アブラムシ類における共生体置換現象に関する進化生物学的研究

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東京大学大学院
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EVOLUTIONARY BIOLOGICAL STUDIES ON REPLACEMENT OF SYMBIOTIC MICROORGANISMS IN APHIDS

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MICROORGANISMS AND BASIC STRUCTURE OF THEIR GALLS: IMPLICATIONS FOR HOST–SYMBIONT COEVOLUTION AND EVOLUTION OF STERILE SOLDIER CASTES

AIMS AND SCOPE

So far, some 4000 Aphididae species have been described, almost all of which harbor prokaryotic intracellular symbionts in the cytoplasm of mycetocytes, specialized cells for this purpose. The symbionts are so specialized to intracellular conditions that they cannot propagate themselves outside the host cells. The aphids are provided by the symbionts with essential nutrients, and therefore cannot reproduce without them. In short, the aphids and intracellular symbionts are intimately mutualistic with each other and are no longer separable. The symbionts are passed to the next generation of the host aphids by ovarial transmission in the manner of maternal inheritance like mitochondria and chloroplasts.

The endosymbiotic associations with microorganisms are quite ubiquitous phenomena amongst invertebrates, plants, algae, fungi, protozoa, and so on, though the extent of interdependence is varied from case to case. Microorganisms often possess special and effective metabolic abilities. The establishment of endosymbiosis, housing a useful microorganism in the body, utilizing its ability under an optimal condition, exploring new ecological niche with its help, and escalating their interdependence, is likely to be one of the most important sources of adaptive evolutionary novelties. In this context, I have been much interested in the possible and potential roles of endosymbiosis in adaptive evolutionary processes, particularly in aphids.

I suppose that mutation, sex, and endosymbiosis are the principal sources of evolutionary novelties. In the scheme of neo-Darwinism, it is no doubt that mutations on DNA are the raw materials of genetic variations on which natural selection acts to pick up adaptive ones. Accumulated genetic variations in conspecific different individuals, some are adaptive by themselves and others are potentially adaptive in combination with other genes, are randomly sampled and combined through sexual reproduction, in which new adaptive gene sets can be created. In addition, by interspecific combination of endosymbiosis, one partner will be able to acquire a whole system that has already established adaptive functions to the other partner, and *vice versa*, which will potentially lead to remarkable evolutionary innovations by leaps.

Mutation and sexual reproduction are daily events in most organisms with relatively

slight evolutionary effects, and their mechanisms have been understood to a considerable extent. All organisms are inevitably vulnerable to mutations as long as they live. Damages on DNA and errors in repairing them are the major cause of mutations, which are facilitated by exposure to radiation and certain chemicals. Most organisms experience genetic rearrangement due to sexual reproduction every generation. Chromosomal shuffling and recombination through the process result in increased genetic variations. In contrast, endosymbiosis, though potentially with great evolutionary effects, is far less frequent event. In a geological time scale it is evident that numerous endosymbiotic associations have been successfully established since they are now found from a wide spectrum of organisms. However, it is still not well understood how often symbiotic associations occur in nature, how often they are successfully established, and what kind of factors are involved in the process.

Then, what can we do first to approach practically to the endosymbiotic evolution? Conceptually, there are several possible ways:

1) To find and observe the process of establishment of endosymbiosis occurring in nature.

2) To construct endosymbiotic system artificially and analyze the process.

3) To reconstruct the process of past endosymbiotic events by comparing the present organisms.

1) Although this is a direct and convincing way to investigate the endosymbiotic evolution, it is apparently very difficult to find the process of endosymbiosis occurring in nature. It would be much more probable to expect to hit jackpots three times a day in Las Vegas than to come across the scene. However, it should be noted that at least one case has been reported. Jeon (1987) witnessed the process that an intracellular pathogenic bacteria of *Amoeba proteus* turned into an essential cell component for it.

2) If *Escherichia coli* micro-injected into cultured cells could be housed in the cells harmoniously, all the evolutionary process from free-living bacteria to endosymbionts could be followed through in laboratory. Though no one can assert this or similar experiment impossible, I doubt whether it is ever possible. There has been no report that artificial endosymbiosis was successfully established.

3) At present stage, this approach, to reconstruct the process of the past endosymbiotic events by comparing the present organisms, seems to be the only generally applicable way to investigate this subject.

Now I decided to take the approach of above 3). In analyzing the endosymbiotic process which occurred in the past in a group of organisms, I have to work on the following aspects:

a) When was the symbiont acquired?

b) What kind of microorganism was the ancestral symbiont?

c) What kind of organism was the ancestral host?

d) What does the symbiont do for the host?

e) What does the host do for the symbiont?

f) What evolutionary effects on the symbiont due to the symbiosis are observed?

g) What evolutionary effects on the host due to the symbiosis are observed?

h) Have any remarkable evolutionary novelties been established in the endosymbiosis?

My ultimate aim is to answer all the questions above. To answer a)–c), phylogenetic analyses are necessary. To answer d) and e), physiological approaches are needed. To answer f)–h), comparative approaches will be effective. Of course, it is not easy to attain the aim, but I will do my best to understand these problems better.

My doctoral thesis is evolutionary biological studies on symbiont–replacement in aphids. In studying symbiotic system of various aphids, I discovered that in a group of aphids, tribe Cerataphidini, there are species with ancestral symbiont and those with newly–acquired symbiont. It was suggested that in an ancestor of this group, replacement of the symbiont from old type to new one must have occurred. Thus, I investigated the process of endosymbiotic evolution (in this case, symbiont–replacement) in this group extensively.

This thesis is composed of three parts. In part I, more than 60 species of aphids were examined for their symbiotic system histologically and immunologically. It was shown that irrespective of aphid species, the primary intracellular symbionts are highly conserved in Aphididae and carry common characters, suggesting that the symbionts have been derived from an symbiotic bacteria that was acquired by the common ancestor of the present aphids. In part II, contrary to the ubiquitous occurrence of the intracellular symbiont, I reported the discovery that a Cerataphidini aphid *Hamiltonaphis* (= *Astegopteryx*) *styraci* possesses neither intracellular

symbionts nor mycetocytes but harbors yeast-like extracellular symbionts in the hemocoel and fat body. The novel symbiont was studied by immunoblotting, light microscopy, antibiotic sensitivity analysis and electron microscopy. This discovery suggested that in an ancestor of Cerataphidini, replacement of symbiont from intracellular prokaryote to extracellular yeast must have occurred. In part III, therefore, symbiotic systems of Cerataphidini aphids were studied extensively. More than 40 species of Cerataphidini and related groups were collected from Japan, Taiwan, Sumatra and Malaysia, and their symbiotic systems are examined histologically and immunologically. Based on the results, I analyzed phylogeny of Cerataphidini aphids, proposed a hypothesis on their evolutionary history of various characters, and discussed possible host-symbiont coevolutionary relationship.

The results of this thesis have been, or will be, published as follows:

PART I

Fukatsu T. and Ishikawa H. (1993) Occurrence of chaperonin 60 and chaperonin 10 in primary and secondary bacterial symbionts of aphids: Implications for the evolution of an endosymbiotic system in aphids. *Journal of Molecular Evolution* 36, 568–577.

PART II

Fukatsu T. and Ishikawa H. (1992) A novel eukaryotic extracellular symbiont in an aphid, *Astegopteryx styraci* (Homoptera, Aphididae, Hormaphidinae). *Journal of Insect Physiology* 38, 765–773.

PART III

Fukatsu T., Aoki S., Kurosu U. and Ishikawa H. (1994) Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: Implications for host-symbiont coevolution and evolution of sterile soldier castes. Submitted.

PART I

OCCURRENCE OF CHAPERONIN 60 AND CHAPERONIN 10 IN PRIMARY AND SECONDARY BACTERIAL SYMBIONTS OF APHIDS: IMPLICATIONS FOR THE EVOLUTION OF AN ENDOSYMBIOTIC SYSTEM IN APHIDS

ABSTRACT

All aphids harbor symbiotrophic prokaryotes ("primary symbionts") in a specialized abdominal cell, the mycetocyte. Chaperonin 60 (Cpn60, symbionin) and chaperonin 10 (Cpn10), which are high and low molecular weight heat shock proteins, were sought in tissues of more than 60 aphid species. The endosymbionts were compared immunologically and histologically. It was demonstrated that (1) there are two types of aphids in terms of the symbiotic system: some with only primary symbionts and others with, in addition, secondary symbionts; (2) the primary symbionts of various aphids are quite similar in morphology whereas secondary symbionts vary; and (3) irrespective of the aphid species, Cpn60 is abundant in both the primary and secondary symbionts, while Cpn10 is abundant in the secondary symbionts but present in small amounts in the primary ones. Based on these results, I suggest that the primary symbionts have been derived from a prokaryote that was acquired by the common ancestor of aphids whereas the secondary symbionts have been acquired by various aphids independently after divergence of the aphid species. In addition I point out the possibility that the prokaryotes under intracellular conditions have been subject to some common evolutionary pressures , and as a result, have come to resemble cell organelles.

INTRODUCTION

So far, some 4000 species of aphids (Aphidoidea) are known, which are divided into three families; Aphididae, Adelgidae and Phylloxeridae. Aphididae (in a narrow sense, only this family is regarded as aphid) is a divergent and predominant group whose life cycle is composed of both viviparous and oviparous phases. Adelgidae and Phylloxeridae are small groups which are oviparous throughout their life cycle [29; see Fig. 1].

Almost all the Aphidoidea species ever examined harbor prokaryotic intracellular symbionts in the mycetocytes or bacteriocytes, huge cells specialized for this purpose. In these aphids, there are two types of intracellular symbionts, which are called primary and secondary symbionts, respectively. The primary symbiont is ubiquitous and predominant in amount among almost all the aphid species, while secondary symbionts are found only in some species and are minor in amount [2]. These intracellular symbionts live only in the host-cell cytoplasm, and have no free-living state. As a consequence, they cannot propagate themselves when taken out of the host cell. Aposymbiotic aphids that have lost their symbionts show retarded growth and become sterile [19, 33]. It has been discovered that the primary symbionts of various aphids provide their hosts with essential amino acid [17, 36]. Thus, the aphid and its symbionts are intimately mutualistic.

One important aspect of the aphid endosymbiosis has been noted through the studies of a protein produced by a primary symbiont, which was named symbionin.Symbionin is the major protein synthesized by the primary symbiont of the pea aphid *Acyrthosiphon pisum* [7, 18], which suggests that it should play some important roles in this intracellular symbiotic system. Recently, symbionin was purified from the pea aphid [11] and shown to be homologous to the *Escherichia coli* GroEL protein [12].

In *E. coli*, GroEL, together with GroES, is encoded by the *groE* operon [37], and the two proteins synergistically chaperone correct folding and assembly of other polypeptides [8, 9]. GroEL and GroES homologs are ubiquitous and highly conserved among prokaryotes and collectively called chaperonin 60 (Cpn60) and chaperonin 10 (Cpn10), respectively [8]. These are all heat shock proteins. Cpn60 is also found in mitochondria and chloroplasts and is required for the folding and assembly process of other polypeptides [4, 15]. Cpn60 of the cell organelles is not encoded by their own genome but by the nuclear one [14, 28]. The organellar systems are also

different from those of bacteria in that the gene encoding Cpn10 has not been found adjacent to the Cpn60 gene and in that no protein immunoreactive to anti-Cpn10 antibody has been detected.

Since, according to the Endosymbiosis Theory [25, 26], an endosymbiont is taken to be an intermediate between a free-living bacterium and a cell organelle, it is interesting to know about the occurrence of Cpn60 and Cpn10 in the aphid intracellular symbionts. In the present studies, with this in mind, the amount and localization of these proteins in both primary and secondary symbionts of more than 60 aphid species were examined immunologically and histologically.

MATERIALS AND METHODS

Materials

Aphid samples were collected in the field in Japan (Table 1). Collected samples were stored at −80°C in plastic tubes for protein analyses. Some fresh specimens were dissected in Bouin's solution and their embryos were fixed in it for immunohistochemical studies.

Antisera

Symbionin was purified from the pea aphid *Acyrthosiphon pisum* [11]. GroEL and GroES were purified from *E. coli* as described by Lecker *et al.* [23] and Chandrasekhar *et al.* [3], respectively. Antisera against these proteins were prepared in male Japan White Rabbits as described by Hara *et al.* [13].

SDS-PAGE immunoblotting

The insect tissues stored at −80°C were homogenized in solubilizing buffer [57.7mM Tris-HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol] on ice. The homogenates (50mg of insects/ml) were boiled at 100°C for 5min and then centrifuged. The supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [22] using 10% (for Cpn60) or 15% (for Cpn10) slab gels. The proteins separated in the gels were transferred to PVDF membranes (Immobilon, Millipore) in transfer buffer [0.125M Tris, 0.96M glycine, 20% (v/v) methanol] with a semidry-type electroblotting apparatus (NA-1512, Nippon Eido Co., Ltd.) at a current of 2mA/cm^2 for 90 min. Immunoblotting was performed using a Vectastain Elite ABC kit (Vector). The membranes were washed with Tween 20-containing Tris-buffered saline [T-TBS: 20mM Tris-HCl (pH 7.4), 150mM NaCl, 0.1% (v/v) Tween 20] and then incubated with 2% normal goat serum in T-TBS (NGS-T-TBS) for 30min to block nonspecific binding of the antibodies. The membranes were then placed into antiserum diluted (anti-symbionin, 1:10000; anti-GroEL, 1:5000; anti-GroES, 1:2500) in NGS-T-TBS for 2h and subsequently washed well in T-TBS. They were then incubated with

secondary antibody (affinity-purified, biotinylated goat anti-rabbit IgG) in NGS-T-TBS for 2h and subsequently washed thoroughly in T-TBS. Finally, the membranes were incubated with avidin-biotin horseradish peroxidase complex (ABC reagent) in T-TBS for 1h. After the extensive washing in T-TBS, bound peroxidase on the membranes was visualized with 4C1N substrate (0.05% 4-chloro-1-naphthol, 0.1% H_2O_2 in TBS). For the control, preimmune sera were used instead of antisera.

Native-PAGE immunoblotting

The insect tissues were homogenized in solubilizing buffer [63mM Tris-HCl (pH 6.8), 15% (v/v) glycerol] (50mg of insects/ml) and centrifuged at 0°C. The supernatants were subjected to non-denatured polyacrylamide gel electrophoresis (Native-PAGE) [5] using 5% slab gels. The proteins separated in the gels were transferred to the membranes and immunoblotted essentially as described, but the transfer buffer contained no methanol in this case.

Immunohistochemistry

The aphid embryos fixed in Bouin's solution were embedded in Paraplast (Monoject), sectioned at 5μm thickness, and mounted on gelatin-coated microscope slides. Immunohistochemistry was performed using a Vectastain Elite ABC kit. The tissue sections were deparaffinized and hydrated through a xylene-ethanol-water series and washed with Tris-buffered saline [TBS: 20mM Tris-HCl (pH 7.4), 150mM NaCl]. They were then incubated with 2% normal goat serum in TBS (NGS-TBS) for 30min to block nonspecific binding of the antibodies. After this incubation, they were placed in antiserum diluted (anti-GroEL, 1:10000; anti-GroES, 1:5000) in NGS-TBS for 2h and subsequently washed well in TBS. The sections were then incubated with secondary antibody (affinity-purified, biotinylated goat anti-rabbit IgG) in NGS-TBS for 2h and washed thoroughly in TBS. Finally they were incubated with avidin-biotin horseradish peroxidase complex (ABC reagent) in TBS for 1h. After extensive washing in TBS, the location of antigens was visualized with Ni-DAB substrate (0.04% 3,3'-diaminobenzidine, 0.1% $NiCl_2$, 0.1% H_2O_2 in TBS). For the control, preimmune sera and antigen-absorbed antisera were used instead of the antisera.

RESULTS

Immunoblot analysis with anti-Cpn60 antisera

The protein samples prepared from 61 aphid species were separated by SDS-PAGE using 10% gels, transferred to PVDF membranes and probe with anti-symbionin antiserum. In all the aphid species examined but *Phylloxerina capreae* (lane 59), the main bands that cross-reacted strongly to anti-symbionin antiserum corresponded to 63kDa proteins (Fig. 2). When immunoblotted with anti-GroEL antiserum, the same profiles were obtained (data not shown). Judging from their cross-reactivity to the antisera and molecular weight, these 63kDa proteins are likely to be Cpn60s, which are the same as symbionin and homologous to GroEL.

To further identify these 63kDa proteins as Cpn60s, I examined whether they are in an oligomeric structure in the native state as are GroEL [16] and symbionin [11], employing Native-PAGE immunoblotting (Fig. 3). In all the aphids examined, the protein bands were strongly cross-reactive to anti-symbionin antiserum, like those of GroEL (lane E) and symbionin (lane A), and exhibited slower mobility on Native-PAGE than thyroglobulin (667kDa).

Immunoblot analysis with anti-Cpn10 antiserum

The protein samples from 61 aphids were separated by SDS-PAGE using 15% gels and probed with anti-GroES antiserum (Fig. 4). In most aphids, no immunoreactive band was found at all, but in several aphids indicated by asterisks, immunoreactive proteins with a molecular mass similar to that of GroES (lane E) were detected.

Immunohistochemistry with anti-Cpn60 antiserum

To examine whether these proteins detected by immunoblotting were really derived from intracellular symbionts, I prepared the tissue sections of 12 aphid species that represent five major subfamilies of Aphididae, and studied these sections immunohistochemically with anti-GroEL antiserum as a probe. The immunoreactions were performed under constant conditions to facilitate direct comparison of the intensity of immunostaining.

As a result, it was revealed that several species contain both the primary and secondary symbionts (Fig. 5A–C,F) whereas the others contain only the primary ones (Fig. 5D,E). In all the aphids examined, both the primary and, if present, secondary symbionts but no other tissues were immunostained (Fig. 5). When preimmune serum or antigen–absorbed antiserum was used, these symbionts were not stained at all (data not shown). The primary symbionts were abundant and morphologically similar to each other, being spherical with a diameter of 2–4μm. In contrast, the secondary symbionts were usually small in number and quite varied in structure; some were spherical or oval (Fig. 5A–C) and others were tubular or thread–like (Fig. 5F). The primary symbionts were also distinguishable from the secondary ones in terms of image of immunostaining. The primary symbionts commonly exhibited granular structures that were densely immunostained, while the secondary symbionts were stained rather uniformly (Fig. 5).

Immunohistochemistry with anti–Cpn10 antiserum

Immunohistochemistry with anti–Cpn10 antiserum as a probe led to results quite different from those obtained with anti–GroEL antiserum. With a short peroxidase–reaction time, the secondary symbionts were immunostained densely, while the primary ones were not stained in any aphid species (Fig. 6A–E). When the reaction time was prolonged, the primary symbionts also stained to an extent (Fig. 6F). Control experiments with preimmune serum and antigen–absorbed antiserum confirmed the specificity of this immunoreaction (data not shown).

