S. Iwai, unpubl. data). These results imply that the host provides at least some of the nutrients to the endosymbiont, irrespective of whether the endosymbiont provides photosynthetic products. From the significant difference between the growth rates of the two organisms, the endosymbionts would be expected to decrease exponentially and to soon be lost under dark conditions. In our results, however, after an exponential decrease, the endosymbionts were not lost but rather increased slightly in number (Fig. 1B, lower panel). This is likely due to the endosymbionts' continued slow growth for a few more days after the host cells arrested theirs (Fig. 1B, grey area, and Table 1), similar to the results observed under the normal light regime.

It has remained unclear whether *P. bursaria* cells cultured in darkness completely eliminate their endosymbionts (Siegel, 1960; Hosoya *et al.*, 1995). Our experimental system revealed that the successive addition of food yeast cells at 5 day intervals to *P. bursaria* in darkness led to a rapid decrease in endosymbionts, which then disappeared within 10–15 days (Fig. 1D); this was even faster than previously reported (Siegel, 1960). Based on this result, it appears that the endosymbionts in *P. bursaria* will be completely eliminated in darkness, provided that food is continuously supplied and the host remains in the log growth phase. The result also implies that *P. bursaria* lacks specific mechanisms to retain a minimum number of endosymbionts under such condi-

tions. It is also unknown whether *P. bursaria* cells that at one point eliminated their endosymbionts in darkness can recover them under light and nutrient conditions. To test this, *P. bursaria* cells were first cultured in darkness for 5 days to reduce endosymbionts to <100 cells per host, then transferred to the normal light regime and provided food yeast cells. Culturing the cells in light for 15–20 days led to recovery of the endosymbionts, which finally attained 600–800 cells per host, irrespective of initial host cell densities (Fig. 1E). This suggests that despite its dynamic behaviour, the endosymbiont population size eventually adjusts to a nearly constant level if *P. bursaria* cells grow in the presence of light and nutrients.

Cell divisions of the host and endosymbionts under light–dark changes

Many ciliates, including *Paramecium* spp., reproduce asexually by binary fission, whereas *Chlorella*-like algae, including endosymbiotic ones, reproduce exclusively by autospore production, in which the algae produce 2, 4, 8 or 16 autospores within a mother cell wall (Kuhl and Lorenzen, 1964). The endosymbiotic algae in *P. bursaria* mostly produce two or four autospores (Reisser, 1992). In addition, the cell cycles of free-living *Chlorella* spp. are often coupled to light–dark changes, usually consisting of autospore production in the light period and their liberation in the dark period (Kuhl and Lorenzen, 1964). To determine whether the cell divisions of *P. bursaria* and the algal endosymbionts within the host cells depend on the light–dark change, the cell divisions of the two organisms were investigated during a light–dark change in the log phase. The ratio of host cells forming cleavage furrows (Fig. 2, upper inset), which are temporally correlated with the progress of nuclear division (Tucker *et al.*, 1980) and can be used as a mitotic index for the ciliate, showed almost no dependence on the light–dark change (Fig. 2, upper panel). In contrast, the ratio of the endosymbiont cells forming two to four autospores (Fig. 2, lower inset), measured as a mitotic index for the *Chlorella*-like algae, showed clear dependence on the light–dark change, with an increase in the light period and a decrease in the dark period (Fig. 2, lower panel). These results suggest that the cell divisions of the host and of the endosymbiont in *P. bursaria* exhibit a different dependence on the light–dark change, probably reflecting the cell division patterns of their respective free-living relatives. Furthermore, there seems to be only slight, if any, coupling between the cell divisions of the two organisms even under the normal light regime. This was also supported by the result that in our system, ratio of the algal cells forming autospores was not significantly different between interphase ($4.4 \pm 2.5\%$, $N = 18$) and dividing ($3.6 \pm 2.4\%$, $N = 18$) host cells within a certain time period (Mann–Whitney *U*-test, $P = 0.3$).

