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Two Intracellular Symbiotic Bacteria from the Mulberry Psyllid *Anomoneura mori* (Insecta, Homoptera)

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We characterized the intracellular symbiotic bacteria of the mulberry psyllid *Anomoneura mori* by performing a molecular phylogenetic analysis combined with in situ hybridization. In its abdomen, the psyllid has a large, yellow, bilobed mycetome (or bacteriome) which consists of many round uninucleated mycetocytes (or bacteriocytes) enclosing syncytial tissue. The mycetocytes and syncytium harbor specific intracellular bacteria, the X-symbionts and Y-symbionts, respectively. Almost the entire length of the bacterial 16S ribosomal DNA (rDNA) was amplified and cloned from the whole DNA of *A. mori*, and two clones, the A-type and B-type clones, were identified by restriction fragment length polymorphism analysis. In situ hybridization with specific oligonucleotide probes demonstrated that the A-type and B-type 16S rDNAs were derived from the X-symbionts and Y-symbionts, respectively. Molecular phylogenetic analyses of the 16S rDNA sequences showed that these symbionts belong to distinct lineages in the γ subdivision of the *Proteobacteria*. No 16S rDNA sequences in the databases were closely related to the 16S rDNA sequences of the X- and Y-symbionts. However, the sequences that were relatively closely related to them were the sequences of endosymbionts of other insects. The nucleotide compositions of the 16S rDNAs of the X- and Y-symbionts were highly AT biased, and the sequence of the X-symbiont was the most AT-rich bacterial 16S rDNA sequence reported so far.

Many insects have established highly elaborate symbiotic associations with specific microorganisms. At all times these specific microorganisms are harbored in the gut rumen, in caeca connected to the gut, inside specialized gut epithelial cells, in the hemocoel, or inside highly developed symbiotic organs called mycetomes in the body cavity (6). Because the microbes are always found in the host insect and are passed from generation to generation by vertical transmission and because the host usually suffers sterility or death when it is deprived of the microbes, the relationships between insects and their specific microorganisms are thought to be obligate and mutualistic in many cases (4, 12).

The Homoptera, including cicadas, planthoppers, aphids, scale insects, psyllids, etc., is an insect group whose endosymbiotic systems are highly developed (6). Because homopteran insects live on nutritionally unbalanced diets consisting of plant sap throughout their lives, it is believed that they need the help of endosymbiotic microorganisms to compensate for nutritional deficiencies. In fact, it has been demonstrated that endosymbiotic microbes of homopterans are involved in metabolic processes, such as the synthesis of essential nutrients and recycling of nitrogenous wastes (5, 11, 12, 32). The endosymbiotic microorganisms have not been cultured in common media, probably because they are highly adapted to special environments inside the host organisms and cannot live outside the hosts (4). Since conventional microbiological methods have been based on isolation of microorganisms, the biological nature of the endosymbionts has been unclear for a long time.

However, recent innovations in molecular phylogenetic techniques have revealed the systematic affinities of fastidious endosymbionts of members of the Homoptera (9, 17, 26–28, 34). In general, microbial DNA fragments, putatively derived from the symbionts, have been amplified by PCR and sequenced from the total DNA of the host insects. Such an approach is, however, often complicated by the diversity and complexity of the endosymbiotic microbiota. When a microbial species is the major symbiont in an insect body, this approach works quite well. However, multiple microbial species commonly coexist in an insect body not only in members of the Homoptera but also in members of many other insect groups (6, 16, 18, 19). Practically, we frequently encounter situations in which 16S ribosomal DNA (rDNA) fragments amplified and cloned from whole insect DNA contain a number of different sequences, which might come from multiple endosymbionts, gut microbes, pathogens, occasional contaminating bacteria, or debris adhering to insect surfaces. In addition, possible biases inherent in PCR amplification and DNA cloning may sometimes result in serious artifacts. Therefore, the microbial DNA sequences obtained must be interpreted in connection with morphological data obtained by using, for example, in situ hybridization with specifically designed probes (3).