Table 2 summarizes the results of the present studies on the 12 aphids. It was revealed that the primary symbionts are immunostained densely with anti–GroEL antiserum but only weakly with anti–GroES antiserum and that the secondary symbionts are stained densely with both anti–GroEL and anti–GroES antisera, irrespective of aphid species.

DISCUSSION

Cpn60 is a major protein common to the aphid intracellular symbionts

In the present studies, immunoblot analyses (Figs. 2 and 3) showed that almost all the aphids examined contain proteins strongly cross-reactive to anti-symbionin and anti-GroEL antisera. It was also demonstrated that these proteins share common properties with Cpn60 in that the molecular mass of the subunit is approximately 63kDa and that the molecular mass in the non-denatured state is larger than that of thyroglobulin. Immunohistochemical studies (Fig. 5) showed that these cross-reactive proteins are localized in the primary and secondary symbiont. From these results, it was concluded that these proteins are Cpn60s and that they are synthesized and localized in the intracellular symbionts of various aphids, as has been shown in the pea aphid *A. pisum* [7, 18].

In SDS-PAGE immunoblotting profiles of some aphids, several immunoreactive bands were detected in addition to the main bands of 63kDa (Fig. 2). Most of these additional bands are likely due to breakdown products of Cpn60 because Coomassie blue staining profiles indicated considerable degradation of the proteins from these species (data not shown).

Among all the aphids examined, only *Phylloxerina capreae* (Fig. 2, lane 59) exhibited no sign of the presence of Cpn60. This is compatible with the histological finding to the effect that the family Phylloxeridae is an exceptional group that lacks the intracellular symbionts [2].

Cpn10 is much smaller in amount in primary symbionts than in secondary symbionts

When anti-GroES antiserum was used as a probe, no immunoreactive proteins were detected in most aphid species examined (Fig. 4). On the other hand, immunohistochemical studies (Fig. 5; Table 2) demonstrated that in all the aphids examined both primary and secondary symbionts were immunostained with anti-GroES antiserum. However, it should be emphasized that the secondary symbionts were immunostained far more densely than the primary ones. These results suggest that Cpn10 does exist in all the aphid species but in most of them in too small an amount to be detected by immunoblotting.

One possible explanation for the difference in immunostainability observed between the

primary and secondary symbionts is that the Cpn10 of the secondary symbionts is more similar to GroES of *E. coli* than Cpn10 of the primary ones is. Recently, however, the gene of Cpn10 in the primary symbiont of the pea aphid was cloned, and its deduced amino acid sequence showed a very high identity, approximately 80%, with that of GroES [35], which does not seem to support this explanation. Therefore, it is more likely that Cpn10 is more abundant in the secondary symbionts than in the primary ones. In this context, it is interesting that in *E. coli* [32] and in a cyanobacterium [40], the expression level of Cpn10 is almost equivalent to that of Cpn60. On the other hand, in cell organelles, Cpn10 has not been detected immunologically, although the presence of Cpn10-like protein has been suggested in a mitochondrion [24]. At this stage it is not clear why in the cell organelles and primary symbionts Cpn10 is disproportionately smaller in amount. One possibility is that, though Cpn10 may be required for some synergistic function with Cpn60, Cpn10 is not as essential as Cpn60 because functions dependent on Cpn60 are more numerous under the intracellular conditions. In fact, even in *E. coli* not all GroEL-dependent process have been shown to require GroES [1, 23]. Another possibility is that in the primary symbiont Cpn60 has acquired a unique role in the long history of symbiosis in addition to the chaperonin function and come to be synthesized selectively [20].

Evolution of the intracellular symbiotic system in aphids

It was revealed that the primary symbionts are quite similar to each other among all the aphid species examined (Fig. 5). Their contents of Cpn60 and Cpn10 were remarkably similar over the species (Table 2), which is unusual among free-living prokaryotes. In contrast, the secondary symbionts were quite varied in shape and structure, and their presence did not seem to reflect the phylogenetic position of the host aphids, at least at the subfamily level. They, like common bacteria, contained both Cpn60 and Cpn10 abundantly (Table 2). Based on these facts, it is conceivable that the primary symbionts have been derived from a prokaryote that was acquired by the common ancestor of aphids whereas the secondary symbionts have been acquired by various aphids independently after divergence of the aphid species. In fact, based on the 16S rRNA sequence data, Unterman *et al.* [38] suggested that the primary symbiont diverged from free-living bacteria earlier than the secondary one. Also, Munson *et al.* [30, 31] claimed that the primary symbionts of various aphids are monophyletic and belong to the same genus *Buchnera*.

The idea that the secondary symbionts are of later acquisition may be supported by another circumstance. In the present studies, the secondary symbiont was not found in the pea aphid *A. pisum* (Table 2). In the meantime, Griffiths and Beck [10] described the secondary symbionts in *A. pisum* as intracellular, while McLean and Houk [27] reported that they are external to the mycetocytes. Douglas and Dixon [6] stated that the secondary symbionts are within the mycetome sheath of the young nymph but only loosely associated with the mycetocytes in the fourth-instar nymph and young adult. These and my contradictory results taken together, it is likely that the secondary symbiont is present in some strains of *A. pisum* but absent in others. Such a variety seems to imply that at least in *A. pisum*, the secondary symbiont is not as essential for the host as the primary one, and that it was a recent addition to the primary one.

Implication for symbiotic evolution

The Endosymbiosis Theory [25, 26], which is now supported by many data at the molecular level, claims that cell organelles such as mitochondria and chloroplasts have been derived from endosymbiotic bacteria. In this context, the primary and secondary symbionts of aphids may be regarded as intermediate forms between free-living bacteria and cell organelles. In the present studies, I demonstrated that in terms of amount of Cpn60 and Cpn10, the primary symbionts, which have symbiosed with aphids for a very long time, are similar to cell organelles whereas the secondary symbionts whose acquisitions by the hosts were relatively recent events are similar to free-living bacteria (Table 3). This finding, though circumstantial, provides supporting evidence for the idea that these two types of intracellular symbionts are intermediates at different stages.

In this connection, it should be noted that in the genes of the primary symbiont the base substitution tend toward a remarkable increase in A + T content [34]. This trend is also generally found in genes of endoparasitic bacteria [41] and mitochondria [21, 39] which all are intracellular. These facts raise the possibility that the prokaryotes kept under the intracellular conditions for a long time have been subject to some common evolutionary pressures and that, as a result, they have come to have common molecular properties, as described here. Although such evolutionary pressures are hypothetical, the two type of intracellular symbionts of aphids may be a good material in which to further study the process and mechanism of endosymbiotic evolution.

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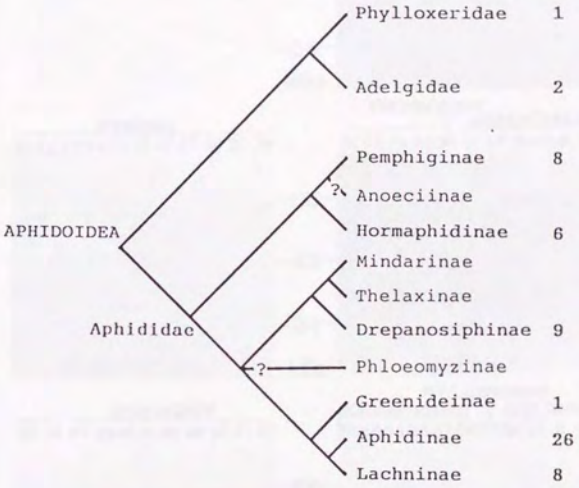

Fig. 1. A phylogenetic relationship of aphid families and subfamilies proposed by Moran [29]. The numbers on the side of family or subfamily names indicate how many species were examined in this study.

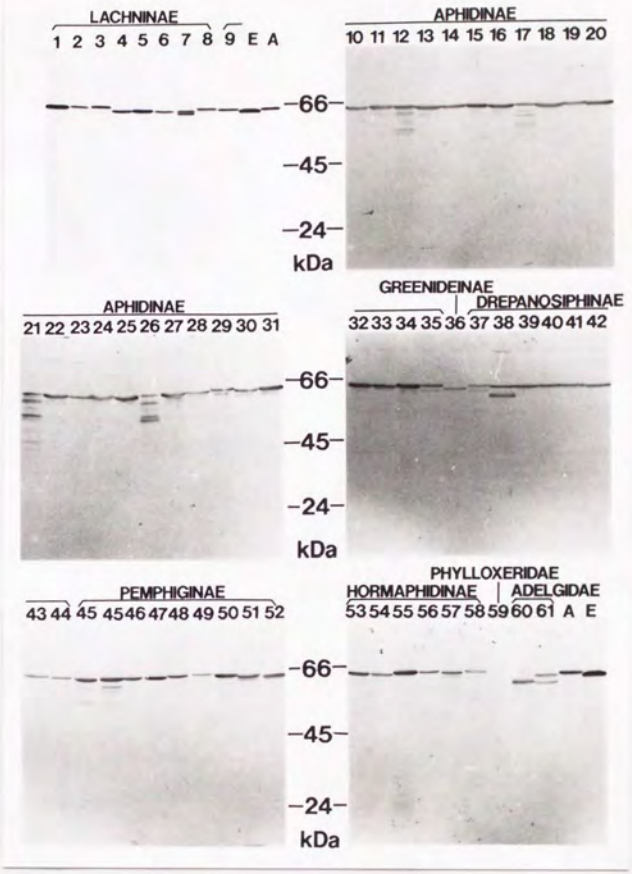

Fig. 2. SDS-PAGE immunoblot analysis of Cpn60. In each lane, proteins equivalent to 250μg of wet body weight of insects were electrophoresed in 10% gel, transfered to PVDF membrane, and probed with anti-symbionin antiserum. The lane numbers correspond to the species numbers in Table 1. Lanes A and E are the controls, *A. pisum* (symbionin) and *E. coli* (GroEL).

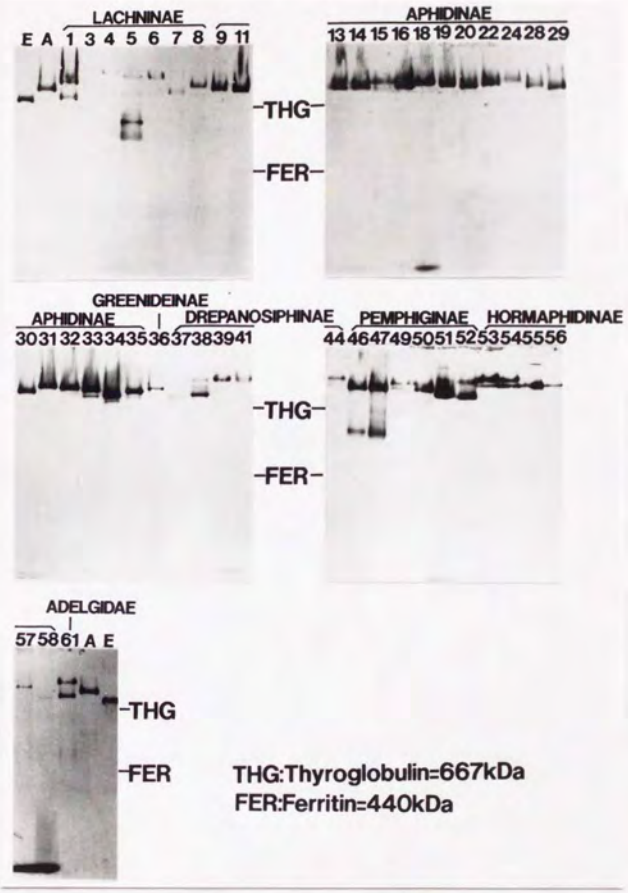

Fig. 3. Native-PAGE immunoblot analysis of Cpn60. In each lane, proteins equivalent to 500μg of wet body weight of insects were electrophoresed in 5% gel, transfered to PVDF membrane, and proved with anti-symbionin antiserum. The lane numbers correspond to the species numbers in Table 1. THG and FER indicate the mobility of thyroglobulin (667kDa) and ferritin (440kDa), respectively.

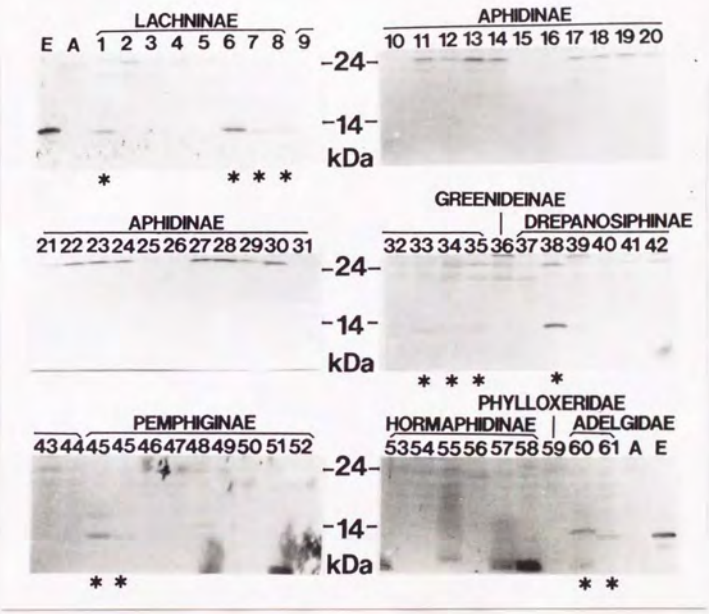

Fig. 4. SDS-PAGE immunoblot analysis of Cpn10. In each lane, proteins equivalent to 500μg of wet body weight of insects were electrophoresed, blotted to PVDF membrane, and probed with anti-GroES antiserum. The lane numbers correspond to the species numbers in Table 1. The bands of approximately 24kDa are nonspecific ones. The dense bands at the bottom of several lanes are due to intrinsic pigments of aphids.

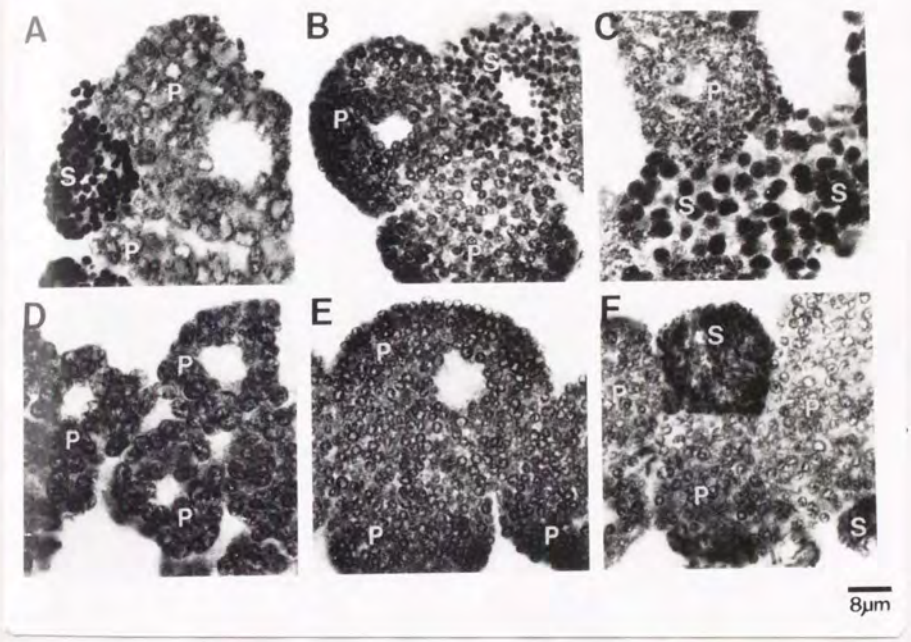

Fig. 5. Immunohistochemistry of Cpn60. Aphid embryos were fixed in Boiun's solution, embedded in paraffin wax, and processed into 5μm tissue sections. The sections were probed with anti-GroEL antiserum to visualize the localization of Cpn60. The procedures of the immunoreactios were kept constant so as to compare the intensity of immunostaining (see Materials and Methods). A, *Nippolachnus piri*; B, *Sitobion ibarae*; C, *Yamatocallis tokyoensis*; D, *Hamamelistes miyabei*; E, *Aphis nerii*; F, *Stomaphis yanonis*. Peroxidase reaction time for visualization was 2min. P and S indicate the primary and secondary symbionts, respectively.

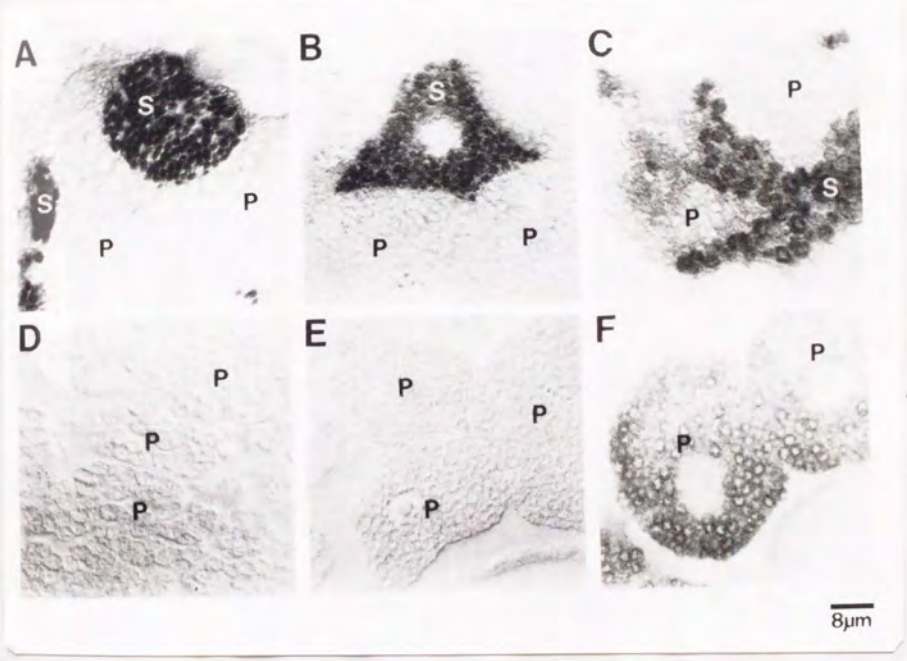

Fig. 6. Immunohistochemistry of Cpn10. The tissue sections were probed with anti-GroES antiserum. A, *N. piri*; B, *S. ibarae*; C, *Y. tokyoensis*; D, *H. miyabei*; E and F, *A. nerii*. Peroxidase reaction time for visualization was 3min in A–E and 30min in F. P and S indicate the primary and secondary symbionts, respectively.