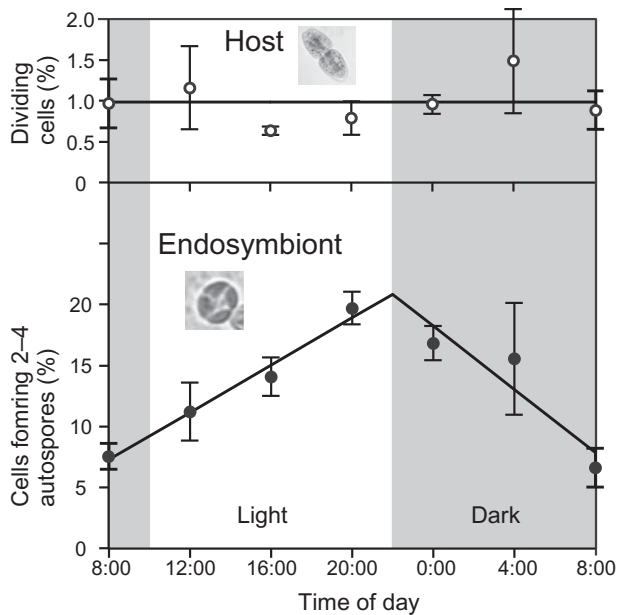


Fig. 2. Cell divisions of *P. bursaria* and the endosymbiotic algae during a light–dark change. Ratio of *P. bursaria* cells forming cleavage furrows (upper panel) and ratio of endosymbiotic algal cells forming two to four autospores (lower panel) are presented as mean $\pm$ standard deviation for three independent measurements and plotted against time of day. To evaluate trends in the data, plots were fitted with a constant function, a linear function or two separate regression lines with a joint point at the light–dark transition time (22:00). AIC (Akaike, 1974) values indicate that constant model (4.7) is better than linear (6.4) or two regression lines (7.9) models for host, and two regression lines model with positive and negative slopes (31.3) is better than constant (46.7) or linear (44.9) models for endosymbiont. The optimal models for each organism are shown as solid lines.

Partitioning of the endosymbionts at host cell division

The way in which endosymbionts are partitioned between daughter cells during host cell division may play an important role in maintaining endosymbiont population size. In dividing *P. bursaria* cells, it appeared that the algal endosymbionts were distributed nearly at random without forming pairs (Fig. 3A). Although some algal cells were still attaching to the cell cortex in the dividing cells, their attachment sites were randomly distributed throughout the cortex (Fig. S1). These results raised the possibility that active processes are not involved in the partitioning of the endosymbionts. To clarify this, the number of the endosymbionts in each daughter cell was directly counted. If n endosymbionts within a mother host cell are partitioned randomly with a probability p of entering into a daughter cell, the probability that the daughter cell receives x endosymbionts is given by a binomial distribution $\binom{n}{x} p^x (1-p)^{n-x}$, assuming that the endosymbionts occupy zero volume in the host cell. Because the number of endosymbionts differs greatly among host cells, we determined the distribution not of x but of the standardized

variable, $z = (x - np) / \sqrt{np(1-p)}$, which asymptotically follows the standard normal distribution if x follows a binomial distribution in each cell. In addition, given that the individual cells of a pair of daughter cells of *P. bursaria* were difficult to distinguish, we assumed $p = 0.5$, which meant that each endosymbiont had an equal chance of entering into either daughter cell. For ease of counting, *P. bursaria* cells were grown in darkness for 4–5 days in an attempt to reduce the number of endosymbionts. The distribution of z roughly fitted a standard normal distribution (Fig. 3B, Appendix S1), indicating that the endosymbiont number in each daughter cell followed a binomial distribution. It follows from this result that the endosymbionts are partitioned nearly at random in *P. bursaria*, similarly to what has been found in green *Hydra* (McAuley, 1990). This random partitioning would result from the random localization of the endosymbionts in the host cell at the onset of cell division, and also from the lack of ordered segregation mechanisms during division (Huh and Paulsson, 2011).

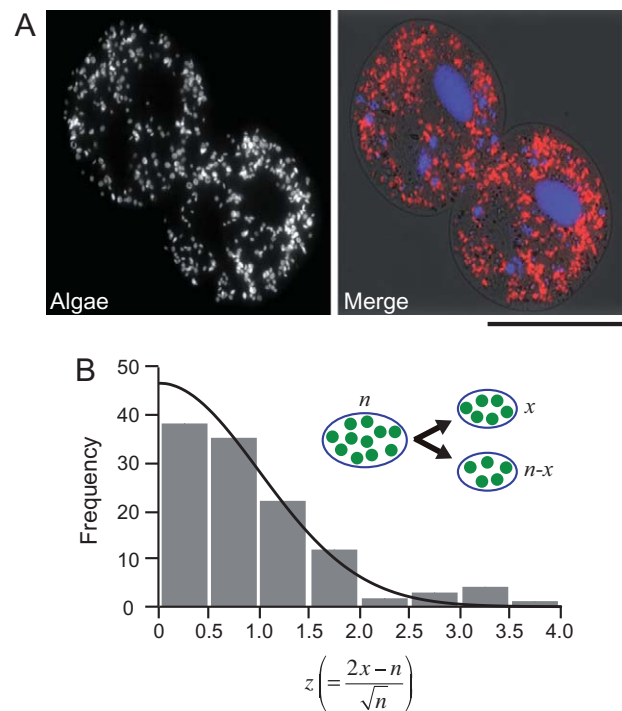


Fig. 3. Partitioning of endosymbiotic algae at *P. bursaria* cell division. (A) Fluorescence micrograph of a *P. bursaria* cell during cytokinesis. (Algae) Chlorophyll fluorescence image representing the distribution of endosymbiotic algal cells. (Merge) Merged image of the chlorophyll fluorescence image (red), DAPI-stained image representing the two large macronuclei of *P. bursaria* (blue), and light microscopic image (grey). The DAPI-stained image also shows the nuclei of yeast cells that were engulfed by *P. bursaria*. Bar, 100 μ m. (B) Distribution of the standardized variable z ($N = 117$ dividing cells). The solid line indicates a standard normal distribution with mean 0 and variance 1.