In the Homoptera, psyllids (Psylloidea) constitute the well-defined group Sternorrhyncha together with aphids (Aphidoidea), scale insects (Coccoidea), and whiteflies (Aleyrodoidea). Only a few histological descriptions are available for the endosymbionts of psyllids. According to previous reports, psyllids have a large, yellow, bilobed mycetome in its abdomen. The mycetome is a complex of the following three types of cells: many round uninucleated mycetocytes, a syncytial tissue surrounded by these cells, and an envelope composed of many flattened cells encasing the whole mycetome. The cytoplasm of the mycetocytes is full of a specific bacterium, called the X-symbiont. The syncytial cytoplasm is also filled with another type of bacterium, called the Y-symbiont (6, 8, 30, 36). Although molecular phylogenetic studies of the intracellular symbiotic bacteria of aphids, scale insects, and whiteflies have been performed (9, 26, 27), no such study has been conducted on the endosymbiotic bacteria of psyllids.

In this study, we characterized the intracellular symbiotic

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bacteria of the mulberry psyllid *Anomoneura mori* by using a molecular phylogenetic approach combined with an in situ hybridization technique.

MATERIALS AND METHODS

Materials. Nymphs of *A. mori*, which had formed colonies covered with plenty of wax, were collected from the undersides of mulberry leaves at Tsukuba, Ibaraki, Japan, in May 1997 and were preserved in acetone.

DNA extraction, PCR, and cloning of 16S rDNA. The insects were repeatedly washed with acetone to remove wax and possible contamination. After the insects were placed on clean tissue paper for a while to remove the preservative, they were subjected to DNA extraction with a QIAamp tissue kit (QIAGEN). From the whole-insect DNA, almost the entire length of bacterial 16S rDNA (about 1.5 kb) was amplified by PCR by using primers 16SA1 (5′-AGAGTTT GATCMTGGCTCAG-3′) and 16SB1 (5′-TACGGYTACCTTGTTACGACTT-3′) and the following temperature profile: 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 70°C for 2 min. The PCR product was purified with a Geneclean II kit (Bio 101, Inc.) and was cloned with TA cloning vector pT7Blue (Novagen) and *Escherichia coli* JM109 competent cells (Takara) by using ampicillin and the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) blue-white selection system.

Typing of cloned 16S rDNA by restriction fragment length polymorphism (RFLP). White colonies that were expected to contain the inserted plasmid were directly subjected to PCR by using primers for the flanking region of the vector's cloning site (primers U-19 [5′-GTTTTCCCAGTCACGACGT-3′] and BT7 [5′-TAATACGACTCACTATAGGG-3′]) in order to check the length of the inserted DNA fragment. If the PCR product was the expected size (about 1.5 kb), it was digested with four base-recognizing restriction endonucleases (*Hin*fI, *Rsa*I, *Sau*3AI, and *Taq*I) and electrophoresed in agarose gels in order to type the cloned 16S rDNA.

DNA sequencing. The white colonies that were determined to contain a 16S rDNA clone were isolated and cultured in 1.5 ml of Luria-Bertani medium supplemented with ampicillin overnight and subjected to plasmid extraction with a QIAprep-Spin miniprep kit (QIAGEN). The purified plasmids, which were eluted with 30 μl of TE buffer, were used as the template DNA for sequencing. A dye terminator-labelled cycle sequencing reaction was performed with a type FS DNA sequencing kit (Perkin-Elmer) and sequencing primers 16SA1, 16SA2 (5′-GTGCCAGCAGCCGCGGTAATAC-3′), 16SA3 (5′-TGCATGGYTGTCG TCAGCTCG-3′), 16SB1, 16SB2 (5′-CGAGCTGACGACARCCATGCA-3′), and 16SB3 (5′-GTATTACCGCGGCTGCTGGCAC-3′) by using the following temperature profile: 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 70°C for 2 min. The reaction products were analyzed with a model 377 ABI PRISM DNA sequencer (Perkin-Elmer).

Database search. A search for homology with the 16S rDNAs described previously was performed by using Ribosomal Database Project (RDP) databases (24). To check the specificity of the probes for in situ hybridization, the SSU Una1 database in the RDP was searched to find sequences that were identical to the probe sequences. The sequence data used for molecular phylogenetic analyses were retrieved from GenBank.