Nos.	Scientific names
	APHIDIDAE
	LACHNINAE
1.	*Cinara pini*
2.	*Eulachnus thunbergii*
3.	*Stomaphis yanonis*
4.	*Lachnus tropicalis*
5.	*Lachnus roboris*
6.	*Tuberolachnus salignus*
7.	*Nippolachnus piri*
8.	Genus sp.
	APHIDINAE
9.	*Rhopalosiphum padi*
10.	*Schizaphis rotundiventris*
11.	*Toxoptera odinae*
12.	*Aphis rumicis*
13.	*Aphis citricola*
14.	*Aphis gossypii*
15.	*Aphis nerii*
16.	*Aphis ichigo*
17.	*Aphis sambuci*
18.	*Melanaphis bambusae*
19.	*Melanaphis japonica*
20.	*Hyalopterus pruni*
21.	*Semiaphis heraclei*
22.	*Brevicoryne brassicae*
23.	*Tuberocephalus sasakii*
24.	*Myzus siegesbeckiae*
25.	*Myzus varians*
26.	*Acyrthosiphon magnoliae*
27.	*Acyrthosiphon nipponicus*
28.	*Indomegoura indica*
29.	*Megoura lespedezae*
30.	*Megoura crassicauda*
31.	*Uroleucon picridis*
32.	*Uroleucon giganteus*
33.	*Uroleucon cephalonopli*
34.	*Sitobion ibarae*
	GREENIDEINAE
35.	*Greenidea nipponica*
	DREPANOSIPHINAE
36.	*Symydobius alniaria*
37.	*Periphyllus californiensis*
38.	*Yamatocallis tokyoensis*
39.	*Tuberculatus fulviabdominalis*
40.	*Tuberculatus capitatus*
41.	*Tuberculatus stigmatus*
42.	*Takecallis arundicolens*
43.	*Tinocallis kahawaluokalani*
44.	*Shivaphis celtis*
	PEMPHIGINAE
45.	*Schlechtendalia chinensis*
46.	*Prociphilus osmanthae*
47.	*Prociphilus oriens*
48.	*Pemphigus* sp.
49.	*Kaltenbachiella japonica*
50.	*Eriosoma lanigera*
51.	*Colophina arma*
52.	*Colophina clematis*
	HORMAPHIDINAE
53.	*Hamamelistes miyabei*
54.	*Hamamelistes kagamii*
55.	*Nipponaphis* sp.
56.	*Ceratovacuna nekoashi*
57.	*Ceratovacuna japonica*
58.	*Pseudoregma panicola*
	PHYLLOXERIDAE
59.	*Phylloxerina capreae*
	ADELGIDAE
60.	*Adelges laricis*
61.	*Adelges japonicus*
	(Control Samples)
A.	*Acyrthosiphon pisum* (Symbionin)
E.	*Escherichia coli* (GroEL and GroES)

Table 1. The list of aphids examined in the present study. Higher taxonomic units are according to Moran [29].

Aphid species	Immunoblotting		Immunohistochemistry			
			P-Sym		S-Sym	
	Cpn60	Cpn10	Cpn60	Cpn10	Cpn60	Cpn10
APHIDINAE						
Acyrthosiphon pisum	+	−	+ + +	+	.....	
Aphis nerii	+	−	+ + +	+	.....	
Brevicoryne brassicae	+	−	+ + +	+	.....	
Megoura crassicauda	+	−	+ + +	+	.....	
Sitobion ibarae	+	+	+ + +	+	+ + +	+ + +
LACHNINAE						
Nippolachnus piri	+	+	+ + +	+	+ + +	+ + +
Stomaphis yanonis	+	−	+ + +	+	+ + +	+ + +
DREPANOSIPHINAE						
Periphyllus californiensis	+	−	+ + +	+	+ + +	+ + +
Yamatocallis tokyoensis	+	+	+ + +	+	+ + +	+ +
PEMPHIGINAE						
Prociphilus osmanthae	+	−	+ + +	+	.....	
Colophina arma	+	−	+ + +	+	+ + +	+ +
HORMAPHIDINAE						
Hamamelistes miyabei	+	−	+ + +	+	.....	

Table 2. The results of immunoblotting and immunohistochemistry on the 12 aphids that were examined immunohistochemically. In immunoblotting columns: "+" and "−" indicate detected or not, respectively. In immunohistochemistry columns: "+++", immunostained very densely within 3min; "++", immunostained densely within 10min; "+", immunostained within 30min, but not so densely. "....." indicates the absence of the secondary symbiont. P−Sym, primary symbionts. S−Sym, secondary symbionts.

Period of endosymbiosis		Cpn60[a]	Cpn10[a]
↓	Free-living bacteria	+ + +	+ + +
	Secondary symbionts	+ + +	+ + +
	Primary symbionts	+ + +	+
	Organelles	+ + +	−

Table 3. The content of Cpn60 and Cpn10 in relation to the length of the endosymbiotic period. "+++", abundant; "+", not abundant; "−", not present.

PART II

DISCOVERY OF EUKARYOTIC EXTRACELLULAR SYMBIONT IN A CERATAPHIDINI APHID, *HAMILTONAPHIS* (= *ASTEGOPTERYX*) *STYRACI* (HOMOPTERA, APHIDIDAE, HORMAPHIDINAE)

ABSTRACT

In studies on the endosymbiotic system of various aphids, immunoblotting with an antiserum against symbionin, an intracellular symbiont-specific protein, revealed that *Astegopteryx styraci* does not have symbionin while all the other aphids examined do. Histological studies showed that *A. styraci* possesses neither mycetocyte nor intracellular symbiont but harbors budding microbes in the hemocoel and fat body. By stepwise density gradient centrifugation, the microbes were successfully isolated from the aphid tissues. When the protein synthesized by isolated microbes was analyzed *in vitro*, it showed antibiotic sensitivity of the eukaryotic type. By electron microscopy, cell organelles such as nuclei and mitochondria were found in the microbial cells. Thus it is concluded that *A. styraci* harbors eukaryotic extracellular symbionts instead of the prokaryotic intracellular symbionts found in most other aphids. The possible function and evolutionary origin of these eukaryotic microbes, and implications for the classification and phylogeny of aphids are discussed.

INTRODUCTION

Aphids (Homoptera, Aphididae), which consist of 10 subfamilies with about 4000 species, form one of the most prolific groups of insects [9, 20]. Almost all aphid species examined have been reported to harbor microbial intracellular symbionts in the cytoplasm of mycetocytes or bacteriocytes, huge cells specialized for this purpose [5]. Judging from their ultrastructure and sensitivity to antibiotics, these symbionts are obviously prokaryotic [10, 13]. The intracellular symbionts have not been propagated when taken out of the host cell [11, 18], and the host aphids show retarded growth and become sterile when deprived of their intracellular symbionts [15, 22]. It has been revealed that the intracellular symbionts provide their hosts with essential amino acids [12, 19, 23]. Thus, the aphids and their intracellular symbionts are intimately mutualistic with each other.

In the mean time, it has been reported that a few aphid species contain neither mycetocyte nor intracellular symbiont but harbor yeast-like microbes in the hemocoel and fat body [4, 16]. However, this type of symbiosis in aphids has scarcely been studied except in the early histological descriptions.

Astegopteryx styraci is an aphid that forms large coral-like galls on twigs of the big leaf storax, *Styrax obassia*, in Japan. This species is also known as an eusocial aphid with second instar soldiers [1]. In the present study, I demonstrate that *A. styraci* lacks the ordinary intracellular symbiotic system as found in other aphids but harbors yeast-like microbes. The nature of the microbe was studied in detail by immunoblotting, light microscopy, antibiotic sensitivity analysis and electron microscopy.

MATERIALS AND METHODS

Insect materials

The galls of *A. styraci* were collected from *S. obassia* at Shomaru Pass, Hanno city, Saitama and at Atoyama-rindo, Nishitama county, Tokyo, Japan.

Other aphid species, *Toxoptera odinae*, *Lachnus tropicalis*, *Nippolachnus piri*, *Shivaphis celtis*, *Prociphilus osmanthae*, *Colophina arma*, *Hamamelistes miyabei*, *Ceratovacuna nekoashi* and *Pseudoregma panicola* were collected in the suburbs of Tokyo and stored at −80°C. *Acyrthosiphon pisum* was an established strain maintained in the laboratory at 15°C on the seedlings of broad bean *Vicia faba*.

Electrophoresis and immunoblotting

The aphid samples were homogenized in SDS-lysis buffer [57.7mM Tris-HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol] on ice. The homogenate (50mg of insects/ml) was boiled at 100°C for 5min and then centrifuged. The supernatants (5μl on each lane) were subjected to SDS-PAGE using a 10% slab gal [17]. The proteins separated in the gel were electrophoretically transferred to a nitrocellulose membrane in transfer buffer [0.125M Tris, 0.96M glycine, 20% (v/v) methanol] at a current of 2mA/cm^2 for 60min and probed with anti-symbionin antiserum using Vectastain Elite ABC kit (Vector) [6]. For the control, preimmune serum was used instead of the antiserum.

Histological procedures for light microscopy

To examine fresh specimens, the aphids were dissected in Tris-buffered saline [20mM Tris-HCl (pH 7.4), 150mM NaCl] on microscope slides.

For paraffin tissue sections, the adult aphids were decapitated and fixed in alcoholic Bouin's solution (33mg picric acid dissolved in 5ml of 80% ethanol, 2ml of formalin and 0.5ml of acetic acid). The fixed insects were washed in 70% ethanol, dehydrated and cleared through ethanol-xylene series and embedded in Paraplast plus (Monoject). Serial tissue sections (3μm)

were prepared on a rotary microtome and mounted on gelatin-coated microscope slides. The sections on the slides were dewaxed and hydrated through xylene-ethanol-water series, and stained with Ehrlich's hematoxylin and eosin.

For semi-ultrathin sections, the embryos dissected from adult aphids were prefixed in 2% paraformaldehyde in 0.1M phosphate buffer for 12h, postfixed in 1% osmium tetroxide in 0.1M phosphate buffer for 2h at 0°C, and embedded in epoxy resin according to the method of Spurr [24]. The tissue sections (0.3-0.5μm) were made on a Porter-Blum MT2 ultramicrotome with glass knives and mounted on gelatin-coated microscope slides. The sections on the slides were stained with toluidine blue solution (0.25% toluidine blue, 0.25% borax) at 60°C for 30sec.

Stepwise density gradient centrifugation

For the density-gradient centrifugation medium, 100% Percoll solution [250mM sucrose, 5% polyethylene glycol 6000, 1% Ficol (Pharmacia Fine Chemicals), 1% bovine serum albumin in Percoll (Pharmacia Fine Chemicals)] was diluted with buffer A [35mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 25mM KCl, 250mM sucrose] to obtain 80, 65, 55, 45, 20 and 10% Percoll solutions. The Percoll gradient was made in 12ml test tubes; 2ml of 80%, 3ml of 65%, 1ml of 55%, 1ml of 45%, 1ml of 20% and 1ml of 10% Percoll solutions were successively layered in this order from the bottom to the top of each tube.

Aphids isolated from a gall were washed briefly with 70% ethanol to remove excreted wax and to sterilize their surface. Then, they were washed well with water, homogenized in approx. 10 volumes of buffer A in a Dounce-type homogenizer on ice and filtered through a nylon mesh. The resultant crude suspension was centrifuged at 1500rpm for 5min at 0°C, and the pellet was resuspended in a minimal volume of buffer A, layered over the Percoll gradient and centrifuged at 1800rpm for 20min at 0°C. The microbes isolated in 65-80% Percoll interphase were collected with a syringe.

Analysis of in vitro protein synthesis

The isolated microbes were washed twice with methionine-free Grace's culture medium ($Grace^{-Met}$) [8] and suspended in an aliquot of $Grace^{-Met}$. Protein synthesis of the isolated mi-

crobes was examined in 1.5ml plastic tubes at room temperature; 100μl of Grace^{-Met}, 10μl of microbe suspension and 5μl of antibiotic solution (200μg/ml chloramphenicol or 200μg/ml cycloheximide) were mixed in each tube, and then 1μl of [^{35}S]methionine (0.38MBq/μl) was added. After 1h of incubation, 20μl of unlabelled methionine (1mg/ml) was added to stop the incorporation of [^{35}S]methionine, and the culture medium was removed by centrifugation. The pelleted microbes were crushed in the SDS–lysis buffer by mixing vigorously with glass beads for 10min at 4°C, boiled at 100°C for 5min and then centrifuged. The supernatants (10μl on each lane) were subjected to SDS–PAGE as described above, and the labelled proteins in the gel were analyzed by fluorography [3] using Enlightening (Biotechnology Systems) and XOMAT–AR film (Eastman Kodak Co., Ltd).

Electron microscopy

The isolated microbes were prefixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1M phosphate buffer for 12h, postfixed in 1% potassium permanganate for 12h at 4°C, and embedded in Spurr's resin as a pelleted form. Ultrathin sections (silver–gray colored) of the microbes were made on a Porter–Blum MT2 ultramicrotome using a diamond knife, mounted on formbar–coated copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEM100CX, JEOL Ltd).

RESULTS

Immunoblotting with anti-symbionin antiserum

In the course of studies on aphid endosymbiosis, the occurrence of symbionin, an intracellular symbiont-specific protein [6, 14], in various aphids was examined by immunoblotting using anti-symbionin antiserum. Figure 1 shows the immunoblotting profiles of 11 aphid species which represent five major subfamilies. Almost all the aphid species exhibited a 63kDa main band due to symbionin, but only *A. styraci* (lanes 8-10) showed no immuno-reactive band. The lack of symbionin was found not only in adult aphids (lane 10) but also in normal nymphs (lane 8) and soldiers (lane 9). This result suggested that *A. styraci* had an unusual symbiotic system.

Histological studies on the symbiotic system

When adult aphids were dissected in Tris buffer and observed freshly, no mycetocytes were found at all although such cells should be easily identified because of their large size. Instead, I found slender oval-shaped cells free from other tissues in the buffer in which the insects were dissected (Fig. 2). Usually, two cells, often of unequal size, were found connected end to end, in a manner reminiscent of budding yeasts. The "microbes" were present in all the individual examined including nymphs and soldiers, which were collected from two distant places (data not shown).

Figure 3(A) is a paraffin section of an adult aphid through the abdominal part. No mycetocytes were found in the area between ovarioles and gut, where huge mycetocytes are located in aphids in general. In the aphid tissues examined, the microbes were stained with eosin; they were chiefly associated with embryos in ovarioles (arrows) but also found in maternal fat body areas (arrowheads). In maternal fat body [Fig. 3(B)], some of them intruded in the cytoplasm of fat body cells while others were in the intercellular spaces. In mature embryos in which many tissues and organs had differentiated [Fig. 3(C)], the microbes were relatively dispersed between the various organs in the abdomen. In contrast, in young embryos [Fig. 3(D)], the microbes formed dense aggregates. Embryos at a very early stage of development, i.e. with only one cell layer, already harbored several microbes [Fig. 3(E)].

Figure 4(A) is a semi-ultrathin epoxy resin section of an embryo through the abdomen which is stained with toluidine blue. This histological technique enabled me to visualize selectively the microbes in aphid tissues. The microbes were found exclusively in the abdomen, located in the hemocoel surrounded by gut, ovarioles and fat body. Unlike the adult insect, the cytoplasm of the embryonic fat bodies was free of microbes. Figure 4(B) is a magnified image of the microbes, some of which clearly appear to be budding.

Antibiotic sensitivity of the microbe

Figure 5 shows crushed aphid tissues fractionated in the Percoll gradient, showing that the microbes could successfully be isolated in the 65-80% Percoll interphase.

The protein synthesis of the isolated microbes was analyzed *in vitro* in the presence and absence of antibiotics. The microbes were suspended in methionine-free Grace's medium, and [^{35}S]methionine incorporation into the proteins were analyzed by fluorography (Fig. 6). With no antibiotic, the microbes synthesized many proteins actively (lane 1). When chloramphenicol, a specific inhibitor of prokaryotic protein synthesis, was added, the protein synthesis showed little inhibition (lane 2). In contrast, in the presence of cycloheximide, a specific inhibitor of eukaryotic protein synthesis, the protein synthesis was inhibited almost completely (lane 3).

Electron microscopy

Figure 7 represents an electron microscopic image of a microbe in the process of division or budding. The adoption of potassium permanganate as post-fixative resulted in ultrathin sections of high quality. In the microbe cell, nucleus, mitochondria, endoplasmic reticula, cell membrane and thick cell wall were obviously observed. Several nuclear pores were observable in the nuclear membrane. Ribosomes were not found because they are easily degraded by potassium permanganate. Electron-dense bodies were often observed close to the nucleus; these might be derived from vacuoles.

DISCUSSION

Almost all aphid species harbor prokaryotic intracellular symbionts in mycetocytes in the abdomen [5]. Generally, these intracellular symbionts contain symbionin abundantly [7]. In the present study, however, *A. styraci* exhibited no cross-reactive protein to anti-symbionin antiserum (Fig. 1). Histological studies (Figs 2–4) revealed that this species possesses neither mycetocytes nor intracellular symbionts but harbors budding microbes chiefly in the hemocoel and also in the fat body. The protein synthesis of the microbes showed an antibiotic sensitivity typical of eukaryotes (Fig. 6). Electron microscopic observation revealed that the microbe possesses cell organelles such as nucleus and mitochondria (Fig. 7). It is therefore concluded that *A. styraci* harbors eukaryotic symbionts instead of the prokaryotic intracellular symbionts found in most other aphid species. Although the symbionts were also found in the cytoplasm of fat body cells, their main location, particularly in the embryonic stages, is in the hemocoel and there are no specialized cells to harbor them. Therefore, these symbionts should be regarded as extracellular.

What do these extracellular symbionts do in the host aphid? At present, there is no direct information on the function of this microbe. It is not even clear whether the extracellular symbiont is really symbiotic or rather parasitic. However, it should be noted that although various aphids have been reported to be seriously harmed when they are deprived of their intracellular symbionts [15, 22], *A. styraci* is inherently free from such intracellular symbionts. It is also notable that the extracellular symbionts were found in all the individuals of *A. styraci* examined, irrespective of instar and morph. These facts suggest that in place of the intracellular symbionts in most aphids, the extracellular symbionts are essential for *A. styraci* by making, for example, nutritional contributions to the host. Since, in general, extracellular symbionts are less fastidious than intracellular ones, the symbiont of *A. styraci* would provide a good opportunity to study the nature of host-symbiont interactions in aphids.

Other aphids which harbor extracellular symbionts instead of intracellular symbionts have already been described by Buchner [4] and Kolb [16] based on light microscopic observations. Through extensive studies on many aphids, they found several species in the subfamily Hormaphidinae exceptional in their symbiotic system. Among three tribes of Hormaphidinae (Hormaphidini, Nipponaphidini and Cerataphidini), these aphids were confined to Cerataphidini. Although many Cerataphidini aphids (e.g. *Pseudoregma*, *Ceratovacuna*) have an ordinary symbi-

otic system, four *Cerataphis* species [*C. fransseni* (= *variabilis*), *C. freycinetiae*, *C. lataniae* and *C. orchidearum*], *Glyphinaphis bambusae*, and an unidentified species were reported to possess neither mycetocytes nor intracellular symbionts but harbor yeast-like microbes in the hemocoel and fat body cells. According to the results of the present study, *A. styraci* should be added to the list. Since the early reports by Buchner and Kolb, this work is the first detailed study on the nature of the extracellular symbiont.