To further investigate the endosymbiont partitioning, the simple binominal distribution model was compared with other models. First, to examine the contribution of the active partitioning mechanisms to produce an even distribution, we considered mixture models of even and binominal distributions. If a fraction r of n endosymbionts in a mother host cell is partitioned evenly between daughter cells and the remainder is partitioned randomly with a probability of entering into a daughter cell p , the probability that the daughter cell receives x endosymbionts is given by

$$\binom{n-nr}{x-nr/2} \cdot p^{x-nr/2} (1-p)^{n-x-nr/2}. \quad (1)$$

Second, to test the possibility of self-volume exclusion (Birky and Skavaril, 1984; Huh and Paulsson, 2011) of the endosymbionts, we considered hypergeometric distribution models, in which a mother host cell containing n endosymbionts has a finite volume with only N spaces per cell for the endosymbionts. In this case, if a fraction p of N spaces is partitioned into a daughter cell, the probability that the daughter cell receives x endosymbionts follows a hypergeometric distribution:

$$\frac{\binom{Np}{x} \cdot \binom{N-Np}{n-x}}{\binom{N}{n}}. \quad (2)$$

Similar to the case for the binominal distribution model, we also assumed $p=0.5$ for these models. Under this assumption, Eq. 2 becomes almost identical to Eq. 1 in Birky and Skavaril (1984). The log likelihood of the data monotonically increased with decreasing r or increasing N for the mixture and the hypergeometric models respectively (Fig. S2). To select an optimal model, we used AIC, which allows for comparison of models with different numbers of parameters (Akaike, 1974). The AIC values showed that the simple binomial distribution model (AIC = 776.5) was better than the mixture models (AIC = 782.4 when $r = 0.05$), suggesting that the contribution of active partitioning mechanisms to produce an even distribution was either small or absent. This result also confirms that the difference in endosymbiont number between daughter cells did not arise only from measurement errors. The binomial model was also preferable to the hypergeometric distribution models (AIC = 780.3 when $N = 10\,000$), suggesting that self-volume exclusion would not occur in *P. bursaria*. Under conditions of $r < 0.05$ or $N > 10\,000$, the contribution of even partitioning is so small and the number of spaces for endosymbionts is so large that the effect of even partitioning or self-volume exclusion can be substantially ignored. Although the distribution of the standardized variable z appeared to roughly fit a standard normal distribution, the variance of z (1.25) was signifi-

cantly larger than 1 (χ^2 test, $P < 0.05$). This suggests that the assumption of $p = 1/2$ was not met in some *P. bursaria* cells (see Appendix S2 for detail). In those cells, the partitioning may have been disordered (Huh and Paulsson, 2011), either by different sizes of daughter cells or by biased localization of the endosymbionts in the host cells at the onset of the divisions. Indeed, such biased distributions of the endosymbionts were occasionally observed in interphase cells (Fig. S3).

Model for maintaining the algal endosymbionts in *P. bursaria*

The population dynamics of the host *P. bursaria* and the endosymbiotic algae demonstrated the significant differences in their growth properties. Although growth rates were markedly different in constant darkness, they were comparable in the mid-log phase under the normal light regime, as previously described (Weis, 1969). However, even under the normal light regime, the growth of the two organisms exhibited time lags, which were the main cause of the dynamic behaviours of the endosymbiont population size in the host cells. Moreover, the cell divisions of the hosts and the endosymbionts exhibited differences in dependence on the light-dark change, as the endosymbiont was coupled to the change, whereas the host was almost not (similarly to a sea anemone in Dimond *et al.*, 2013). These results suggest both that there is only slight, if any, coupling between the cell cycles of the two organisms and that direct regulation of endosymbiont cell division plays only a limited role in *P. bursaria*, with the endosymbionts likely to be regulated by some indirect mechanism. This notion is consistent with the finding that *P. bursaria* lacks active mechanisms for retaining a minimum number of endosymbionts in darkness and for the partitioning of endosymbionts between daughter cells at host cell division. It had previously been concluded that the cell cycle of the endosymbiont was dependent on that of the host, on the basis of the finding that the numbers of endosymbionts in dividing host cells were roughly twice those in the host cells before the divisions (Kadono *et al.*, 2004). However, we suggest that this only reflected a similar growth rate of the two organisms in a case in which the number of endosymbionts per host was nearly uniform before the host cell division. In addition, apparently similar growth rates of two organisms do not necessarily indicate a coupling of their cell cycles.

Despite the lack of direct regulatory mechanisms, the endosymbiont population size in the host cells was eventually adjusted to a nearly constant level once they were grown with light and nutrients. To account for this apparent regulation, we here propose an indirect mechanism based only on the different growth properties of the two organisms as follows:

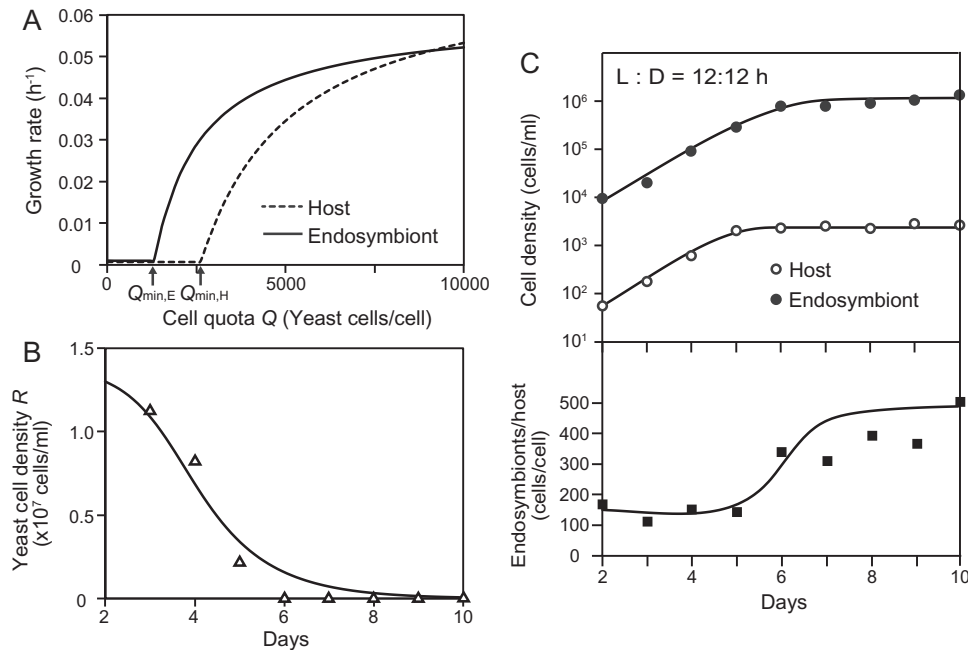


Fig. 4. Simulation of one representative population dynamics of *P. bursaria* and endosymbiotic algae based on the mathematical model consisting of Eqs 3–6. The parameters used are summarized in Table 2. (A) Assumed growth rates of *P. bursaria* (dashed line) and endosymbiotic algae (solid line) based on the model. (B) Consumption of yeast cells in the culture. The solid line is based on the model. (C) Simulations of growth of *P. bursaria* and endosymbiotic algae (upper panel) and mean number of endosymbionts per host (lower panel) from the log (day 2) to the stationary phase under a 12 h/12 h light/dark cycle. The solid lines are based on the model.

- (i) The growth of the host is mostly dependent on external food, which contains multiple elements as a package and is acquired by phagocytosis.
- (ii) In light, the growth of the algal endosymbionts is limited not by carbon but by other elements such as nitrogen and phosphorus, which are obtained by relatively free access to nutrient pools in the host cells. This assumption is consistent with our finding that the host provides nutrients to the endosymbionts regardless of obtaining photosynthetic products in return, and also the finding that cell division of the endosymbiont is limited by the host's nutritional conditions during early infection process (Kodama and Fujishima, 2012).
- (iii) In early and mid-log phase, the host and the endosymbiont grow at similar rates, roughly maintaining endosymbiont numbers in the host cell. If the hosts start their growth with a shorter lag than the endosymbionts (as in our culture system), endosymbiont population size in host cells decreases.
- (iv) In late log and early stationary phase, as the food is consumed and the nutrient pool in the host is exhausted, the growth of the hosts is arrested first because they require a larger amount of nutrients, followed by the endosymbionts. As a result of this time lag, the endosymbiont population size catches up.

We suggest that similar growth rates between the host and the endosymbiont described previously (Karakashian and Karakashian, 1965; Muscatine and Pool, 1979; Reisser, 1986) are not enough to maintain endosymbiont numbers in the host cell because the growth rates of both organisms tend to be variable depending on various growth conditions. Under such conditions, the different nutrient requirements for the growth between the two organisms are likely to play important roles in maintaining the endosymbionts.

To further analyse the proposed mechanism, we developed a mathematical model to describe the population dynamics of the host and the endosymbiont, on the basis of the proposed growth properties of these organisms. Let H and E be the cell densities of the host and the endosymbiont in culture respectively. To express their growth properties, we use the Droop equation (cell-quota model), which is suited to deal with phytoplankton growth particularly in transient situations (Droop, 1968). According to the equation, the growth rate of the host is related to the cell quota, or the internal nutrient content, of the host cell Q :

$$\frac{dH}{dt} = \mu_{\max,H} \left(1 - \frac{Q_{\min,H}}{Q} \right) H, \quad (3)$$

where $\mu_{\max,H}$ is a theoretical maximum growth rate with an infinite quota, and $Q_{\min,H}$ is the minimum quota at which

Table 2. List of variables and parameters used for the simulation of the population dynamics of *P. bursaria* and endosymbiotic algae.