Molecular phylogenetic analysis. Multiple alignment of 16S rDNA sequences was performed by the methods of Feng and Doolittle (15) and Gotoh (20) with a computer. The final alignment was inspected and corrected manually. Ambiguously aligned regions were excluded from the phylogenetic analysis. Nucleotide sites that included an alignment gap(s) were also omitted from the aligned data set. A neighbor-joining tree (31) was constructed with Kimura's two-parameter distance (23) by using the program package Clustal W (33). A bootstrap test (14) was performed with 1,000 resamplings. A maximum-likelihood tree (13) was constructed by using the program package fastDNAML (29). In heuristic searches for an optimal tree with the best log-likelihood score, we adopted jumbled orders for taxon addition, used empirical base frequencies, and repeated independent searches at least three times. A bootstrap test for the maximum-likelihood tree was performed by the approximate method, local bootstrap probability, with the program package MORPHY, version 2.3 (1).

Histology. Histological preparation, in situ hybridization, and enzymatic probe detection were performed essentially as described by Fukatsu et al. (19). The insects preserved in acetone were transferred to alcoholic formalin (ethanol-formalin, 3:1), dissected to obtain their abdomens, and kept in the fixative overnight. Then the abdomens were dehydrated and cleared through an ethanol-xylene series and embedded in paraffin. Serial tissue sections (thickness, 5 μm) were cut with a rotary microtome and were mounted on silane-coated glass slides. The sections were dewaxed with a xylene-ethanol series and air dried prior to in situ hybridization.

In situ hybridization. The oligonucleotide probes DIG-Kiji16SA (5′-digoxigenin-GCTGCCTTCCTTGAAAGT-3′) and DIG-Kiji16SB (5′-digoxigenin-GCTGCCTCCCATAGGAGT-3′) were used in this study (see Fig. 2). About 150 μl of hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) containing 50 pmol of probe per ml was applied to the tissue section, which was covered with a coverslip and incubated in a humidified chamber at room temperature overnight. To eliminate nonspecific binding of the probe, the tissue section was washed in washing buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 40% formamide) for 10 min at 42°C. Under these wash conditions, even a single-base mismatch results in dissociation of the probes. After the tissue section was washed with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), it was subjected to detection of bound probe. To confirm the specificity of hybridization, the following control experiments were conducted: no-probe control, RNase digestion control, and competitive suppression control containing excess unlabelled probe (19). Control experiments were also performed with a widely used general eubacterial 16S rRNA probe, digoxigenin-labelled EUB338 (2, 19).

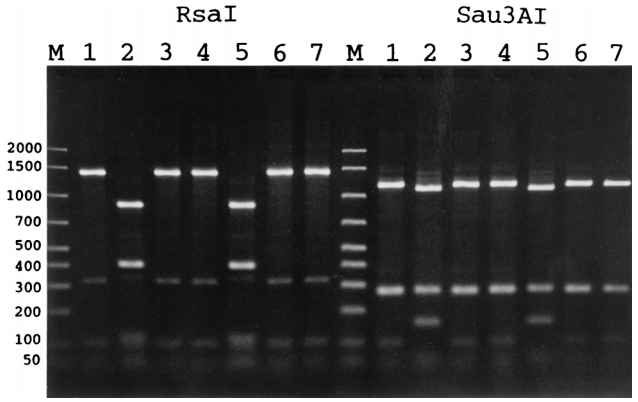

FIG. 1. RFLP analysis of bacterial 16S rDNAs amplified and cloned from total DNA of *A. mori*. Lanes 1 through 7 contained cloned 16S rDNA fragments digested by *Rsa*I (left) or *Sau*3AI (right) and resolved on a 2.5% agarose gel. Lanes 1, 3, 4, 6, and 7 contained A-type clones, whereas lanes 2 and 5 contained B-type clones. Lanes M contained DNA size markers, whose sizes (in base pairs) are indicated on the left. RFLP profiles of *Hin*fI and *Taq*I digests also agreed with the typing data (data not shown).