Buchner [4] reported that the *Astegopteryx* species he examined contained typical intracellular symbionts, in contrast of my results. However, since *A. styraci* lacks morphs on the secondary host plant which have been used in the recent classification of Cerataphidini, the present taxonomic position of this species is still tentative [1]. The occurrence of extracellular symbionts support the idea that *A. styraci* should be transferred to a group distinct from *Astegopteryx*. Recently, in fact, a new genus *Hamiltonaphis* was elected to accept this species [2].

An interesting question is the origin of this unusual symbiont in these aphids. One possibility is that aphids with these eukaryotic symbionts constitute a distinct sister group from all the other aphids with intracellular symbionts. However, this is quite unlikely since the former aphids obviously belong to the Cerataphidini because of their morphology and life cycle. An alternative possibility is that these aphids originally harbored intracellular symbionts but acquired yeast-like microbes, which then took over. This idea is supported by the following: 1) Data of 16S-rRNA sequences suggest that the primary intracellular symbiont was acquired by the common ancestor of the present aphids [21]. 2) Notwithstanding this, some species of the Cerataphidini harbor eukaryotic symbionts while the others contain intracellular symbionts. 3) The mechanism of ovarial transmission and the location in the aphid body of the eukaryotic symbionts are quite similar to those of the intracellular symbionts [4, 16]. Plausibly, the acquisition of the yeast-symbiont occurred only once in the common ancestor of Cerataphidini aphids which now harbor yeasts. If so, the aphids with eukaryotic symbionts in the Cerataphidini would constitute a monophyletic group, which may contribute to the classification and phylogenetic studies of Hormaphidinae aphids in general.

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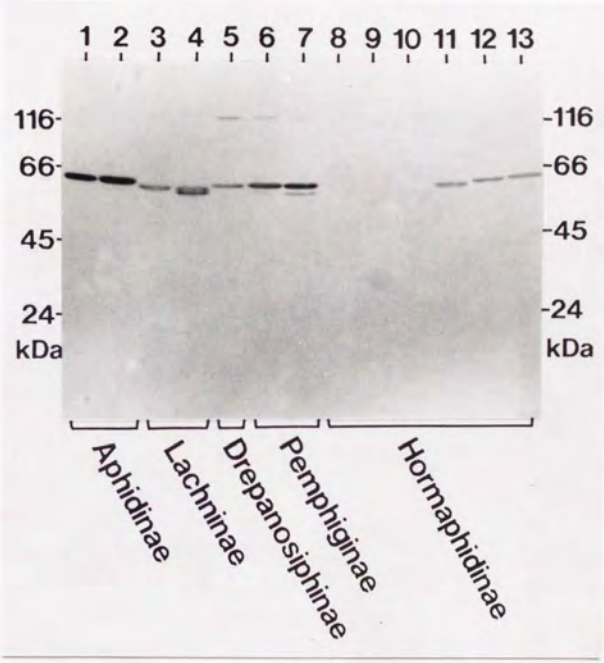

Fig. 1. Immunoblotting with anti-symbionin antiserum. Proteins extracted from 11 aphids, including *A. styraci*, were separated by SDS-PAGE and probed with antiserum against symbionin. Lane 1, *Ac. pisum*; lane 2, *T. odinae*; lane 3, *L. tropicalis*; lane 4, *N. piri*; lane 5, *S. celtis*; lane 6, *P. osmanthae*; lane 7, *C. arma*; lane 8, *A. styraci*, normal nymphs; lane 9, *A. styraci*, soldiers; lane 10, *A. styraci*, adults; lane 11, *H. miyabei*; lane 12, *Cer. nekoashi*; lane 13, *Ps. panicola*. Subfamily names are shown beneath the lanes.

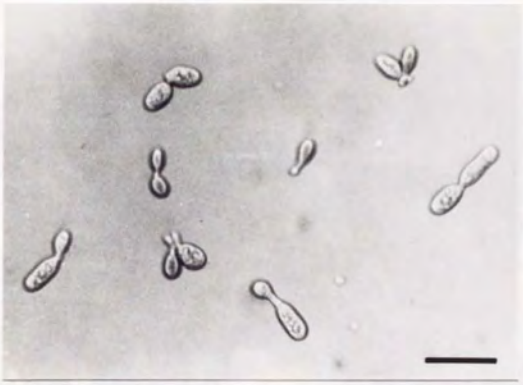

Fig. 2. The microbes found in the buffer in which an adult of *A. styraci* was dissected. Bar represents 20μm.

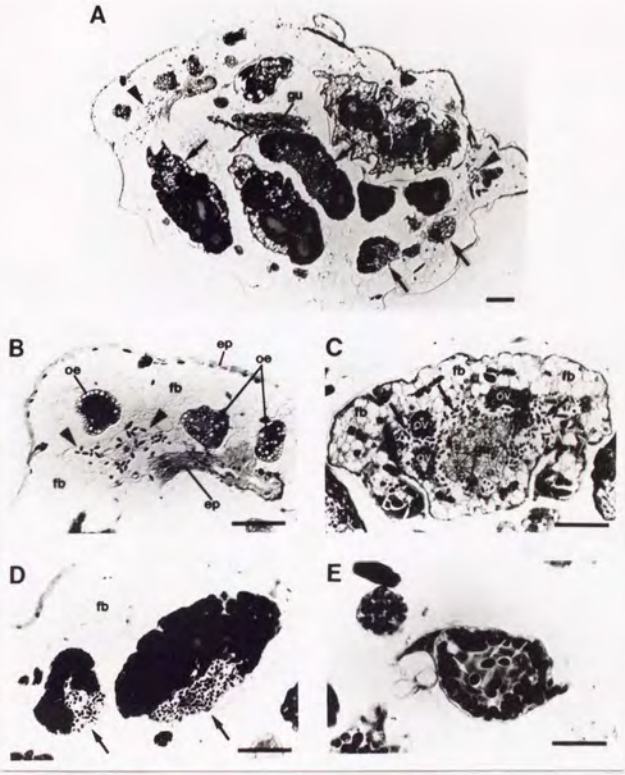

Fig. 3. Paraffin tissue sections (3μm) of *A. styraci*, stained with Ehrlich's hematoxylin and eosin and observed under a Normarski differential interference microscope. (A) A cross section through the abdomen of an adult aphid; (B) fat body area of the adult where the microbes are located; (C) a mature embryo cross-sectioned through the abdominal part; (D) early embryos with dense microbe mass; (E) a very early embryo with only one cell layer, which harbors several microbes. Arrows indicate the microbes associated with embryos while arrowheads show those found in the fat body areas. Bars represent 50μm in (A)-(D) and 20μm in (E). Abbreviations: ep, epithelial tissue; fb, fat body; gu, gut; oe, oenocyte; ov, ovariole.

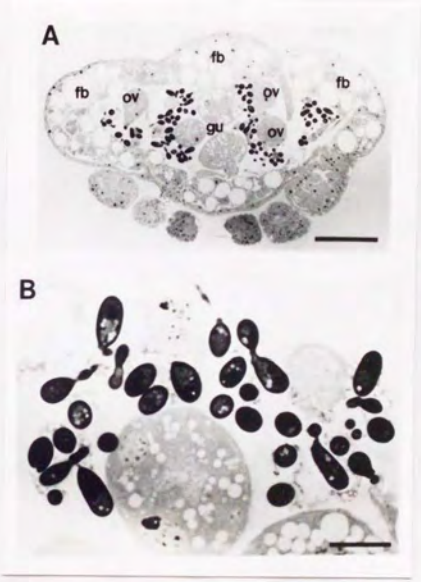

Fig. 4. Semi-ultrathin sections of a dissected embryo stained with toluidine blue, in which the microbes were stained selectively. (A) A cross-section through the abdomen; (B) a magnified image of the microbes. Bars represent 50μm in (A) and 20μm in (B). Abbreviations are the same as in Fig. 3.

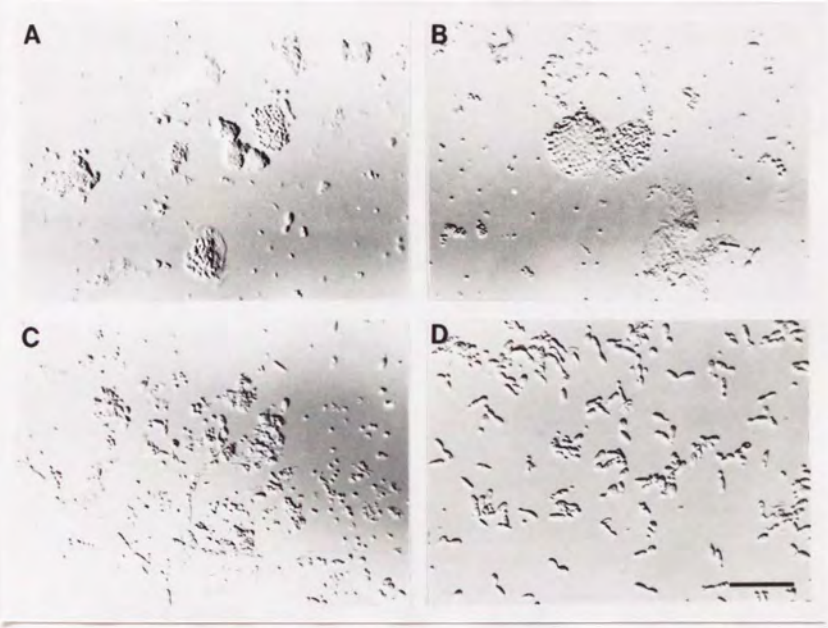

Fig. 5. Crushed aphid tissues fractionated in the Percoll stepwise density-gradient observed under a Normarski differential interference microscope. (A) 25-45%, (B) 45-55%, (C) 55-65% and (D) 65-80% Percoll interphase fractions, respectively. Bar represents 50μm.

Fig. 6. *In vitro* protein synthesis and antibiotic sensitivity of the isolated microbes. The same amount of the microbes suspended in Grace's medium (methionine-free) were incubated with [^{35}S]methionine in the presence or absence of antibiotics, and the extracted proteins from them were analyzed by fluorography. Lane 1, without antibiotic; lane 2, with chloramphenicol (8.6μg/ml); lane 3, with cycloheximide (8.6μg/ml).

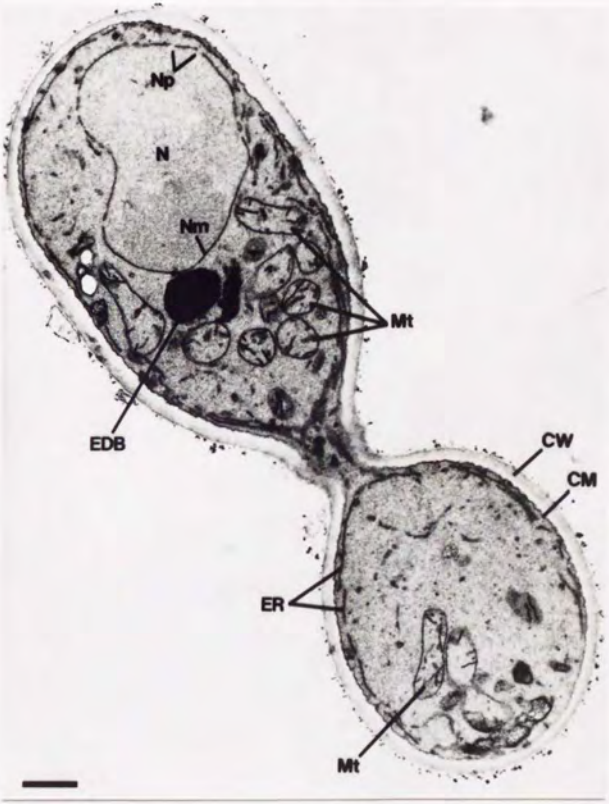

Fig. 7. A transmission electron microscopic image of the microbe. Bar represents approx. 0.5μm. Abbreviations: CM, cell membrane; CW, cell wall; EDB, electron dense body; ER, endoplasmic reticulum; Mt, mitochondria; N, nucleus; Nm, nuclear membrane; Np, nuclear pore.

PART III

PHYLOGENY OF CERATAPHIDINI APHIDS REVEALED BY THEIR SYMBIOTIC MICROORGANISMS AND BASIC STRUCTURE OF THEIR GALLS: IMPLICATIONS FOR HOST–SYMBIONT COEVOLUTION AND EVOLUTION OF STERILE SOLDIER CASTES

ABSTRACT

I collected more than 40 species of aphids of Cerataphidini and related groups, which cover all the Cerataphidini genera ever described, and examined their symbiotic system histochemically. The Cerataphidini aphids were divided into two groups in terms of their symbiotic system; species with prokaryotic intracellular symbionts in mycetocytes which are typical of other Aphididae species in general, and those with yeast-like extracellular symbionts in hemocoel and fat body which are quite exceptional in Aphididae. The species with yeast-like symbionts, 12 out of 38 species examined, were further divided into three groups in terms of morphology of their symbionts; 5 species with slender yeasts, 6 with roundish yeasts, and 1 with large yeasts with multiple budding sites. This division based on the symbionts coincided well with the division of genera based on morphology of the insects, with the only exception of "*Cerataphis*" *bambusifoliae*. By character argumentation on the symbiont types, I suggest that harboring the yeast-like symbiont is an apomorphic state, and evolved only once in Cerataphidini. It is also pointed out that the galls of Cerataphidini aphids fall into two types, single cavity galls and multiple cavity galls, considering its basic plan and structure. By character argumentation on the gall types, I suggest that the multiple cavity gall is apomorphic and of single origin in Cerataphidini. Based on these results, I analyzed phylogenetic relationship of Cerataphidini aphids, and suggest that the Cerataphidini is constituted by two major sister monophyletic groups; one characterized by a synapomorphy, harboring yeast-like symbionts, and the other characterized by a synapomorphy, multiple cavity galls. A phylogenetic hypothesis on the evolutionary history of various characters such as two types of sterile soldier castes, symbionts, galls, etc., in this group is proposed. Also I discuss possible origin and function of the yeast-like symbiont, evolutionary effects of the symbiont-replacement on the host aphids, and taxonomic treatment of problematic taxa in Cerataphidini.

INTRODUCTION

So far, some 4000 species of aphids (Homoptera, Aphididae) have been described. Almost all of them harbor microbial intracellular symbionts in the cytoplasm of mycetocytes, huge cells in the abdomen specialized for this purpose [15]. Judging from their ultrastructure and sensitivity to antibiotics, the symbionts are evidently of prokaryotic nature [32, 35]. They are passed from one to the next generation of the host insects by ovarial transmission and have no free-living state [15, 35]. Thus, they can be regarded as a maternally inherited genetic element like mitochondria. The symbionts are not propagated when taken out of the host cells [33, 48]. The aphids show retarded growth and become sterile when deprived of their symbionts [38, 59]. It has been demonstrated that the symbionts serve as an important component in the pathway of amino acid metabolism [19, 60, 61, 62, 63]. Thus, the aphids and their intracellular symbionts are intimately mutualistic with each other. Morphological, histological, biochemical and molecular biological evidences have consistently suggested that the intracellular symbionts of various aphids are of single origin; they have been derived from a bacterial species that was acquired by the common ancestor of the present aphids [15, 27, 53, 56]. Molecular phylogenetic analyses revealed that the symbionts constitute a monophyletic group *Buchnera*, a genus closely related to *Escherichia coli* [57].

Although the prokaryotic intracellular symbionts are highly conserved amongst Aphididae, several aphids have been reported to be exceptional with respect to their symbiotic system. Four *Cerataphis* species [*C. fransseni* (= *variabilis*), *C. lataniae*, *C. freycinetiae* and *C. orchidearum*], *Glyphinaphis bambusae* and *Hamiltonaphis styraci* have been shown that they contain neither mycetocytes nor intracellular symbionts but harbor yeast-like eukaryotic extracellular symbionts in hemocoel and fat body [11, 14, 25, 42]. All these exceptional aphids belong to the same group Cerataphidini, which prompted me to investigate the symbiotic system of Cerataphidini aphids extensively.

Figure 1 is a phylogenetic relationship among subfamilies of Aphididae based on morphological characters [31, 52]. Aphididae is composed of many subfamilies, one of which is Hormaphidinae. This subfamily is divided into three tribes, one of which is Cerataphidini [20, 30]. Though most aphid species are predominant in temperate regions [17], Cerataphidini is a prosperous group chiefly occurring in tropical-subtropical area of south-eastern Asia [20, 29].

So far, ten genera have been placed under the tribe Cerataphidini, although there are problems about their taxonomic treatments as will be mentioned later.

Figure 2 is the life cycle of Cerataphidini aphids. Typically, they seasonally shift between two host plants, as is the case with Aphididae in general. Their host plant-specificity is generally very strict; for example, for *Ceratovacuna nekoashi*, *Styrax japonica* and *Microstegium vimineum* are the only available primary and secondary hosts, respectively. The primary host plants of Cerataphidini, on which sexuales, fertilized eggs and fundatrices occur, are invariably *Styrax* trees [Fig. 2(A)]. On the primary host, they deform the plant tissue into gall and reproduce in it [Fig. 2(B)]. In the gall, they usually produce two different types of 2nd instar nymphs; one is normal nymph which grows to adult and reproduces, and the other is soldier which does not molt anymore but is specialized for defending and cleaning the gall [Fig. 2(C)]. When the gall matures, winged emigrants begin to disperse to the secondary host plants. Their secondary hosts are in many cases Gramineae (bamboos and grasses), but other plants are also utilized by some species [Fig. 2(D)]. On the secondary host, their morphology dramatically changes from that of the gall generation. In many species, a pair of sharp horns are on the head of the secondary host generation, after which Cerataphidini was named (Cerat = horn) [Fig. 2(E)]. Several species of *Pseudoregma* and *Ceratovacuna* produce two types of 1st instar nymphs also on the secondary host; normal nymphs and soldiers [Fig. 2(F)]. They form open colonies on the secondary host. When the colony matures, winged sexuparae appear and return to the primary host. Sexual females and males from them copulate on the primary host and deposit fertilized eggs. This is the typical life cycle of Cerataphidini aphids. However, there are species or populations which complete their life cycle only on either primary or secondary host plant.