Variables (units)	Symbol	
Cell density of the host in culture (<i>P. bursaria</i> cells per millilitre)	H	
Cell density of the endosymbiont in culture (Algal cells per millilitre)	E	
Cell quota (internal nutrient content) of the host cell (Yeast cells/ <i>P. bursaria</i> cell)	Q	
Cell density of the yeast (resource) in culture (Yeast cells ml ⁻¹)	R	
Parameters (units)	Symbol	Value
Theoretical maximum growth rate with infinite quota (h ⁻¹)		
For host	$\mu_{\max,H}$	0.072
For endosymbiont	$\mu_{\max,E}$	0.060
Minimum cell quota at which growth rate is zero (Yeast cells/ <i>P. bursaria</i> cell)		
For Host	$Q_{\min,H}$	2.6×10^3
For endosymbiont (= $Q_{\min,H}/2$ in this study)	$Q_{\min,E}$	1.3×10^3
Maximum uptake rate of yeast cells by <i>P. bursaria</i> (Yeast cells/h/ <i>P. bursaria</i> cell)	$v_{\max}$	1.0×10^3
Half saturation ratio of yeast cells to <i>P. bursaria</i> cell for uptake (Yeast cells/ <i>P. bursaria</i> cell)	K_m	3.0×10^4
Cell quota consumed per endosymbiont growth (Yeast cells/Algal cell)	Q_E	9.0

Our measurements of the uptake rate of yeast cells by *P. bursaria* using GFP-expressing yeast cells indicated that the above assumption for $v_{\max}$ was relevant (T. Miura and S. Iwai, unpubl. data).

the growth rate is zero (Fig. 4A). Given that the cell can proliferate only if $Q > Q_{\min,H}$, we interpret $Q_{\min,H}$ as the minimum amount of nutrients required to maintain two host cells. Under the assumption that the endosymbionts have free access to the nutrient pool in the host cell, the cell quota of the host can also be regarded as a nutrient store for the endosymbionts. Therefore, we assume that the growth rate of the endosymbiont is also related to the cell quota of the host:

$$\frac{dE}{dt} = \mu_{\max,E} \left(1 - \frac{Q_{\min,E}}{Q} \right) E, \quad (4)$$

where $\mu_{\max,E}$ and $Q_{\min,E}$ are the maximum growth rate and the minimum quota for the endosymbiont respectively. For simplicity, here we assume $Q_{\min,E} = Q_{\min,H}/2$, which corresponds to the minimum amount of nutrients required to maintain a single host cell (Fig. 4A). Even when $Q < Q_{\min,H}$, the host (and the endosymbiont) is functionally immortal due to the algal photosynthesis. The only parameter common to the growth rates of the two organisms is Q , reflecting their independent proliferations. To describe the uptake of prey yeast cells by the ciliate *P. bursaria*, we use Holling's prey-predator approach, with dependence on the ratio of the prey population size to the predator population size (Abrams and Ginzburg, 2000). This ratio-dependent uptake shows good agreement with the consumption of yeast cells (Fig. 4B). In the absence of nutrients for yeast, the yeast (resource) cell density R can only decrease by host uptake:

$$\frac{dR}{dt} = - \frac{v_{\max} \cdot R/H}{R/H + K_m} \cdot H, \quad (5)$$

where $v_{\max}$ is the maximum uptake rate per host and K_m is the half-saturation ratio of yeast cells to *P. bursaria* cells. It follows from Eqs 3–5 that the change in the host cell

quota, which is obtained via uptake, subdivided upon host proliferation, and consumed by endosymbiont growth, is given by

$$\begin{aligned} \frac{dQ}{dt} = & \frac{v_{\max} \cdot R/H}{R/H + K_m} - \mu_{\max,H} \left(1 - \frac{Q_{\min,H}}{Q} \right) Q \\ & - \mu_{\max,E} \left(1 - \frac{Q_{\min,E}}{Q} \right) Q_E \cdot \frac{E}{H}, \end{aligned} \quad (6)$$

where Q_E is the quota consumed per endosymbiont growth, and E/H corresponds to the number of endosymbionts per host. Digestion or expulsion of the endosymbionts is not included as they are assumed to have only minor effects on endosymbiont maintenance in *P. bursaria*. The deterministic model consisting of Eqs 3–6 qualitatively reproduced the population dynamics of the host and the endosymbiont from the log to the stationary phase under the normal light regime (Fig. 4C; parameters in Table 2), supporting the proposed mechanism for maintaining the algal endosymbionts. In addition, the model qualitatively reproduced the recovery of the endosymbiont population size in hosts in which the endosymbionts were at one point reduced to <50 cells per host (Fig. S4). Reproduction of the recovery was however often partial, possibly because the slow growth of the endosymbionts during their stationary phase could not be reproduced. This suggests that processes other than the proposed one are involved in the slow endosymbiont growth, which might determine an upper limit for the endosymbiont population size.

Our model of endosymbiont maintenance provides insights into the evolution of the *P. bursaria*–algae symbiosis. Our results suggest that active processes such as cell cycle coupling, partitioning of the endosymbionts at host cell division or retaining a minimum number of endosymbionts have not evolved in this symbiotic relation-

ship. It is relevant that such direct regulatory mechanisms are not essential for maintaining the endosymbionts in *P. bursaria*, which usually contains several hundreds of endosymbionts. This can be understood from a simple calculation. If a host cell containing >100 endosymbionts divides and the endosymbionts are randomly partitioned between daughter cells with an equal chance of entering into each cell as shown for *P. bursaria*, the probability of either cell receiving less than 10 endosymbionts will be quite rare ($<10^{-17}$). In contrast to such direct mechanisms, the different nutrient requirements for the growth between the two organisms are suggested to play an important role in the maintenance. Given the cellular sizes of the two organisms and the autotrophic growth of algae, these different growth properties would be inherent to their respective free-living relatives. These considerations suggest that the requirements for maintaining the *P. bursaria* symbiosis are relatively simple, and the symbiosis can be readily achieved and may have evolved repeatedly. This view is consistent with the finding that the endosymbiotic algae in *P. bursaria* derive from multiple origins (Hoshina and Imamura, 2008; Pröschold *et al.*, 2011).