Detection of digoxigenin-labelled probe. The probe was detected by using a DIG nucleic acid detection kit (Boehringer Mannheim) essentially as recommended by the manufacturer. Each tissue section was washed with buffer 1 (0.1 M maleic acid–NaOH [pH 7.5], 0.15 M NaCl) and incubated with buffer 2 (blocking solution) for 30 min. An anti-DIG-AP conjugate solution (150 mU of anti-digoxigenin–alkaline phosphatase conjugate per ml in buffer 2) was applied to the slide, which was then incubated overnight. After the preparation was washed with buffer 3 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$), a nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate solution was added to the slide to stain the bound digoxigenin-labelled probe deep blue. The tissue section was washed well with distilled water, mounted in glycerol, and observed with a differential interference microscope.

Nucleotide sequence accession numbers. The 16S rDNA sequences of the X- and Y-symbionts of *A. mori* reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB013086 and AB013087, respectively.

RESULTS

General observations on the endosymbiotic system. A large part of the *A. mori* abdomen was occupied by a large, orange, bilobed mycetome which was easily recognized under a dissecting microscope. When examined histologically, the mycetome was found to be composed of a number of round mycetocytes and a syncytial cytoplasm surrounded by them. These observations are in agreement with a previous report (36).

Identification of two types of 16S rDNA. Almost the entire length of 16S rDNA was successfully amplified from the whole-insect DNA by PCR. Since it was expected that more than one sequence, derived from different microorganisms, would be in the product, the amplified DNA fragments were subjected to cloning. RFLP analysis of the cloned fragments revealed two sequences, tentatively designated A-type and B-type. The A-type clones were obtained more frequently than the B-type clones (Fig. 1).

The nucleotide sequences of the A-type and B-type clones were determined (Fig. 2). Two clones of each type were subjected to sequencing, and the sequences obtained were identical. The lengths, without the regions of amplifying primers,