Cerataphidini is an aphid group in which several very interesting events have probably occurred in the course of its evolution. The origins of two types of soldiers, prokaryotic intracellular symbionts and eukaryotic extracellular ones, and elaborated gall-formation in this group are all intriguing and challenging subjects from evolutionary biological points of view. In order to analyze these subjects, it is necessary to know the phylogenetic relationship among the genera and species in Cerataphidini. However, there are a number of difficulties in the phylogenetic analysis of aphids based on morphological characters. Since body plan of aphids is simple and their exoskeleton is not well sclerotized, morphological characters are relatively scant in this group. Many of the available characters are so trivial and unstable as to be vulnerable to parallel

and/or reversal evolutions. Moreover, the characters are often very variable in response to environmental factors. The same aphid species represents various morphs throughout their complex life cycle; alatae (winged) and apterae (unwinged), asexuals and sexuales, primary-host generation and secondary-host one, sterile soldiers and so on [51]. It makes the problem more complex that in many aphids, including Cerataphidini, the primary-host generation and the secondary-host one of the same species are quite different in morphology (ex. see Fig. 2). Thus, when I attempt to analyze a group of aphids in which some are known as primary-host generation and the others are as secondary-host one, it is difficult to make meaningful comparison between them. So far, therefore, the only phylogenetic analysis of the aphids was at family or subfamily level ([31]; see Fig. 1). At lower levels, there have been no phylogenetic studies on the Cerataphidini aphids.

In the present study, I examine symbiotic system of Cerataphidini aphids extensively, and demonstrate that they are divided into two groups; species with intracellular symbionts and those with yeast-like ones. Based on the symbiont types and basic structure of the galls, which are shown to be stable but polymorphic characters in Cerataphidini, I analyze phylogenetic relationship in this group. In addition, evolution of two types of soldiers, effects of symbiont-replacement to the hosts, and other problems with Cerataphidini will be discussed.

MATERIALS AND METHODS

Insect materials

Thirty-eight species of Cerataphidini aphids were collected from Japan, Taiwan, Sumatra and Malaysia (Table 1). For the outgroup of Cerataphidini, two Hormaphidini and two Nipponaphidini species were also collected. The collected insects were immediately fixed and preserved in alcoholic formalin (ethanol: formalin: acetic acid = 16:6:1).

Histochemical procedures

The fixed insects were decapitated in 70% ethanol, dehydrated and cleared through ethanol-xylene series, and embedded in Paraplast plus (Monoject). Tissue sections of 3-5μm thick were prepared on a rotary microtome and mounted on gelatin-coated microscope slides.

The tissue sections were immunohistochemically stained with anti-symbionin antiserum [26] by which prokaryotic intracellular symbionts were specifically stained in deep brown. They were also subjected to periodic acid-Schiff (PAS) staining [49] to visualize selectively yeast-like extracellular symbionts in deep red or purple. The stained tissue sections were observed under a Normarski differential interference microscope.

RESULTS AND DISCUSSION

I. *Histochemical examination of symbiotic microorganisms of various Cerataphidini aphids*

Among 38 Cerataphidini species I examined histochemically, a number of species were revealed to harbor yeast-like extracellular symbionts instead of intracellular ones. In all species of *Pseudoregma*, *Ceratovacuna*, *Ceratoglyphina*, *Chaitoregma*, *Astegopteryx* and *Aleurodaphis*, and *Cerataphis bambusifoliae* and "*Astegopteryx*" *vandermeermohri*, typical intracellular symbionts were found as in other aphids (Fig. 3). On the other hand, the rest of the species, *Hamiltonaphis*, *Cerataphis*, *Tuberaphis*, "*Astegopteryx*" *leeuweni* and "*Astegopteryx*" *sumatrana* possessed neither intracellular symbionts nor mycetocytes but PAS-positive budding particles in the hemocoel and fat body (Fig. 4). They were apparently yeast-like extracellular symbionts. There were no species that harbored both the intracellular symbionts and extracellular ones.

I found that 12 out of 38 Cerataphidini species had yeast-like symbionts. They were divided into three groups in terms of morphology of their symbionts (Fig. 5). The symbionts of *Hamiltonaphis* and *Cerataphis* showed slender shape [Fig. 5(A)] while those of *Tuberaphis*, "*Astegopteryx*" *leeuweni* and "*Astegopteryx*" *sumatrana* were roundish [Fig. 5(B)]. Distinct from both of them, the symbionts of *Glyphinaphis* were larger in size and of rather irregular shape with multiple budding sites [Fig. 5(C)].

These results are summarized in Table 2 with the information of host plants, basic structure of the gall and production of the soldiers.

II. *Occurrence of yeast-like extracellular symbionts in Cerataphidini: congruence and discrepancy with present classification*

I found that there are a number of Cerataphidini species with yeast-like extracellular symbionts in place of prokaryotic intracellular symbionts typical of Aphididae in general (Fig. 4). Thus, there are two symbiont types in Cerataphidini. The symbiont type agrees well with the present classification at genus level. *Pseudoregma*, *Ceratovacuna*, *Ceratoglyphina*, *Chaitoregma*, *Astegopteryx* and *Aleurodaphis* are genera with intracellular symbionts while *Hamiltona-*

phis, *Cerataphis*, *Glyphinaphis* and *Tuberaphis* are those with yeast symbionts (Table 2). Morphology of the yeast symbionts also reflects the taxonomic division. *Hamiltonaphis* and *Cerataphis* are with slender yeasts, *Tuberaphis* with roundish ones and *Glyphinaphis* with large ones (Fig. 5).

However, the only discrepancy in these rules is found in *Cerataphis bambusifoliae*. *Cerataphis* is characterized by several distinctive morphological features. Their body of the apterous adult on the secondary host is flat disc-shaped and highly sclerotized. On the head there are two horns without hairs. Marginal wax glands are linearly arranged all along the edge of the body, which gives them characteristic appearance that their brown disc-shaped body is fringed with radially excreted white wax [58]. Based on external structures, one cannot help placing *Cerataphis bambusifoliae* under the genus *Cerataphis* as has been so [65]. However, this species possesses intracellular symbionts in mycetocytes while the other *Cerataphis* species all harbor yeast symbionts and are without mycetocytes. This means that *Cerataphis bambusifoliae* is quite unlikely to belong to *Cerataphis* because, if it does, we have to assume either multiple evolution of yeast symbionts or reversal evolution from yeast symbionts to intracellular symbionts, either of which is quite unlikely as will be discussed in later sections (III, IV). Therefore, the morphological resemblance is likely due to parallelism or remaining plesiomorphic characters, though further studies on this species are necessary to make final conclusion. In the following sections, this species will be called tentatively "*Cerataphis*" *bambusifoliae*.

The classification of Cerataphidini species has been exclusively based upon morphological characters of their secondary host generation [30, 58]. Therefore, species whose secondary host generation is lacking or unknown were not subjected to classification, so they have been tentatively placed under the genus *Astegopteryx*. Among the species examined in this study, "*Astegopteryx*" *vandermeermohri*, "*Astegopteryx*" *leeuweni* and "*Astegopteryx*" *sumatrana* are such problematic aphids (see Table 2). Notably, "*Astegopteryx*" *leeuweni* [Fig. 4(E)] and "*Astegopteryx*" *sumatrana* [Fig. 4(F)] harbor roundish yeast symbionts which are specifically found in *Tuberaphis* species, suggesting that they should also belong to the genus. "*Astegopteryx*" *leeuweni* has lost its secondary host generation and entirely depends on the primary host throughout its life cycle [12]. Thus, it is difficult to classify this aphid morphologically since it is impossible to examine its secondary host generation, which should justify the classification based on the symbionts. As for "*Astegopteryx*" *sumatrana*, though its secondary host has been unknown, 1st

instar nymphs deposited by the alate emigrants, which should be the first generation on the secondary host, were observed to carry *Tuberaphis*-like characters [47]. In considering these facts, at this stage it will be reasonable to transfer these species to *Tuberaphis*, although further studies on them are necessary.

The taxonomic position of "*Cerataphis*" *bambusifoliae* and "*Astegopteryx*" *vandermeermohri* have not been settled yet, and will be discussed in section IX.

III. *Occurrence of yeast-like extracellular symbionts in Aphididae: yeast symbiont is likely to have been acquired only once by an ancestor of Cerataphidini*

Endosymbiosis with microorganisms has been found from wide spectra of insects and other invertebrates [15]. Particularly Homoptera, including Aphididae, is one of the groups that possess the most highly developed symbiotic system. It has been demonstrated that almost all the aphid species harbor prokaryotic microorganisms in the cytoplasm of specialized cells, mycetocytes, in the abdomen [15, 27]. The intracellular symbionts of various aphids hold a number of characters in common irrespective of phylogenetic or taxonomic position of their host. The mycetocytes that house them are large, round and uninucleated, and located between gut and ovarioles forming cell-aggregates called mycetomes [15]. The symbionts are globular with a diameter of 3-5μm and gram-negative with thin rudimentary cell wall [27, 34]. They also represent peculiar characters at the molecular level that are not found among related free-living bacteria. When the proteins of the symbionts were analyzed, only one protein species, chaperonin 60 or symbionin, predominates in amount [36], though chaperonin 10, encoded by the same operon, is disproportionally smaller in amount [27, 41]. All these characters found in common among intracellular symbionts of various aphids support the idea that the intracellular symbiosis of aphids is of single origin; it was acquired only once in the course of evolution of Aphididae.

This idea is also supported by recent molecular phylogenetic analyses. Based on 16S rRNA sequences, the symbionts of various aphids constitute a monophyletic group in γ-subdivision of Proteobacteria [56, 71], and a genus *Buchnera* was erected to accept them [57]. Whiteflies (Aleyrodidae) and mealybugs (Pseudococcidae) are sister groups of aphids, constituting Sternorrhyncha, and also possess bacterial symbionts [15]. However, molecular phylogenetic

data indicated that their symbionts are evidently distinct from those of aphids [13]. These studies consistently suggest that the intracellular symbionts of aphids have been derived from a prokaryote that was acquired by the common ancestor of the present Aphididae species. The minimum age of the symbiotic association was estimated as old as 160–280 million years ago by 16S rRNA molecular clock calibrated by fossil records of aphids [53].

On the basis of these studies, it is inferred that the common ancestor of Cerataphidini must have harbored prokaryotic intracellular symbionts, plesiomorphy of the symbiotic system. In fact, its sister groups, Hormaphidini and Nipponaphidini, possess intracellular symbionts (Fig. 3; Table 2). In the present study, however, I found a number of Cerataphidini aphids which possess yeast-like extracellular symbionts instead of the intracellular ones (Fig. 4; Table 2). This means that replacement of symbionts from intracellular prokaryotes to extracellular yeasts occurred in the course of evolution of Cerataphidini. Although hundreds of aphids which cover most of major groups of Aphididae have been examined for their symbiotic system [15, 24, 27], occurrence of yeast symbionts was confined to Cerataphidini [14, 25, 42]. Probably, replacement of symbiotic microorganisms is a quite rare event in aphids. It seems unlikely that the symbiont replacement happened several times only in Cerataphidini. The localization and distribution of the yeasts in the tissue through host's post-embryonic development and the mode of their ovarial transmission are quite similar among various Cerataphidini species with yeasts [14, 24, 42], favoring the single origin of the yeast symbionts. The only available evidence that might support their multiple origin is that their shape represents several different types (Fig. 5), but it is also plausible that the differences in shape have evolved from a common ancestral form. All these evidences taken together, the yeast symbiont is considered to be acquired only once by an ancestor of the Cerataphidini species that now house yeasts.

IV. *Phylogenetic information deduced from yeast-like symbionts*

In aphids, yeast symbionts are obviously apomorphic whereas intracellular ones are plesiomorphic. Thus, it is deduced that the Cerataphidini aphids with yeasts should constitute a monophyletic group. Of course, this phylogenetic argumentation rests on the premises that the yeast symbionts have been acquired only once (without parallel evolution) and that reversal

replacement of symbionts from extracellular yeasts to intracellular prokaryotes has never occurred (without reversal evolution). In the present study, I assume that these premises are true in the light of parsimony and many circumstantial evidences discussed in section II, but admit that they should be verified by molecular phylogenetic analysis in future.

In morphology there were three types of yeast symbionts; slender ones in *Hamiltonaphis* and *Cerataphis*, roundish ones in *Tuberaphis* and large ones in *Glyphinaphis* (Fig. 4). These symbiont types are sufficiently distinct from one another and consistent with morphologically well-defined genera as *Cerataphis*, *Glyphinaphis* and *Tuberaphis*. Thus, I can make an assumption that the symbiont types reflect phylogenetically related aphid groups. However, polarity of the characters, which of the three types is plesiomorphic, remains unknown.

V. *Basic plans of gall structure in Cerataphidini: single cavity gall and multiple cavity gall*

All the Cerataphidini aphids whose primary host generation has been described have been shown to form well-developed galls on *Styrax* trees. Size, structure and appearance of their galls represent conspicuous diversity. There are various types of galls which look like banana-bundles, flowers, corals, beanpods, curled hairs, cauliflowers and so on (Fig. 6). Such curious deformations of plant tissues by aphids have attracted great interest of many researchers, so a number of reports on Cerataphidini galls have been published [18, 66, 67, 68, 69]. However, most of the reports describe only external appearance of the galls and are often uncertain of identification of the aphids. In short, works on the Cerataphidini galls have been fragmentary and simply descriptive.

Recently, through a series of ecological studies on many Cerataphidini aphids by Kurosu and Aoki, the correspondence of aphid species to their galls, external and internal structure of the galls, and in several cases detailed gall-forming process have come to be well understood, which allowed me to attempt systematic comparative study on the Cerataphidini galls. I found that they fall into two categories, *single cavity gall* and *multiple cavity gall*, based on their basic structure and the mode of gall formation.

Figure 7(A) schematically shows the process of the formation of single cavity galls. When a fundatrix attacks the host plant, the plant tissues around the insect grow out and encase it

finally. Then the rudimentary gall becomes larger and often highly deformed, with the aphids reproducing in it. As a result, the gall has only one cavity and the fundatrix is confined in the room. The galls of *Hamiltonaphis* [4], *Cerataphis* [64], *Tuberaphis* [8, 9, 47] and "*Astegopteryx*" *vandermeermohri* [18, 47] are of single cavity (Table 2), although in some cases the gall is highly deformed and very complex in appearance (ex. *Tuberaphis takenouchii*, "*Astegopteryx*" *vandermeermohri*) and the unity of space in the gall is not easy to recognize. This type of gall formation process has been studied in detail in *Hamiltonaphis styraci* [4] and *Tuberaphis takenouchii* [47].

The formation process of multiple cavity gall is distinct from that described above [Fig. 7(B)]. When a fundatrix attacks the host plant, the plant tissues around the insect grow out and encase it. Subsequently a number of projections of plant tissues, called subgall rudiments, are differentiated from it. Each subgall rudiment transforms into a sac-like structure, or a subgall, with a small entrance through which parthenogenetic offsprings of the mature fundatrix enter the subgalls. Then the gall grows with the aphids reproducing in every subgall, leaving the fundatrix outside. Thus, the gall has many cavities, each corresponding to a subgall, and the fundatrix is left outside the rooms. The galls often look like a banana-bundle or flower because a number of subgalls are radially connected at a point. The galls of this type are found among *Pseudoregma* [5, 7], *Ceratovacuna* [6, 43], *Ceratoglyphina* [10, 45] and *Astegopteryx* [46], although in *Ceratoglyphina* the gall is so highly deformed that multiple cavity structure is recognizable only at very early stages. Detailed studies on the formation process of this type of galls have been performed in *Ceratovacuna nekoashi* [43], *Astegopteryx bambucifoliae* [46] and *Ceratoglyphina styracicola* [45].

VI. *Phylogenetic information deduced from gall structure*

As stated above, I demonstrated that the galls of Cerataphidini aphids are, though at a glance their appearances represent great diversity, divided into two types, single cavity galls and multiple cavity galls, on account of their basic plan and structure. Since there are many species whose primary host generation is unknown (see Table 2), my knowledge on their galls is still incomplete. However, the type of the gall is always identical at intragenus level, suggesting that

this character is stable to a sufficient extent. Moreover, I found a close relationship between symbiont-type and gall-type; the species and genera with intracellular symbionts always form multiple cavity gall whereas those with yeast symbionts form single cavity gall, with only one exception of "*Astegopteryx*" *vandermeermohri* which harbors intracellular symbionts and forms single cavity gall (Table 2). These facts suggest that the types of the gall may serve as a good character for phylogenetic analysis. As for the symbiont types, intracellular symbiont is plesiomorphic. Notwithstanding this, character argumentation of the gall types, as follows, strongly supported the contrary view, that is, single cavity gall is plesiomorphic.

Although gall formation has been found among divergent groups of aphids [22, 73], multiple cavity galls are confined to Cerataphidini. Most of aphid galls are single cavity type. This is probably because it is the most simple shape of closed gall and thus easy to evolve. The structure and formation process of multiple cavity gall are apparently more complex and elaborated than those of single cavity gall (see Fig. 7). Therefore it is more acceptable that in Cerataphidini multiple cavity gall evolved in an ancestor which had already formed single cavity gall than that multiple cavity gall evolved earlier.

The apomorphy of multiple cavity gall is also supported by outgroup comparison. The aphids of Hormaphidini and Nipponaphidini, constituting subfamily Hormaphidinae with Ceratapidini, always form galls on their primary host plants, *Hamamelis* and *Distylium*, respectively [29, 73]. Thus, it is quite likely that the ability of gall formation has already evolved in the common ancestor of Hormaphidinae. The basic structure of the galls is single cavity and the fundatrix is found inside the room in both Hormaphidini and Nipponaphidini [54]. Since they are sister groups of Cerataphidini and thus regarded as its outgroups, it is deduced that single cavity gall should be plesiomorphy of gall structure in Cerataphidini. There is no convincing evidence supporting that multiple cavity galls have evolved several times in Cerataphidini.

Under the circumstances discussed above, the Cerataphidini aphids that form multiple cavity galls are considered to constitute a monophyletic group. However, I note that the phylogenetic argumentation has a weak point. The information of their galls is incomplete. Particularly, it is disappointing that in *Chaitoregma*, "*Cerataphis*" *bambusifoliae* and *Glyphinaphis* no gall generation has been discovered. I presume that the first and second taxa above are multiple cavity gall formers while the last one is a single cavity gall former, based on the congruence of symbiont type and gall type. In the present stage, I suggest that multiple cavity gall in Cerataphi-

dini is apomorphic and of single origin and that *Chaitoregma* and "*Cerataphis*" *bambusifoliae* belong to the monophyletic group characterized by it. However, this view needs to be further verified by molecular phylogenetic analysis in future.

VII. *Phylogenetic analysis of Cerataphidini aphids*

In the above discussion, I have demonstrated that among Cerataphidini aphids the types of their symbionts and the basic structure of their galls are conservative but polymorphic characters (see sections III, V). I reasonably determined the polarity of these characters by outgroup comparison and other circumstantial evidences (see sections IV, VI). Based on these results, I constructed phylogenetic relationship among Cerataphidini genera and proposed a phylogenetic hypothesis on their evolutionary history.