Conclusions

The population dynamics of the host *P. bursaria* and its algal endosymbionts suggest that endosymbiont population size in *P. bursaria* is regulated and maintained not by active processes such as cell division coupling, partitioning of the endosymbionts at host cell division or retaining a minimum number of endosymbionts, but rather by some indirect means. We proposed one such simple mechanism based only on the different growth properties of the two organisms, in which the endosymbiont requires a smaller amount of nutrients than the host for its respective growth and thereby grows even after the arrest of the host growth. This mechanism is likely to be important in maintaining the endosymbionts under various growth conditions, in which growth rates of the two organisms tend to be variable. The mathematical model based on these different growth properties quantitatively reproduced the population dynamics, supporting the proposed mechanism. Our model provides insights into the evolution of the *P. bursaria* symbiosis and suggests that the symbiotic relationship may have evolved readily and repeatedly. Although it remains to be determined whether the proposed model can fully explain the maintenance of endosymbionts in *P. bursaria*, and also whether it can be applied to other endosymbiosis such as in protists, the model is simple enough that it may provide a useful basis for understanding the mechanisms underlying the maintenance of algal endosymbionts.

Experimental procedures

Strains and culture of *P. bursaria*

Paramecium bursaria strains HA1, HA3 and HA4 were collected from ponds in the cities of Hirosaki, Tsukuba and Kyoto in Japan respectively. The *Chlorella*-like endosymbiotic algae in the three strains were morphologically similar, i.e., they were spherical to ellipsoidal in shape and 4–7 μm in size, containing a parietal chloroplast with a pyrenoid and producing two or four autospores. HA1 (syngen 3, mating type IV) was mainly used in this study, whereas the reproducibility of the results was qualitatively confirmed using the other strains. All these strains are available from the National Bio-Resource Project, Japan (<http://nbrpcms.nig.ac.jp/paramecium/>). To obtain bacteria-free cultures of *P. bursaria*, single cells were isolated, individually washed at least nine times with 1 ml of artificial pond water (AW; 0.3 mM NaCl, 0.1 mM KCl, 0.1 mM MgSO_4 , 0.5 mM CaCl_2) containing 500 $\mu\text{g ml}^{-1}$ ampicillin, and cultured in mini-Petri dishes containing yeast cells as described below. To culture *P. bursaria*, *S. cerevisiae* BY4741 cells grown in yeast peptone dextrose medium were harvested, washed with AW and finally suspended at a concentration of $\sim 1.4 \times 10^7$ cells ml^{-1} in sterilized AW supplemented with 500 $\mu\text{g ml}^{-1}$ ampicillin. *Paramecium bursaria* cells were grown in this culture at 24°C under a 12 h/12 h light/dark cycle, and irradiated at an intensity of $\sim 40 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a fluorescent lamp, unless otherwise noted. For growth measurements, cells were grown in 20 ml of cultures in 50 ml Erlenmeyer flasks capped with a sponge plug (Silicosen; Shin-Etsu Polymer). These culture flasks were gently agitated once daily. Cultures were routinely checked for bacterial contamination by plating aliquots onto standard agar plates.

Measurements of cell densities

To measure *P. bursaria* cell density, 50–500 μl of aliquots were collected from cultures and transferred to an acrylic chamber with the addition of 1 mM NiCl_2 to stop cell movement. The sedimented cells were counted using an inverted microscope. Measurements were repeated three times to obtain mean values at each time point for each culture. To measure the total density of algal cells, 0.5 or 1 ml of aliquots were collected from the cultures, and sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% to lyse *P. bursaria* cells. This concentration of SDS did not affect algal cell appearance at least during the measurements. The lysates were centrifuged to sediment the algal cells, and defined volumes of the supernatants were removed to concentrate cells, which were counted using a haemocytometer. For counting the algal cells lying outside *P. bursaria* cells, a 1 ml of aliquot of culture was passed through an 11 μm nylon net filter (Millipore) to eliminate *P. bursaria* cells, then centrifuged to concentrate and count the algal cells. For the detection of cell divisions during a light–dark change, *P. bursaria* cells were first grown to the log phase for 5 days under a 12 h/12 h light/dark cycle. The *P. bursaria* and algal cells samples were prepared as described above, and ratios of *P. bursaria* cells forming cleavage furrows and algal cells forming two to four autospores in their cell walls were determined by light microscopy.

Direct counting of endosymbiotic algae per paramecium

The number of endosymbiotic algae per paramecium was directly counted by rupturing *P. bursaria* cells according to Karakashian (1963), with the modification that fluorescence microscopy was used for cells containing low numbers of algae. For counting the algae in each daughter cell after host cell division, *P. bursaria* cells were first grown in constant darkness for 4–5 days to reduce the number of endosymbionts. The culture was transferred to Petri dishes to identify dividing cells under a dissecting microscope. Cells forming cleavage furrows were individually collected and incubated in 1 ml of AW at room temperature until each cell completed the division, typically for <10 min. Immediately after division, each daughter cell was separately placed onto a glass slide and ruptured to count the algae in each daughter cell using fluorescence microscopy.