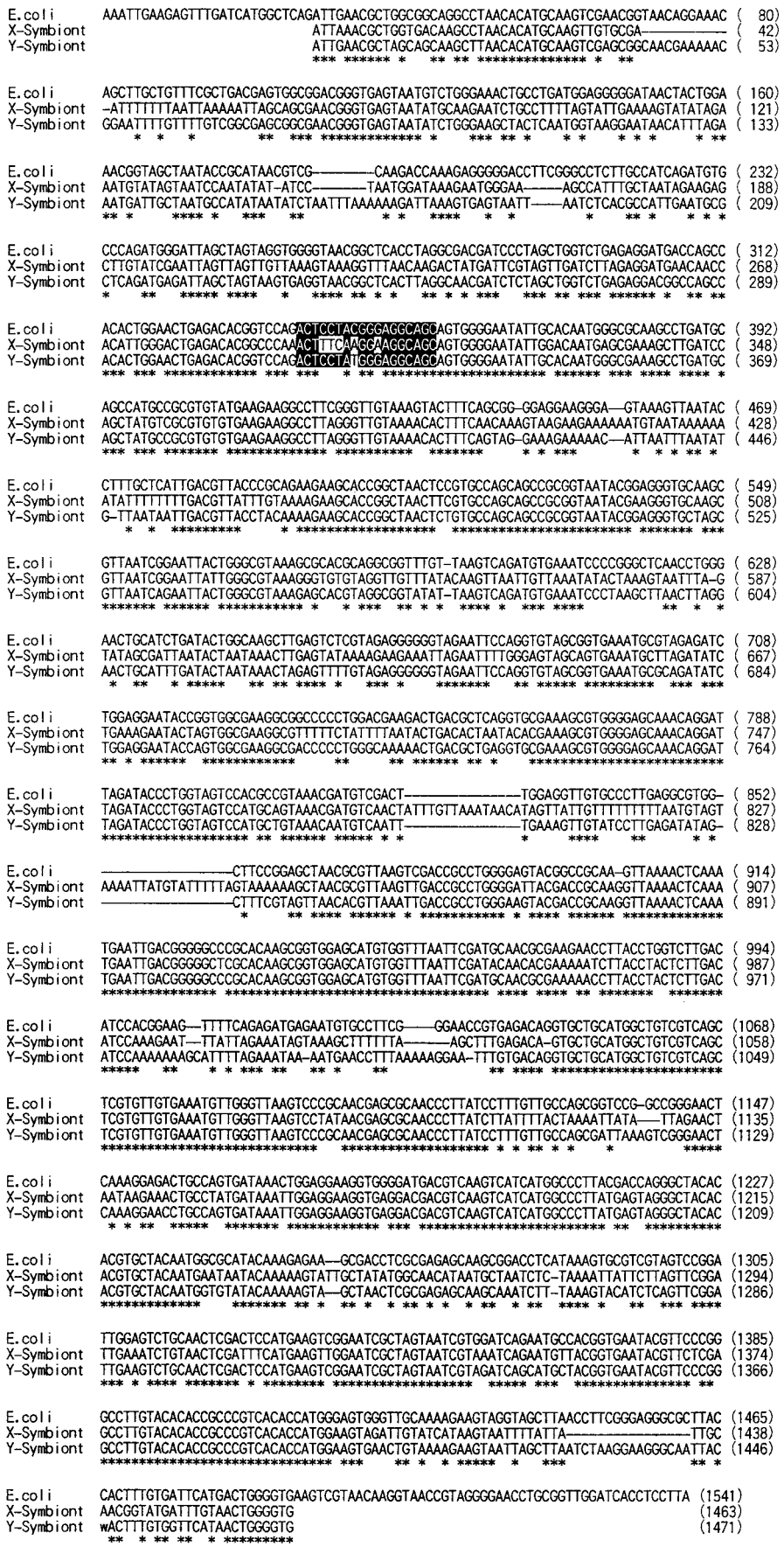

FIG. 2. 16S rDNA sequences of the X-symbiont (A-type) and the Y-symbiont (B-type) aligned with the sequence of *E. coli*. The nucleotide regions complementary to the probes used for in situ hybridization are highlighted. Asterisks represent matched nucleotide sites, and dashes represent alignment gaps.