The tribe Cerataphidini is composed of two large monophyletic groups; one, characterized by yeast-like extracellular symbionts, contains *Hamiltonaphis*, *Cerataphis*, *Glyphinaphis* and *Tuberaphis* species whereas the other, characterized by multiple cavity galls, embraces *Pseudoregma*, *Ceratovacuna*, *Ceratoglyphina*, *Chaitoregma*, *Astegopteryx* and "*Cerataphis*" *bambusifoliae*. "*Astegopteryx*" *vandermeermohri* keeps both characters plesiomorphic, constituting a third group alone. The monophyletic group with yeasts is further divided into three subgroups based on the symbiont types; *Hamiltonaphis* and *Cerataphis* with slender yeasts, *Tuberaphis* with roundish ones, and *Glyphinaphis* with large ones.

Figure 8 is a phylogenetic tree of Cerataphidini aphids. Beside each taxon, character states on its symbiont and gall are indicated. When important evolutionary events occurred are estimated and also indicated on the tree. Let us follow the evolutionary course of the Cerataphidini on the phylogeny I proposed. In the common ancestor of the family Aphididae, the root of the tree, prokaryotic intracellular symbiont was acquired [Fig. 8(A)]. Then the subfamily Hormaphidinae branched off from the other aphids. In the common ancestor of Hormaphidinae, the ability of gall formation on the primary host plant must have evolved; the gall was single cavity type [Fig. 8(B)]. The Hormaphidinae diverged into three tribes, Hormaphidini, Nipponaphidini and Cerataphidini. The common ancestor of Cerataphidini acquired the primary host *Styrax* on which it formed a single cavity gall [Fig. 8(C)]. Then, the Cerataphidini diverged into two major mono-

phyletic groups, multiple cavity gall group and yeast-like symbiont group. In the common ancestor of the former group, the ability to form multiple cavity gall evolved in place of single cavity one [Fig. 8(D)], keeping the intracellular symbiont. In the common ancestor of the latter group, replacement of symbionts from intracellular bacteria to extracellular yeasts occurred [Fig. 8(E)], keeping the single cavity gall. The latter group was further diverged into three subgroups which harbor slender, roundish and large yeast symbionts, respectively.

VIII. *Evolution of two types of sterile soldier castes in Cerataphidini*

Eusocial insects, which possess the reproductive division of labor into reproductive castes and sterile working castes, have been found from Hymenoptera (bees, wasps and ants), Isoptera (termites), Thysanoptera (thrips) and Homoptera (aphids) [16, 39, 72]. In aphids, it is thought that eusociality has evolved at least four times; once in *Colophina* (Pemphiginae, Eriosomatini), once in *Pemphigus* (Pemphiginae, Pemphigini) and twice in Cerataphidini (Hormaphidinae) [1]. In the eusocial aphids, two types of nymphs are produced, normal nymphs and soldiers. The soldiers have the following characteristics: 1) They attack predatory intruders, often in a self-sacrificing manner. 2) They are 1st or 2nd instar nymphs and do not molt further, so that they do not reproduce. 3) They morphologically differ from the conspecific normal nymphs of the same instar [1].

Among all eusocial insects ever known, Cerataphidini is particularly interesting in that it is the only group that has evolved two boldly distinct types of sterile castes in the different stages of life cycle (see Fig. 2). Figure 9 shows the characteristics of the two types of soldiers. On the primary host plant, many Cerataphidini species produce 2nd instar soldiers, which are characterized by sclerotized exoskeleton, in the gall. The soldiers attack the intruder by stinging with the stylets, and clean their gall by pushing honeydew globules and shed skins off [Fig. 9 (A)]. On the other hand, some species of *Pseudoregma* and *Ceratovacuna* produce 1st instar soldiers on the secondary host. They are armed with a pair of sharp horns on the head and powerful fore-legs. When encountering with a predator, they clutch it by the fore-legs and pierce its body with the horns [Fig. 9(B)]. Differences in instar, morphology and attacking behavior between the two types of soldiers strongly suggest that the two independently evolved. Not only the soldiers of

the former type but those of the latter hold similar characters in common irrespective of aphid species, suggesting that each of them is of single origin. Considering the fact that 2nd instar soldiers are found ubiquitously whereas 1st instar soldiers are restricted to two genera of Cerataphidini, it was pointed out that the former should be of more ancient origin than the latter [2].

Now I can reconstruct the evolution of the two types of soldiers in Cerataphidini on account of the phylogeny. Beside each taxon of the phylogenetic tree, occurrence of the soldiers is indicated (Fig. 8). The 2nd instar soldiers are found from a number of genera covering the two major monophyletic groups and "*Astegopteryx*" *vandermeermohri*. Thus, it is concluded that the 2nd instar soldier evolved in the common ancestor of the present Cerataphidini aphids [Fig. 8(F)]. In contrast, the 1st instar soldier occurs only in *Pseudoregma* and *Ceratovacuna*. This suggests that it evolved after the two major monophyletic groups had diverged, probably in the common ancestor of the two genera [Fig. 8(G)].

Why did the soldier on the primary host evolve so early and be conserved among many taxa whereas soldier on the secondary host did later in a restricted group? As discussed in former sections (V, VI), the common ancestor of Cerataphidini is estimated to have formed single cavity galls on the primary host plant *Styrax*. Once successfully formed, gall provides the aphids with plenty of food, refuge from predators and stable environment, which will enable the insects to reproduce efficiently. It is no doubt that gall formation was a very adaptive character for the ancestral aphid. On the other hand, aphid gall, which is a large biomass composed of insects and soft plant tissues, is extremely attractive food resource for predators. In addition, since gall is large and conspicuous, predators can easily find it. Once it is broken by intruders, it is impossible to repair or recover it. I suppose that these factors may have facilitated an early evolution of 2nd instar soldier in Cerataphidini to defend precious gall. It is also notable that in Cerataphidini a single fundatrix founds a gall, at least in several cases [4, 43, 45, 46, 64]. Relatedness among the colony members in a gall will be extremely high as long as gall foundation by multiple females and/or intercolony migration do not occur frequently [28]. This factor may also have facilitated the evolution of the altruistic sterile caste by way of kin selection [74]. In contrast, circumstances of the secondary host generation are quite different. The secondary host plants of Cerataphidini are chiefly bamboos or grasses which are in general far more abundant than the primary hosts *Styrax* trees. Since alate emigrants from a gall disperse to many secondary hosts, colonies on the secondary host are far larger in number than those on the primary host.

Also, since on the secondary host they form open colonies of relatively small population size, the colonies are less conspicuous for predators but easily destroyed once detected by them. Under these conditions, it seems that the benefit of defending colony by soldier production at the expense of intrinsic rate of natural increase is much lower on the secondary host than on the primary host, which may explain the restricted occurrence of the soldiers on the secondary host.

IX. *Treatment of problematic taxa, "Astegopteryx" vandermeermohri, "Cerataphis" bambusifoliae, and Aleurodaphis*

In this study, I demonstrated that Cerataphidini is composed of two major monophyletic groups, one is characterized by harboring yeast symbionts and the other by forming multiple cavity galls. However, "*Astegopteryx*" *vandermeermohri* belongs to neither group because it keeps both characters plesiomorphic; it forms single cavity gall and harbors intracellular symbionts (Table 2). Figure 10 shows three possible phylogenetic position of this species. In case of Fig. 10(A) it belongs to the former monophyletic group while in case of Fig. 10(B) it does to the latter. If Fig. 10(C) is the case, it constitutes an outgroup against all the other Cerataphidini species. In all the three cases, a new genus should be erected to accept this species although its secondary host generation has not yet been examined.

"*Cerataphis*" *bambusifoliae* is the most problematic species in this study. Its morphology strongly suggests that it should belong to the genus *Cerataphis*, thus to the monophyletic group with yeast symbionts. However, it harbors not yeast symbionts but intracellular symbionts (see section II). Regarding the cellular level character state of symbionts as more important, I suggest that this species is placed in the clade with multiple cavity galls and intracellular symbionts, though its gall has been unknown.

So far, it has been controversial whether the genus *Aleurodaphis* really belongs to Cerataphidini because it possesses peculiar morphological features among Cerataphidini genera [9]. Cerataphidini aphids have the following characteristics in common: 1) They form galls on the primary host *Styrax*. 2) They produce 2nd instar soldiers on the primary host. 3) Their secondary hosts are monocots, most of which are Gramineae (with several exceptions). 4) They have sharp horns on the head (with several exceptions). These characters are, however, lacking or unknown

in *Aleurodaphis* species. Aoki and Usuba [9] suggested that "*Astegopteryx*" *takenouchii* is the primary host generation of an *Aleurodaphis* species based on the morphology of its nymphs. If this were true, it would be justified that *Aleurodaphis* is included in Cerataphidini. However, recent discovery of its secondary host generation [47] and examination of its symbionts in this study demonstrated that "*Astegopteryx*" *takenouchii* belongs to *Tuberaphis*. At present, it is uncertain whether *Aleurodaphis* is a member of Cerataphidini, and therefore excluded it from the phylogenetic analysis (see Fig. 8).

The phylogenetic analysis in this study, as seen in Fig. 8, still contains a number of uncertainties. There are several problematic taxa as mentioned above. Phylogenetic relationships among the genera within the major monophyletic groups are to be further analyzed. I admit that the present character argumentation rests on several assumptions that should be verified using different approaches. In order to settle these problems and to understand host–symbiont coevolution better, molecular phylogenetic analyses of both the symbionts and hosts in Cerataphidini are now in progress.

X. *On the origin of yeast–like symbiont*

As discussed in former sections (III, IV), a kind of yeast–like microorganism is considered to have taken over the original bacterial intracellular symbionts in the common ancestor of Cerataphidini aphids that now house yeasts. This scenario raises a lot of questions: What kind of fungus was it? How did it infect the insect and take over the original symbiont? Did the yeast replace the original symbiont within one or several generations, or did they coexist for a considerable period? Why is there no species with both yeast and bacterial symbiont now? Although there is little evidence to answer these questions, it may be useful to speculate possible scenarios and develop hypotheses.

The extracellular symbionts of Cerataphidini look like budding yeasts. However, it is difficult to specify fungi related to them because budding unicellular fungi are known from various groups of Eumycetes [50]. One way to investigate this will be to determine the sequences of 18S rRNA of the yeast symbionts to construct molecular phylogeny.

One possible explanation for the origin of the yeast symbiont is that it was derived from a

parasitic or pathogenic fungus. In various insect groups, fungi have been reported to be one of the most common parasites. It is conceivable that in an ancestral Cerataphidini species a parasitic fungus lost its harmful effects on the host and became a symbiont, just as has been supposed by many researchers to be plausible as the explanation of the origin of endosymbiotic associations in general [21, 40].

Interestingly, Kolb [42] reported occasional occurrence of a parasitic fungus in *Pseudoregma panicola*. In the specimens of this species from Java, she found yeast-like microbes in addition to the typical intracellular symbionts. They were localized in hemolymph and fat body, but not in embryos. The superfluous yeasts were apparently not symbiotic but parasitic since they were found from only a part of the individuals she examined and systematic ovarial transmission of them was not observed. In fact, I failed to discover such yeasts although I examined many specimens of *Pseudoregma panicola* collected in Japan [24]. However, it is conceivable that such a parasitic fungus might have been the original form of the yeast symbionts found among present Cerataphidini aphids.

In spite of my extensive studies on the symbiotic system of Cerataphidini, there was no species that harbored both the intracellular and extracellular symbionts. This suggests that the newcomer yeasts soon or later took over the original intracellular symbionts. It seems that there are several possible factors that facilitated the symbiont replacement. A candidate is the difference in transmission efficiency of the symbionts. The symbionts of aphids are passed from one to the next generation by ovarial transmission in which embryos at a very early stage are infected by the symbionts of a small number [15]. Therefore, if the yeast symbionts were more efficient in the transmission than the intracellular ones, the former would replace the latter as the host's generation went on. Since this process is of exponential nature, the replacement might have completed within a small number of generations. The hypothetical difference in efficiency is, though circumstantial, supported by the following anatomical observations: Since intracellular symbionts are confined in the cytoplasm of mycetocyte, they have to go out of the cell into hemocoel to infect embryos. In contrast, yeast symbionts are always located in the hemocoel, thus more accessible to the embryos to be infected. Even if there is no difference in the mean efficiency, random drift may result in fixation into either type. The other candidates are direct interactions between the two types of symbionts. The two symbionts have to compete for resource and energy in the same host insect. If one can utilize them better, it will overwhelm or

expel the other. Usually microorganisms produce antibiotic substances. Actually, it has been suggested that symbiotic yeasts of planthoppers, *Laodelphax striatellus* and *Nilaparuata lugens*, produce antibiotics that prevent their host from infection by pathogens [23]. Likewise, the yeast symbionts might have chemically expelled the intracellular ones.

XI. *On the function of yeast-like symbiont*

At present, there is no direct information on the function of the yeast-like extracellular symbionts of Cerataphidini aphids. However, it should be noted that all the Aphididae species ever examined harbor symbiotic microorganisms without exception; most of them are with intracellular prokaryotes and only some Cerataphidini species are with extracellular yeasts. When the intracellular symbionts are destroyed, the host aphids show retarded growth and become sterile [38, 59]. It has been demonstrated that the intracellular symbionts work as an important component in the pathway of amino acid metabolism [19, 60, 61, 62, 63]. These facts indicate that symbiotic microorganisms are essential for aphids in general. In some Cerataphidini aphids, since they are free from the intracellular symbionts, it is quite likely that extracellular yeasts is also essential for their host aphid by making, for example, nutritional contributions to the host. To prove this, however, physiological studies on the yeast symbionts and their hosts will be necessary. In order to investigate these problems, I am attempting to develop the system of rearing of Cerataphidini aphids on artificial diets.

XII. *Hypotheses on the effects of symbiont-replacement in Cerataphidini: implications for possible evolutionary interaction between host and symbiont*

In an ancestor of Cerataphidini, symbiont-replacement from intracellular bacteria to extracellular yeasts occurred. Considering the aphid symbionts are so essential for the hosts as to be comparable to cell organelles [37], it seems likely that some effects of the symbiont-replacement on the host aphids should be observed in this group. Comparison between the two sister groups one of which is subjected to the symbiont replacement while the other is not could pro-

vide clues to those effects. In other words, the former group provides an experiment data set (species subjected to the symbiont replacement) while the latter provides a control data set (species not subjected to it). When various characters were compared between the two groups, a remarkable difference was found in the type of secondary host plants between the two groups. As for the secondary host, majority of Cerataphidini aphids live on Gramineae (bamboos and grasses) whereas there are minorities which depend upon Zingiberaceae (gingers), Palmae (palms), Loranthaceae (mistletoes), Orchidaceae (orchids) and other several plants. As shown in Table 2, most species with intracellular symbionts live on Gramineae exclusively, but several on Palmae and Zingiberaceae. In contrast, the species with yeast symbionts (underlined in Table 2) infest the exceptional secondary hosts other than Gramineae (also underlined in the Table) with only one exception of *Glyphinaphis bambusae*.

To confirm this remarkable correlation between symbiont type and secondary host, I summarized all the reports on the secondary host of Cerataphidini aphids in Table 3. It is evident that species with intracellular symbionts live mostly (38/45) on Gramineae while those with yeasts do exclusively (15/16) on various plants other than Gramineae.

Why have the aphids with yeast symbionts found their way into various plants whereas those with intracellular ones have stuck fast to Gramineae? Here I propose hypotheses by which the difference in the host plant range could be explained in relation to the symbiont type.

(1) *Host range radiation hypothesis*

The common ancestor of Cerataphidini utilized Gramineae as secondary host plant. The fact that most of Cerataphidini aphids live on Gramineae can be interpreted accordingly. Its symbiont was prokaryotic intracellular one. Let us imagine what happened in an ancestor of the Cerataphidini aphids which now house yeasts when symbiont-replacement from intracellular bacteria to extracellular yeasts was established in it. Since intracellular symbionts of various aphids have been shown to serve as an important component in the pathway of amino acid metabolism in general [19, 60, 61, 62, 63], it is plausible that the newcomer yeasts took over the function, which is the minimal requirement to be satisfied by it. However, it is more likely that further changes were accompanied with the process. The original symbiont was prokaryote but the new one was eukaryote. Therefore, metabolic properties of the symbiont must have drastical-

ly changed through the process. Then, it is conceivable that available host range of the aphid was broadened owing to changed metabolic properties with the yeast symbiont. The exceptional secondary host plants utilized by the species with yeasts, mistletoes, pothos, etc., seem to be free of other aphids in general. The ability to utilize these "empty" plants allowed the yeast-harboring ancestor to radiate into various ecological niches and promoted speciation, which have resulted in the present characteristic host range of the Cerataphidini aphids with yeasts.

Based on this hypothesis, the difference of utilized secondary host plants between the two sister monophyletic groups is successfully explained in relation to the symbiont-replacement and the physiological function of the symbiont. However, there is no direct evidence that aphid symbionts are involved in the availability or preference of the host plant. It is also unclear whether yeast symbionts are so superior to intracellular ones as to enable the host aphid to utilize new plants it could not feed on before.

(2) *Radiation on Gramineae hypothesis*

The secondary host plant which the common ancestor of Cerataphidini utilized was not Gramineae. The two lines with intracellular symbionts and with yeast-like symbionts diverged, and speciation has progressed in each line, radiating to new host plants. In the process, a line with intracellular symbionts, which had acquired Gramineae as secondary host, succeeded for some reason. This line succeeded and flourished, having experienced frequent speciation so as to occupy more than a half of the present Cerataphidini species.

In this hypothesis, available host range has no correlation to the symbiont type. Gramineae is apomorphic secondary host. This hypothesis assumes that the line utilizing Gramineae have experienced frequent speciation, but why? One possibility is that multiple cavity gall which had evolved in this line was evolutionarily so favorable that radiation into new niches and speciation were facilitated. In fact, multiple cavity galls seem better than single cavity ones in that since the subgalls are separated and each of them is functionally independent of the others, the multiple cavity galls is resistant to partial destruction and intrusion by predators.

I admit that these hypotheses rest on a number of assumptions and speculations and thus should be further verified. To test which of these ideas is more appropriate, I can employ physiological and molecular phylogenetic approaches, although evidences will be indirect and circumstantial.

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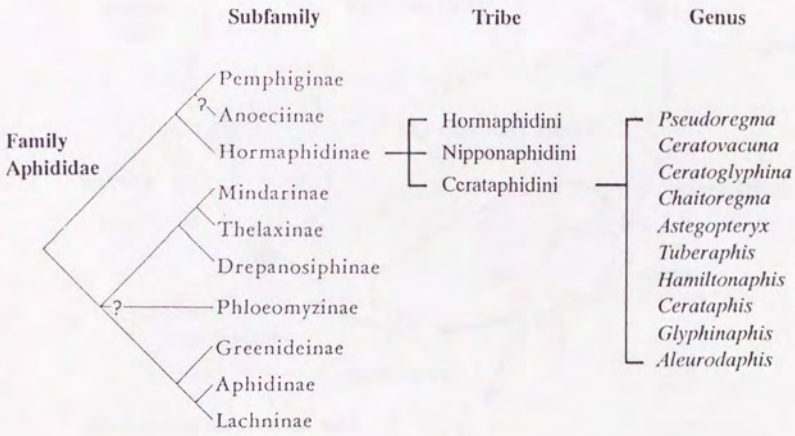


Fig. 1. Phylogeny of Aphididae and position of Cerataphidini.