Fluorescence microscopy

For observation of dividing cells, *P. bursaria* cells grown to mid-log phase were stained with 1 µg ml⁻¹ 4',6-Diaminidino-2-phenylindole (DAPI) in Petri dishes. Cells forming cleavage furrows were collected, placed onto glass slides and compressed with coverslips for optimal observation. Localizations of endosymbiotic algae were not significantly perturbed by the compression. The ruptured or dividing cells were observed with an Olympus BX51 microscope equipped with a 40× UPlanSApo objective and a mercury lamp as a light source. U-MWIG3 (Olympus) and XF06 (Omega Optical) filter sets were used to observe chlorophyll autofluorescence and DAPI respectively. Fluorescence images were collected on a charge-coupled device camera (WAT-120N+; Watec) and processed using IMAGEJ (<http://imagej.nih.gov/ij/>).

Acknowledgements

We thank Takashi Miura, Fumiaki Sakurada and Yoshiki Fujimori for technical assistance. We also thank National Bio-Resource Project-Paramecium of MEXT, Japan, for providing several *P. bursaria* strains. This work was supported by a grant from the Faculty of Education, Hirosaki University.

References

- Abrams, P.A., and Ginzburg, L.R. (2000) The nature of predation: prey dependent, ratio dependent or neither? *Trends Ecol Evol* **15**: 337–341.
- Akaike, H. (1974) A new look at the statistical model identification. *IEEE Trans Autom Contr* **19**: 716–723.
- Birky, C.W., and Skavaril, R.V. (1984) Random partitioning of cytoplasmic organelles at cell division: the effect of organelle and cell volume. *J Theor Biol* **106**: 441–447.
- Davy, S.K., Allemand, D., and Weis, V.M. (2012) Cell biology of cnidarian–dinoflagellate symbiosis. *Microbiol Mol Biol Rev* **76**: 229–261.
- Dimond, J.L., Pineda, R.R., Ramos-Ascherl, Z., and Bingham, B.L. (2013) Relationships between host and symbiont cell cycles in sea anemones and their symbiotic dinoflagellates. *Bio Bull* **225**: 102–112.
- Douglas, A., and Smith, D.C. (1984) The green hydra symbiosis. VIII. Mechanisms in symbiont regulation. *Proc R Soc Lond B Biol Sci* **221**: 291–319.
- Droop, M.R. (1968) Vitamin B12 and marine ecology. IV. The kinetics of uptake, growth and inhibition in *Monochrysis lutheri*. *J Mar Biol Assoc UK* **48**: 689–733.
- Esteban, G.F., Fenchel, T., and Finlay, B.J. (2010) Mixotrophy in ciliates. *Protist* **161**: 621–641.
- Fitt, W.K. (2000) Cellular growth of host and symbiont in a cnidarian–zooxanthellar symbiosis. *Bio Bull* **198**: 110–120.
- Görtz, H.D. (1982) Infections of *Paramecium bursaria* with bacteria and yeasts. *J Cell Sci* **58**: 445–453.
- Gu, F., Chen, L., Ni, B., and Zhang, X. (2002) A comparative study on the electron microscopic enzyme-cytochemistry of *Paramecium bursaria* from light and dark cultures. *Eur J Protistol* **38**: 267–278.
- Hoshina, R., and Imamura, N. (2008) Multiple origins of the symbioses in *Paramecium bursaria*. *Protist* **159**: 53–63.
- Hosoya, H., Kimura, K., Matsuda, S., Kitaura, M., Takahashi, T., and Kosaka, T. (1995) Symbiotic algae-free strains of the green paramecium *Paramecium bursaria* produced by herbicide paraquat. *Zoolog Sci* **12**: 807–810.
- Huh, D., and Paulsson, J. (2011) Random partitioning of molecules at cell division. *Proc Natl Acad Sci USA* **108**: 15004–15009.
- Johnson, M.D. (2011) The acquisition of phototrophy: adaptive strategies of hosting endosymbionts and organelles. *Photosynth Res* **107**: 117–132.
- Kadono, T., Kawano, T., Hosoya, H., and Kosaka, T. (2004) Flow cytometric studies of the host-regulated cell cycle in algae symbiotic with green paramecium. *Protoplasma* **223**: 133–141.
- Karakashian, M.W., and Karakashian, S.J. (1973) Intracellular digestion and symbiosis in *Paramecium bursaria*. *Exp Cell Res* **81**: 111–119.
- Karakashian, S.J. (1963) Growth of *Paramecium bursaria* as influenced by the presence of algal symbionts. *Physiol Zool* **36**: 52–68.
- Karakashian, S.J., and Karakashian, M.W. (1965) Evolution and symbiosis in the genus *Chlorella* and related algae. *Evolution* **19**: 368–377.
- Karakashian, S.J., and Rudzinska, M.A. (1981) Inhibition of lysosomal fusion with symbiont-containing vacuoles in *Paramecium bursaria*. *Exp Cell Res* **131**: 387–393.
- Kodama, Y., and Fujishima, M. (2009) Localization of perialgal vacuoles beneath the host cell surface is not a prerequisite phenomenon for protection from the host's lysosomal fusion in the ciliate *Paramecium bursaria*. *Protist* **160**: 319–329.
- Kodama, Y., and Fujishima, M. (2012) Cell division and density of symbiotic *Chlorella variabilis* of the ciliate *Paramecium bursaria* is controlled by the host's nutritional conditions during early infection process. *Environ Microbiol* **14**: 2800–2811.
- Kuhl, A., and Lorenzen, H. (1964) Handling and culturing of *Chlorella*. *Methods Cell Biol* **1**: 159–187.
- McAuley, P.J. (1982) Temporal relationships of host cell and algal mitosis in the green hydra symbiosis. *J Cell Sci* **58**: 423–431.