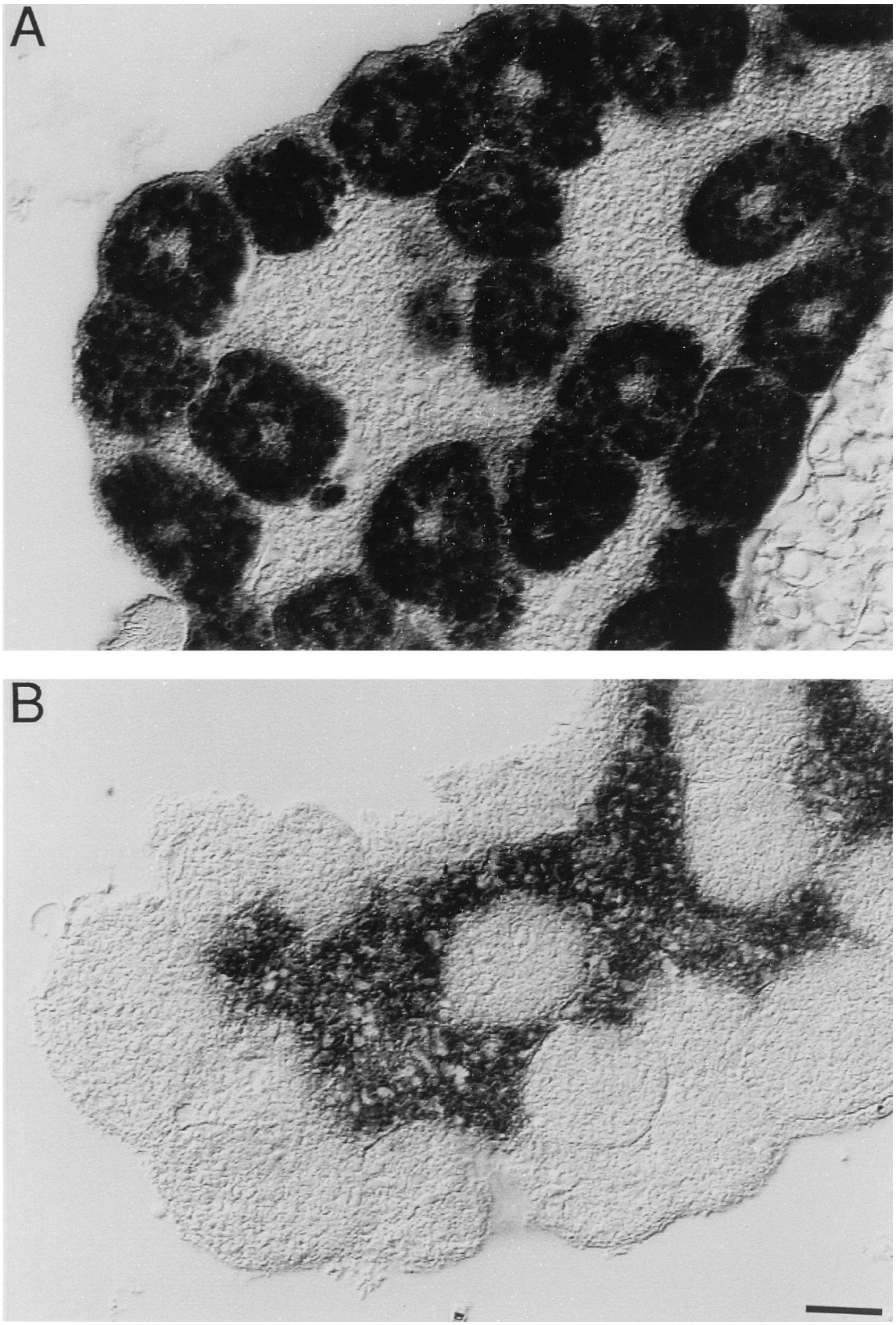

FIG. 3. In situ hybridization of intracellular symbiotic bacteria in the mycetome of *A. mori*. (A) Probe DIG-Kiji16SA targets the A-type sequence in the round mycetocyte, where the X-symbionts are located. (B) Probe DIG-Kiji16SB targets the B-type sequence in the syncytium, where the Y-symbionts are harbored. Bar = 20 μm.