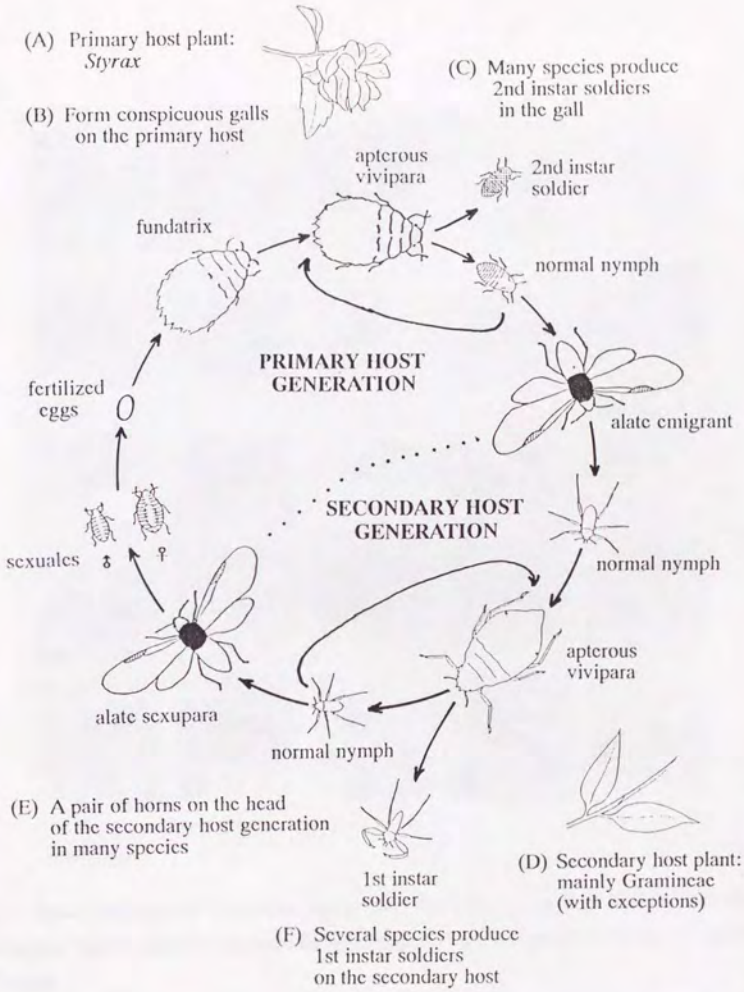


Fig. 2. Life cycle of Cerataphidini aphids. (A)–(F) indicate the important characters that are characteristic of Cerataphidini.

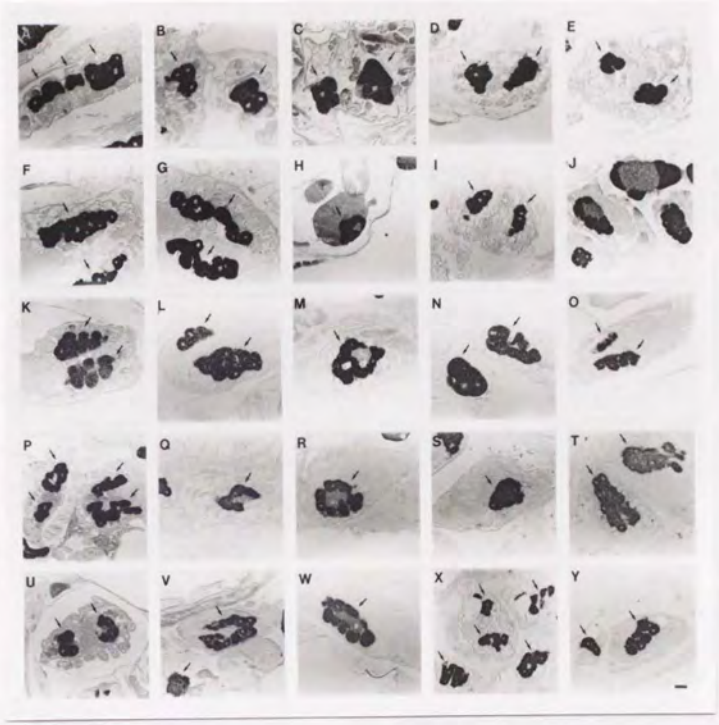


Fig. 3. Tissue sections of Cerataphidini aphids with prokaryotic intracellular symbionts in mycetocytes. Arrows indicate intracellular symbionts stained immunohistochemically. Bar represents 25μm.

A, *Pseudoregma alexanderi*; B, *P. koshunensis*; C, *P. bambucicola*; D, *P. panicola*; E, *P. nicolaiae*; F, *P. sundanica*; G, *Ceratovacuna lanigera*; H, *C. nekoashi*; I, *C. longifila*; J, *Ceratoglyphina styracicola*; K, *Chaitoregma tattakana*; L, *Astegopteryx bambucifoliae*; M, *A. unimaculata*; N, *A. singaporensis*; O, *A. muiri*; P, *A. rappardi*; Q, *A. minuta*; R, *A. basalis*; S, *A. rhapidis*; T, "*Astegopteryx*" *vandermeermohri*; U, "*Cerataphis*" *bambusifoliae*; V, *Aleurodaphis blumeae*; W, *Al.* sp.; X, *Hormaphis betulae*; Y, *Nipponaphis distyliicola*.

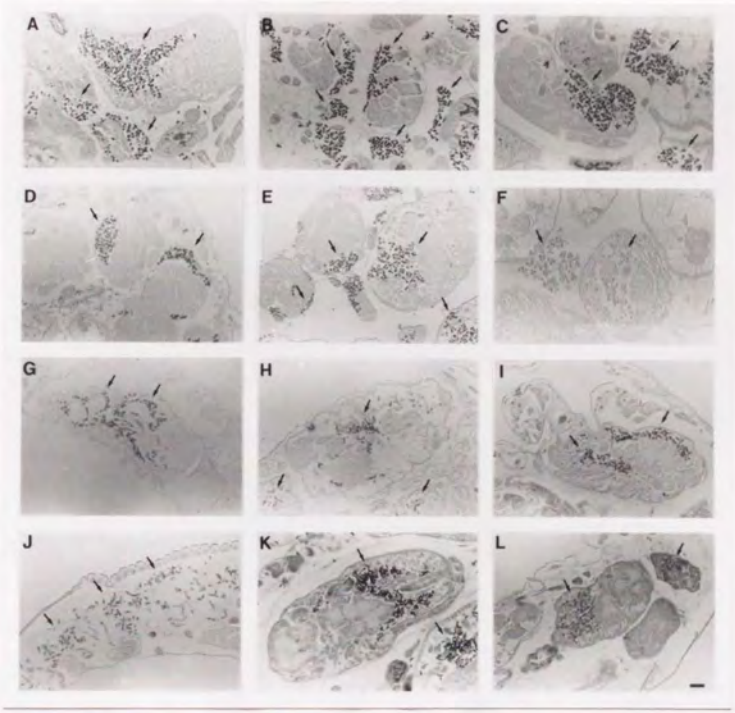


Fig. 4. Tissue sections of Cerataphidini aphids with yeast-like extracellular symbionts. Arrows indicate yeast-like symbionts visualized by PAS staining. Bar represents 25μm.

A, *Tuberaphis coreana*; B, *T. taiwana*; C. *T. loranthi*; D, *T. takenouchii*; E, "*Astegopteryx*" *leeuweni*; F, "*A.*" *sumatrana*; G, *Hamiltonaphis styraci*; H, *Cerataphis fransseni*; I, *C. lataniae*; J, *C. freycinetiae*; K, *C. pothophila*; L, *Glyphinaphis bambusae*.

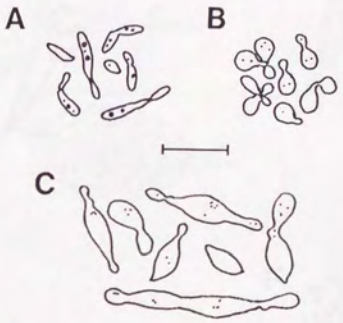


Fig. 5. Three types of yeast-like symbionts found in Cerataphidini. A, slender type (*Hamiltonaphis styraci*); B, roundish type (*Tuberaphis sumatrana*); C, large irregular type (*Glyphinaphis bambusae*). Bar represents 20μm.

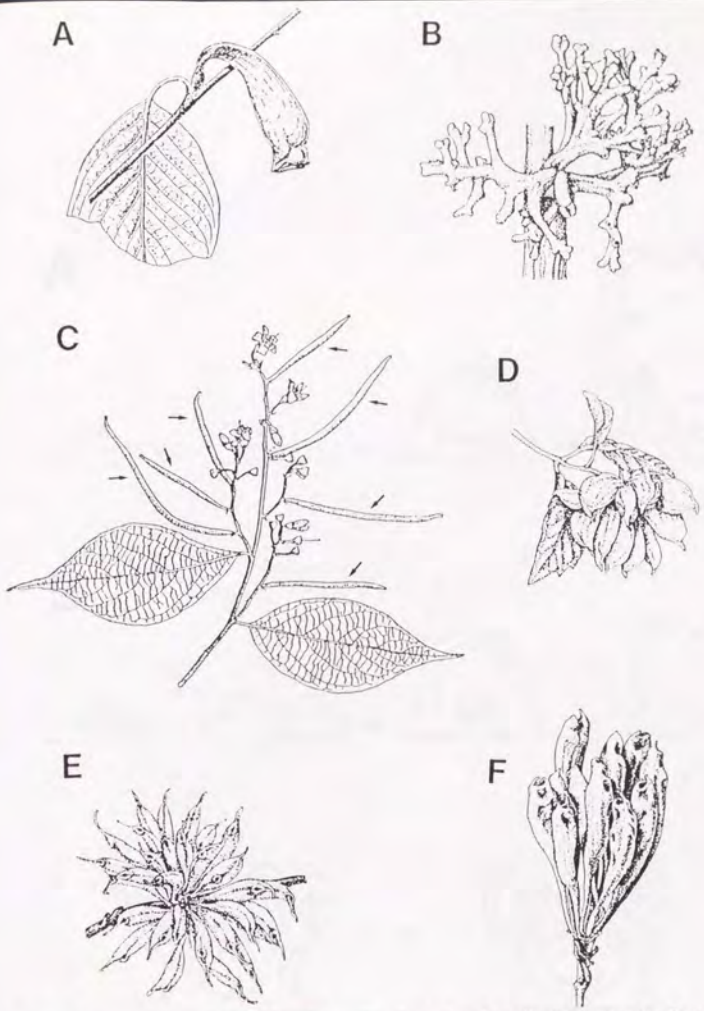


Fig. 6. Various types of Cerataphidini galls. A, A simple gall of *Cerataphis fransseni* on *Styrax benzoin*, from [70]; B, a coral-like gall of *Tuberaphis taiwana* on *Styrax formosana*, from [8]; C, *Tuberaphis* (= "*Astegopteryx*") *leeuweni* forms beanpod-like galls (arrows) on peduncles of *Styrax* sp., from [18]; D, a banana-bundle gall of *Ceratovacuna nekoashi* on *Styrax japonica*, from [43]; E, a flower-like gall of *Astegopteryx bambucifoliae* on *Styrax superifolia*, from [46]; F, a gall of *Pseudoregma bambucicola* on *Styrax superifolia*, from [7]. A–C are single cavity galls while D–F are multiple cavity ones.

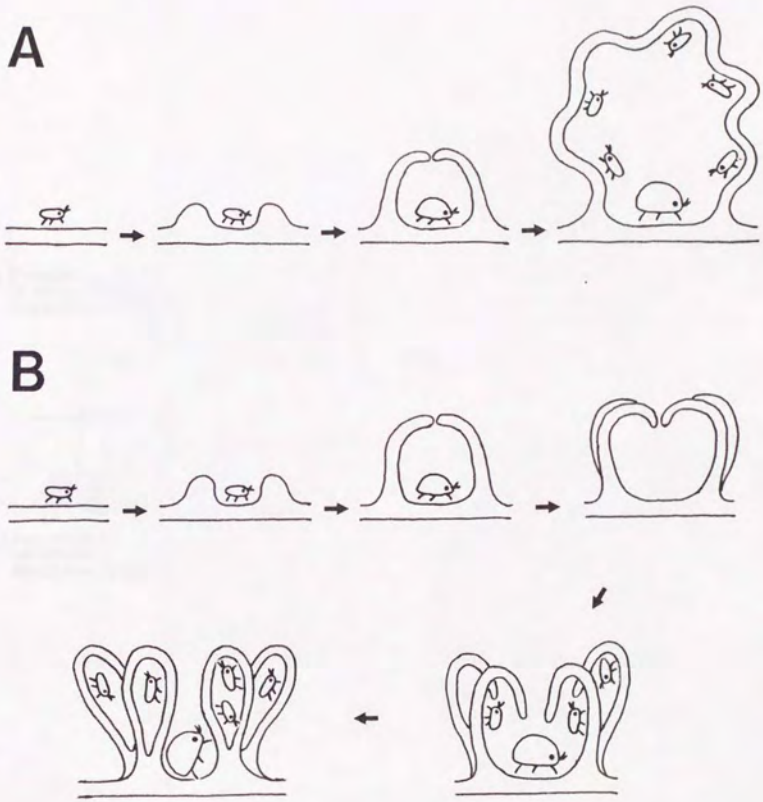


Fig. 7. Formation process of (A) single cavity gall and (B) multiple cavity gall of Cerataphidini.

Fig. 8. Phylogenetic relat
and the basic structure of

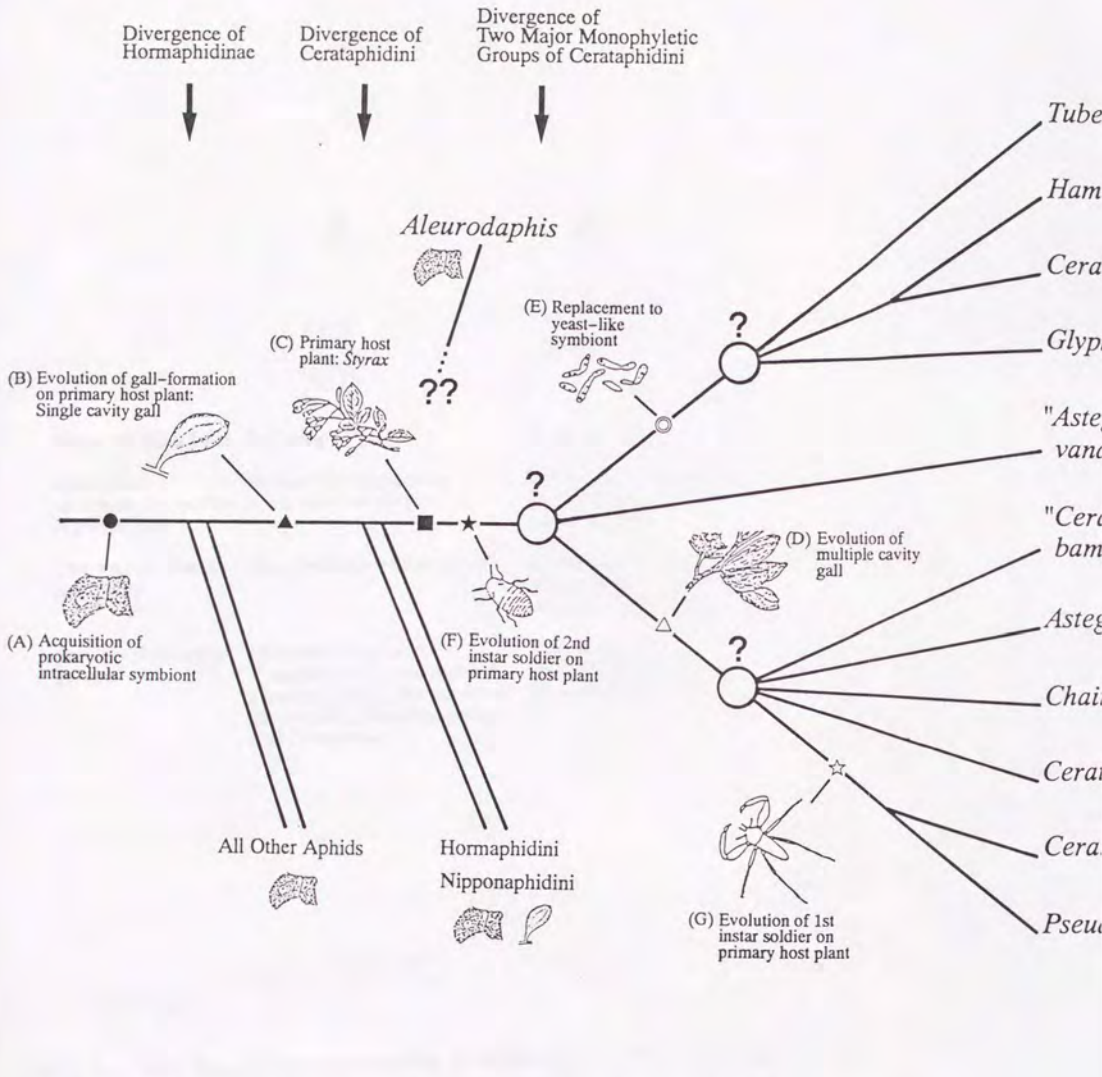
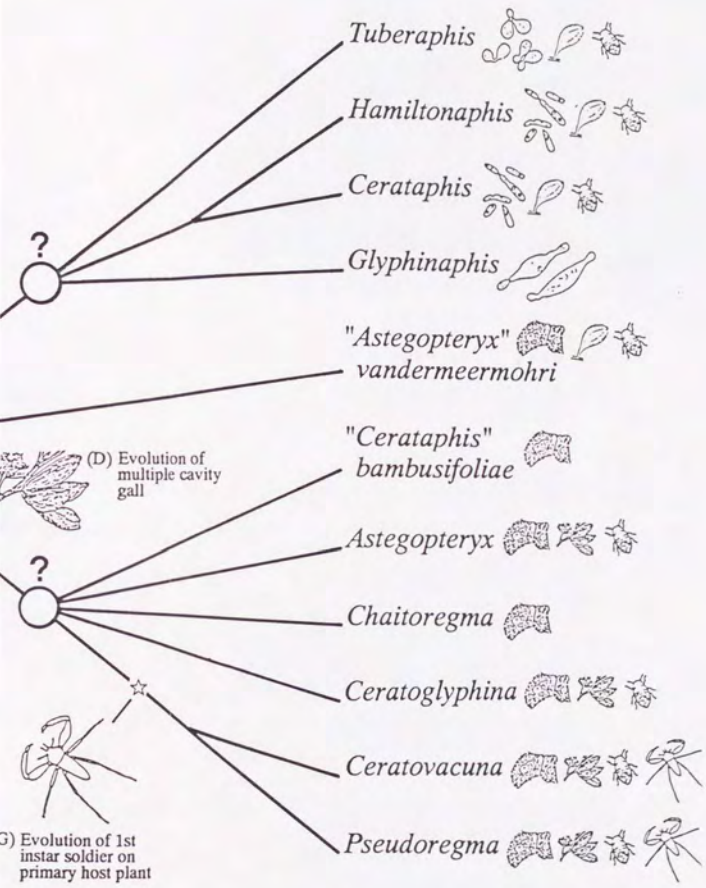


Fig. 8. Phylogenetic relationship among Cerataphidini aphids based on the types of symbionts and the basic structure of galls.



of symbionts

	A	B
		
Instar of the soldier	2nd instar	1st instar
Host plant on which the soldier is produced	Primary host generation in the galls on *Styrax*	Secondary host generation on Gramineae or Zingiberaceae
The way to attack predators	Sting predator with stylet	Clutch predator with enlarged fore legs and pierce its body with horns
Soldier-producing genera	More than 7 genera[a)] *Pseudoregma*, *Ceratovacuna*, *Ceratoglyphina*, *Astegopteryx*, *Tuberaphis*, *Hamiltonaphis*, and *Cerataphis*	Only 2 genera *Pseudoregma* and *Ceratovacuna*

Fig. 9. Two types of soldiers of Cerataphidini. A, soldiers produced on the primary hosts; B, soldiers produced on the secondary hosts.

a) "*Astegopteryx*" *vandermeermohri* also produces 2nd instar soldiers [47].