- McAuley, P.J. (1985) The cell cycle of symbiotic *Chlorella* I. The relationship between host feeding and algal cell growth and division. *J Cell Sci* **77**: 225–239.
- McAuley, P.J. (1990) Partitioning of symbiotic *Chlorella* at host cell telophase in the green hydra symbiosis. *Philos Trans R Soc Lond B Biol Sci* **329**: 47–53.
- McAuley, P.J., and Darrah, P.R. (1990) Regulation of numbers of symbiotic *Chlorella* by density-dependent division. *Philos Trans R Soc Lond B Biol Sci* **329**: 55–63.
- Muscatine, L., and Neckelmann, N. (1981) Regulation of numbers of algae in the *Hydra–Chlorella* symbiosis. *Ber Dtsch Bot Ges* **94**: 571–582.
- Muscatine, L., and Pool, R.R. (1979) Regulation of numbers of intracellular algae. *Proc R Soc Lond B Biol Sci* **204**: 131–139.
- Omura, G., Ishida, M., Arikawa, M., Khan, S.M.M.K., Suetomo, Y., Kakuta, S., *et al.* (2004) A bacteria-free monoxenic culture of *Paramecium bursaria*: its growth characteristics and the re-establishment of symbiosis with *Chlorella* in bacteria-free conditions. *Jpn J Protozool* **37**: 119–130.
- Pröschold, T., Darienko, T., Silva, P.C., Reisser, W., and Krienitz, L. (2011) The systematics of *Zoochlorella* revisited employing an integrative approach. *Environ Microbiol* **13**: 350–364.
- Reisser, W. (1986) Endosymbiotic associations of freshwater protozoa and algae. *Progr Protistol* **1**: 195–214.
- Reisser, W. (1992) Endosymbiotic associations of algae with freshwater protozoa and invertebrates. In *Algae and Symbioses*. Reisser, W. (ed.). Bristol, UK: Biopress, pp. 1–19.
- Siegel, R.W. (1960) Hereditary endosymbiosis in *Paramecium bursaria*. *Exp Cell Res* **19**: 239–252.
- Stoecker, D.K., Johnson, M.D., de Vargas, C., and Not, F. (2009) Acquired phototrophy in aquatic protists. *Aquat Microb Ecol* **57**: 279–310.
- Taylor, C.E., Muscatine, L., and Jefferson, D.R. (1989) Maintenance and breakdown of the *Hydra–Chlorella* symbiosis: a computer model. *Proc R Soc Lond B Biol Sci* **238**: 277–289.
- Tucker, J.B., Beisson, J., Roche, D.L.J., and Cohen, J. (1980) Microtubules and control of macronuclear 'amitosis' in *Paramecium*. *J Cell Sci* **44**: 135–151.
- Weis, D. (1969) Regulation of host and symbiont population size in *Paramecium bursaria*. *Experientia* **25**: 664–666.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Distributions of the endosymbiotic algal cells beneath the cell cortex in a dividing *P. bursaria* cell. Dividing cells were fixed in 3% formaldehyde in 50 mM K-phosphate buffer (pH 7.2) for 10 min, washed with AW and observed without compressing the cells.

Fig. S2. Log likelihoods for the different endosymbiont partitioning models.

Fig. S3. Biased distributions of the endosymbiotic algal cells toward the posterior in a *P. bursaria* cell in interphase. (Algae) Chlorophyll fluorescence image representing the distribution of endosymbiotic algal cells. (Merge) Merged image of the chlorophyll fluorescence image (red) and DAPI-stained image representing a macronucleus of *P. bursaria* (blue).

Fig. S4. Simulation of recovery of the endosymbiotic algae number in *P. bursaria* in which endosymbionts were at one time reduced to <50 cells per host. Those cells were assumed to be cultured under a 12 h/12 h light/dark cycle with an initial cell density of 100 or 10 cells ml⁻¹. The simulation was based on the mathematical model consisting of Eqs 3–6 with the parameters summarized in Table 2.

Appendix S1. χ^2 Goodness-of-fit test for standard normal distribution of the standardized variable *z*.

Appendix S2. Analysis of the variance of the standardized variable *z*.