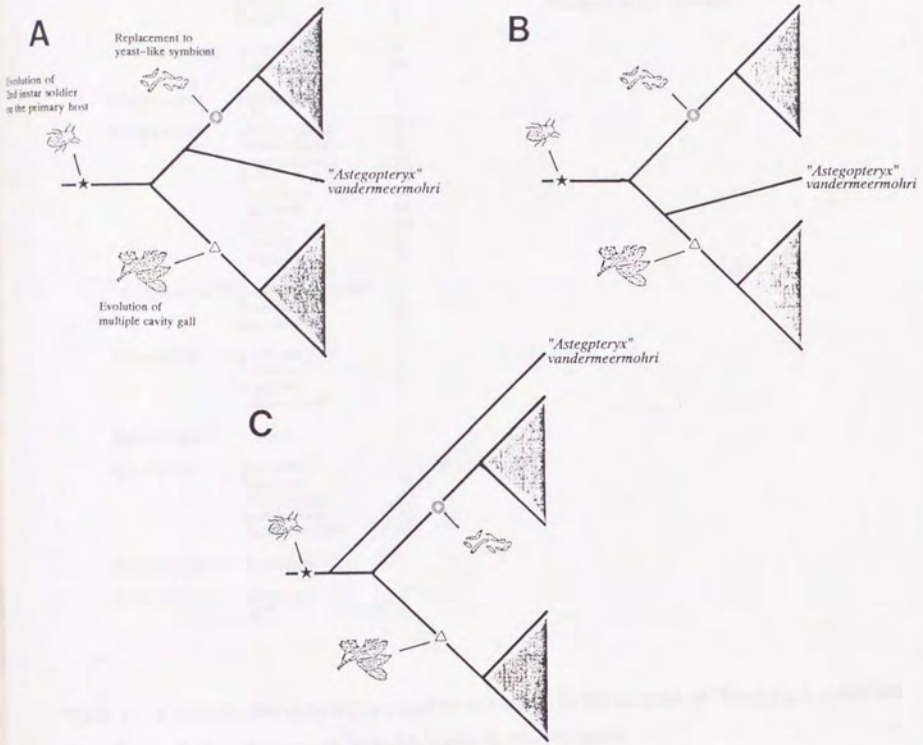


Fig. 10. Three possible phylogenetic relationships among the two large monophyletic groups and "*Astegopteryx*" *vandermeermohri*. See text for A, B and C.

Genus	Species	Origin
(CERATAPHIDINI)		
Pseudoregma	*alexanderi*	T
	koshunensis	J, T
	bambucicola	J
	panicola	J
	nicolaiae	M
	sundanica	M, S
Ceratovacuna	*lanigera*	T, S
	japonica	J
	nekoashi	J
	longifila	T
Ceratoglyphina	*styracicola*[a)]	T
	bambusae	M
Chaitoregma	*tattakana*	T
Astegopteryx	*bambucifoliae*	J, T
	unimaculata	T
	singaporensis	M
	muiri	M
	bambusae	M
	rappardi	M
	minuta	M
	basalis	S
	rhapidis	S
"*Astegopteryx*"[b)]	*vandermeermohri*	S
	leeuweni	S
	sumatrana	S
Tuberaphis[c)]	*coreana*	J
	taiwana	T
	loranthi	S
	takenouchii[d)]	T
Hamiltonaphis[e)]	*styraci*	J
Cerataphis	*fransseni*[f)]	J, T, M
	lataniae	M
	freycinetiae	M
	pothophila	M
	bambusifoliae	T
Glyphinaphis	*bambusae*	T
Aleurodaphis	*blumeae*	J
	sp.[g)]	J

Genus	Species	Origin
(HORMAPHIDINI)		
Hormaphis	*betulae*	J
Hamamelistes	*miyabei*	J
(NIPPONAPHIDINI)		
Nipponaphis	*distyliicola*	J
Neothoracaphis	*yanonis*	J

Table 1. Cerataphidini aphids examined in this study. In the column of "Origin", J, collected from Japan; T, from Taiwan; M, from Malaysia; S, from Sumatra.

a) This species, previously called "*Astegopteryx*" *styracicola*, was once regarded as the primary host generation of *Ceratovacuna bambusae* [3], but it turned out to be distinct from *C. bambusae* [47]. Here, thus, we call this species *C. styracicola*. b) Species whose secondary host generation has been unknown are, because difficult to classify, placed under "*Astegopteryx*" tentatively. c) *Rappardiella* [58] is a junior synonym of *Tuberaphis*. d) This species, previously called "*Astegopteryx*" *takenouchii*, was transferred to *Aleurodaphis* based on the morphology of 1st instar exules [9]. However, its secondary host generation was found on Loranthaceae [47]. This species undoubtedly belongs to *Tuberaphis*. e) A new genus to accept previous "*Astegopteryx*" *styraci* [11]. f) *C. variabilis* and *C. palmae* turned out to be the secondary host generation of *C. fransseni* [64]. g) Described in Moritsu [55].

Genus[a]	Species[b]	Symbiont[c]	Gall[d]	Primary Host	Secondary[e] Host	2nd instar Soldier	1st instar Soldier
(CERATAPHIDINI)							
Pseudoregma	*alexanderi*	intracellular	----	----	Gramineae	----	present
	koshunensis	intracellular	multiple	*Styrax*	Gramineae	present	present
	bambucicola	intracellular	multiple	*Styrax*	Gramineae	present	present
	panicola	intracellular	----	----	Gramineae	----	present
	nicolaiae	intracellular	----	----	Zingiberaceae	----	----
	sundanica	intracellular	----	----	Zingiberaceae	----	present
Ceratovacuna	*lanigera*	intracellular	----	----	Gramineae	----	----
	japonica	intracellular	multiple	*Styrax*	Gramineae	present	present
	nekoashi	intracellular	multiple	*Styrax*	Gramineae	----	----
	longifila	intracellular	----	----	Gramineae	----	present
Ceratoglyphina	*styracicola*	intracellular	multiple	*Styrax*	Gramineae	present	----
	bambusae	intracellular	----	----	Gramineae	----	----
Chaitoregma	*tattakana*	intracellular	----	----	Gramineae	----	----
Astegopteryx	*bambucifoliae*	intracellular	multiple	*Styrax*	Gramineae	present	----
	unimaculata	intracellular	----	----	Gramineae	----	----
	singaporensis	intracellular	----	----	Gramineae	----	----
	muiri	intracellular	----	----	Zingiberaceae	----	----
	bambusae	intracellular	----	----	Gramineae	----	----
	rappardi	intracellular	----	----	Palmae	----	----
	minuta	intracellular	----	----	Gramineae	----	----
	basalis	intracellular	----	----	Gramineae	----	----
	rhapidis	intracellular	----	----	Palmae	----	----
"Astegopteryx"	*vandermeermohri*	intracellular	single	*Styrax*	----[f]	present	----
	leeuweni	roundish yeast	single	*Styrax*	absent[f]	present	----
	sumatrana	roundish yeast	single	*Styrax*	----	present	----
Tuberaphis	*coreana*	roundish yeast	----	----	Loranthaceae	----	----
	taiwana	roundish yeast	single	*Styrax*	----	present	----
	loranthi	roundish yeast	single	*Styrax*	Loranthaceae	present	----
	takenouchii	roundish yeast	single	*Styrax*	Loranthaceae	present	----
Hamiltonaphis	*styraci*	slender yeast	single	*Styrax*	absent[f]	present	----
Cerataphis	*fransseni*	slender yeast	single	*Styrax*	Palmae	present	----
	lataniae	slender yeast	----	----	Palmae	----	----
	freycinetiae	slender yeast	----	----	Pandanaceae	----	----
	pothophila	slender yeast	----	----	Araceae	----	----
	orchidearum[g]	slender yeast	----	----	Orchidaceae	----	----
	bambusifoliae	intracellular	----	----	Gramineae	----	----
Glyphinaphis	*bambusae*	large yeast	----	----	Gramineae	----	----
Aleurodaphis	*blumeae*	intracellular	----	----	Compositae	----	----
	sp.	intracellular	----	----	Balsaminaceae	----	----
(HORMAPHIDINI)							
Hormaphis	*betulae*	intracellular	single	*Hamamelis*	Betulaceae	----	----
Hamamelistes	*miyabei*	intracellular	single	*Hamamelis*	Betulaceae	----	----
(NIPPONAPHIDINI)							
Nipponaphis	*distyliicola*	intracellular	single	*Distilium*	Fagaceae	----	----
Neothoracaphis	*yanonis*	intracellular	single	*Distilium*	Lauraceae	----	----

Table 2. (Caption on the next page)

Secondary host plants	Species with intracellular symbionts	Species with yeast-like symbionts	Total
Gramineae	38	1	39
Zingiberaceae	3	0	3
Palmae	3	3	6
Pandanaceae	1	1	2
Orchidaceae	0	1	1
Araceae	0	1	1
Loranthaceae	0	7	7
monoecy on the primary host	0	2	2
Total	45	16	61

Table 3. Secondary host plants and symbiont type of Cerataphidini aphids. All the species whose secondary host plant has been described are listed up and counted. The list in my hand contains 69 Cerataphidini species, 8 of which were omitted because their secondary host has been unknown.

Table 2. (Last page) Symbiont type, basic plan of the gall, host plants, and occurrence of soldiers of the Cerataphidini aphids examined.

a) Genera with yeast symbionts are underlined; b) Species with yeast symbionts are underlined; c) Yeast symbionts are underlined; d) Multiple, with multiple cavity gall; single, with single cavity gall. The latter is underlined; e) Secondary host plants other than Gramineae are underlined; f) Monoecy on the primary host plant; g) This species was not examined in this study, but described by Buchner [14].

PERSPECTIVE

In studying the process of the symbiont-replacement occurred in the past, at least the following questions should be answered:

a) When was the yeast symbiont acquired?

b) What kind of microorganism was the ancestor of the yeast symbiont?

c) What kind of organism was the ancestor of the host?

d) What does the yeast symbiont do for the host?

e) What does the host do for the symbiont?

f) What evolutionary effects on the yeast symbiont due to the symbiosis are observed?

g) What evolutionary effects on the host due to the symbiosis are observed?

h) Have any remarkable evolutionary novelties been established in the endosymbiosis?

To my regret, at this stage my understanding on this subject is still insufficient to give satisfactory answers to these questions. Here, therefore, I will represent a overview of attainments of the present studies: What problems have been well understood? What problems are left to be studied further? To answer the unsolved questions, what kind of approach will be necessary?

a) By phylogenetic analyses, it was strongly suggested that replacement to yeast symbiont occurred in the common ancestor of the Cerataphidini aphids which now house yeasts. At present, the definite age of the symbiont-replacement is unclear. However, this problem will be settled by molecular phylogenetic analyses. I am now sequencing mitochondrial cytochrome oxidase I (CoxI) genes of Cerataphidini aphids and chaperonin 60 (Cpn60) genes of their intracellular symbionts in order to construct molecular phylogeny of both the hosts and symbionts. Moran *et al.* (1993) estimated the base substitution rate of 16S rRNA gene of aphid intracellular symbionts as 1-2% per 50 million years. Thus, using this value, I can calculate the substitution rate of CoxI and Cpn60. Once these values become available, by applying them on the molecular phylogeny, the definite age of the symbiont-replacement will be estimated. Similarly, the ages of evolution of other characters can be calculated.

b) The newly acquired symbiont in Cerataphidini is undoubtedly unicellular fungus which just looks like yeasts. Since budding unicellular fungi have been known from various groups of Eumycetes, at present it is difficult to point out fungi related to the symbiont. I am now sequencing 18S rRNA gene fragments of the yeast symbionts of Cerataphidini aphids in order to find out their relatives.

c) The ancestral host in which symbiont-replacement occurred was undoubtedly a Cerataphidini aphid with intracellular symbiont, probably just like the present species.

d) Although it is quite likely that the yeast symbiont provides the host aphid with essential nutrients in place of intracellular symbiont, there is no direct information on the function of the yeast symbiont. To study its nutritional physiological aspects further, experimental manipulation of Cerataphidini aphids with yeasts is necessary. For this purpose we are attempting to rear Cerataphidini aphids on artificial diets. In our preliminary results, it has been successful to maintain *Hamiltonaphis styraci* at least for several generations on an artificial diet designed for the pea aphid. Also, we are trying to culture isolated yeast symbionts *in vitro*.

e) It has been generally believed that the host insect provides the endosymbiont with substances and energy. It will be also applied to Cerataphidini aphids with yeast symbionts. However, it is difficult to verify this idea experimentally though it seems quite likely.

f) Although free-living bacteria closely related to intracellular symbionts of aphids have thick cell wall, the cell wall of the intracellular symbionts is much reduced. In contrast, the yeast symbionts of Cerataphidini aphids have thick cell wall like free-living yeasts. This may be be cause of relatively recent origin of this symbiotic association. Anyway, I should discuss the specializations of the yeast symbiont due to the symbiosis in future when free-living fungi related to the symbiont will be identified.

g)h) The Cerataphidini aphids with yeast symbiont are not essentially different from those with intracellular symbiont. They possess common morphological characters to such an extent that they are reasonably classified to the same tribe Cerataphidini. They all utilize *Styrax*

trees as the primary host plant and form large galls. They always produce 2nd instar soldiers to defend the gall. In short, it is likely that in Cerataphidini symbiont-replacement from bacteria to yeasts did not have drastic effects on the host aphids. Probably, the yeast symbiont simply took over the functions that had been done by former intracellular symbiont.

However, the yeast symbiont is eukaryote while the intracellular one is prokaryote. They must be of quite different nature. Thus, it seems quite unlikely that the yeast completely complemented the works of the intracellular one. The symbiont-replacement might have brought new favorable or unfavorable characters. One candidate of innovation due to the replacement is available range of the secondary host plants. The cerataphidini aphids with intracellular symbiont extensively utilize Gramineae as the secondary host. In contrast, those with yeast symbiont live on various plants. This difference can be explained when it is assumed that the acquisition of yeast symbiont enabled the host aphid to utilize various plants other than Gramineae.

This hypothesis rests on a number of assumptions and speculations. There are two approaches to verify this hypothesis. One is physiological approach. In analyzing the metabolic functions of their symbiont, it might be shown that the type of the symbiont is responsible for the availability of the host plant. Another is comparative approach. Let us assume that occurrences of symbiont-replacement are also discovered in other Homopteran insects. Then, if changes of available host range are always accompanied with the symbiont-replacement processes, we can reasonably deduce that there is a correlation between the replacement and host range.

In this context, I am much interested in the diversity of endosymbiotic system in Homoptera. Homoptera is one of the groups with most highly developed endosymbiotic systems. It has been reported that some Homopteran insects harbor even four or five different types of symbionts in their body. Thus, a number of impressive aspects of endosymbiotic evolution will be revealed by extensive survey of endosymbiotic system in Homoptera. If I can discover several other cases of symbiont-replacement just as that found in Cerataphidini, the hypothesis on the correlation between symbiont-replacement and host plant range would be verified by comparative method.

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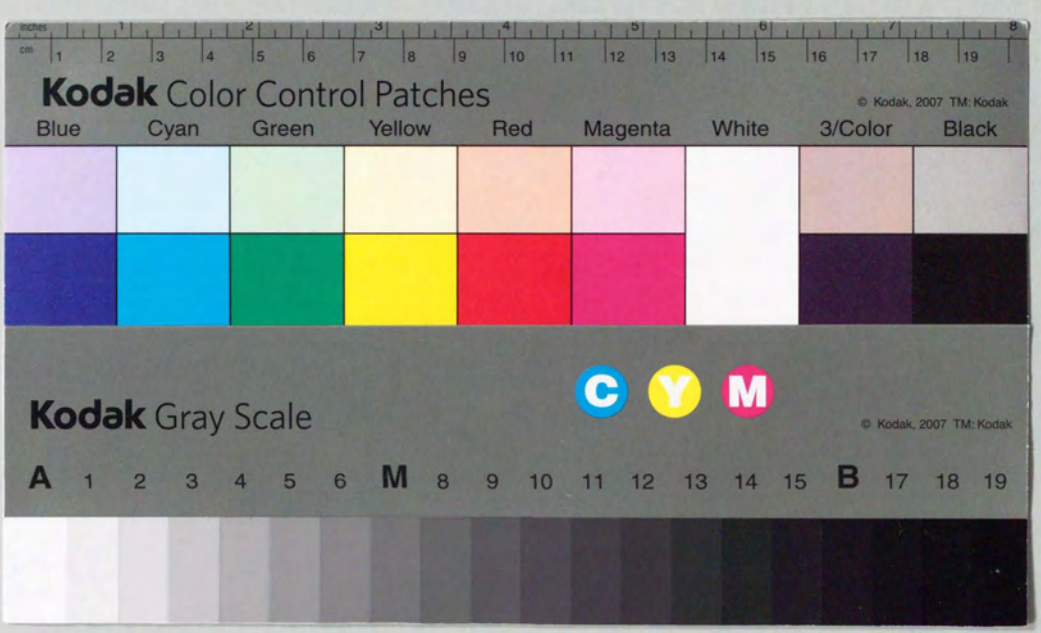
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