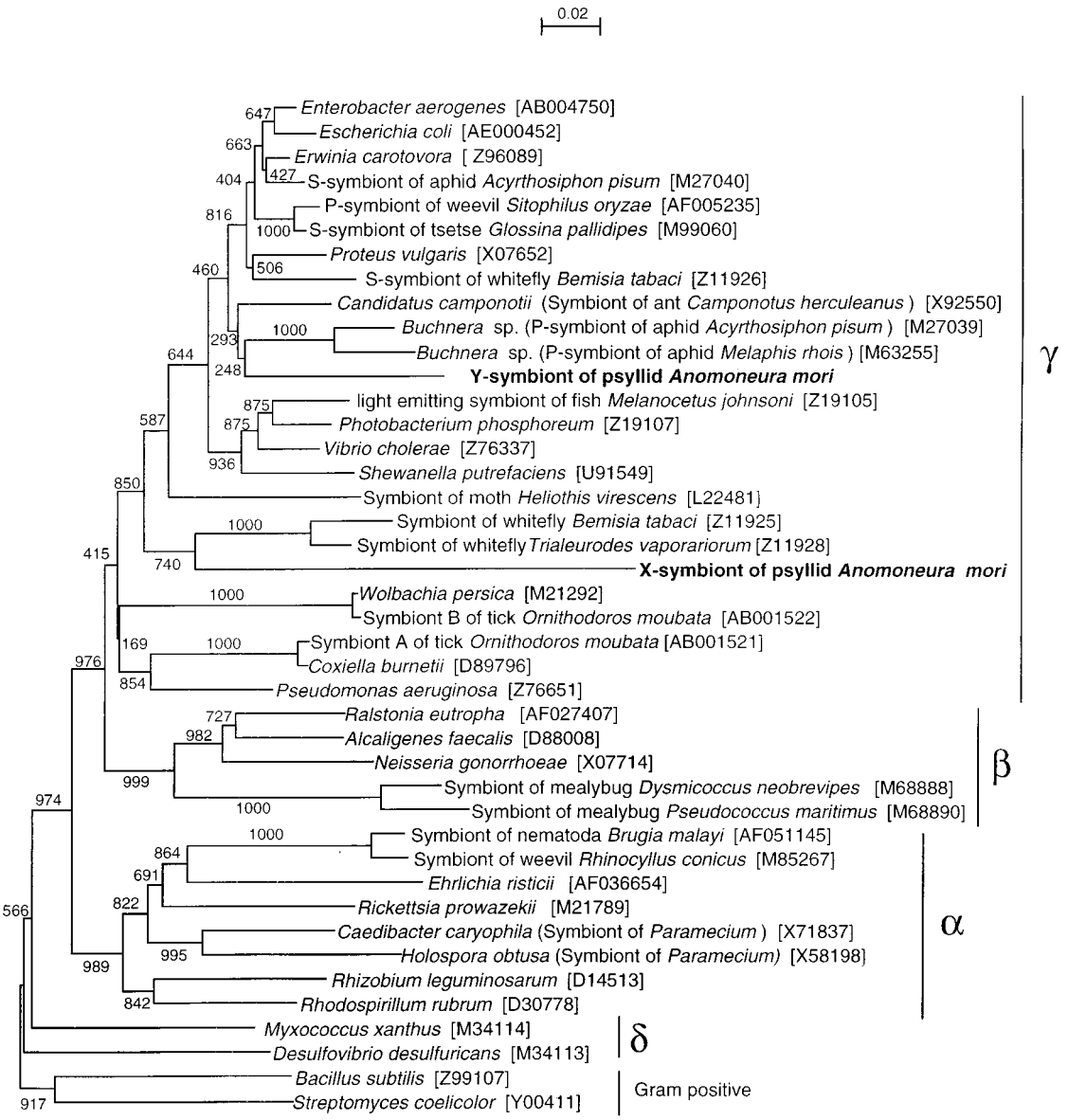

FIG. 4. Phylogenetic positions of the X- and Y-symbionts of *A. mori*. The 16S rDNA sequences of the X- and Y-symbionts, representatives of the *Proteobacteria*, and two gram-positive bacteria (as an outgroup) were analyzed by the neighbor-joining method by using Kimura's two-parameter correction. A total of 1,141 unambiguously aligned nucleotide sites were subjected to the analysis. The bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are accession numbers.

were 1,463 bases for the A-type clones and 1,471 bases for the B-type clones. The RFLP profiles expected from the sequences agreed with the patterns observed.

In situ hybridization. It seemed likely that the two types of 16S rDNA were derived from the X- and Y-symbionts of *A. mori*. To confirm this, we performed 16S rRNA-targeted in situ hybridization with oligonucleotide probes DIG-Kiji16SA and DIG-Kiji16SB, which were specifically designed for A-type and B-type, respectively (Fig. 2).

Figure 3 shows the in situ hybridization results. When probed with DIG-Kiji16SA, the cytoplasm of the round mycetocytes was specifically visualized (Fig. 3A). The syncytial cytoplasm surrounded by the mycetocytes was not stained at all, indicating that the 16S rDNA sequence of A-type was derived from the intracellular symbiotic bacterium of the round mycetocytes, the so-called X-symbiont. In contrast, when hybridized with DIG-Kiji16SB, the syncytium was stained, whereas the round mycetocytes gave no signal (Fig. 3B), indicating that the sequence of B-type could be attributed to the endosymbiotic bacterium of the syncytium, the so-called Y-symbiont. A series of control experiments confirmed the specificity of these results (data not shown). Under stringent hybridization-wash conditions under which *Escherichia coli* and *Buchnera* sp. of the pea aphid were clearly detected, probe EUB338 gave no signal with the tissue sections of *A. mori* (data not shown).

Molecular phylogenetic analysis. Figure 4 is a neighbor-joining tree showing the phylogenetic positions of the X- and Y-symbionts of *A. mori* based on the 16S rDNA sequence analysis. The two symbionts were found to belong to distinct lineages in the γ subdivision of the *Proteobacteria*. The X-sym-

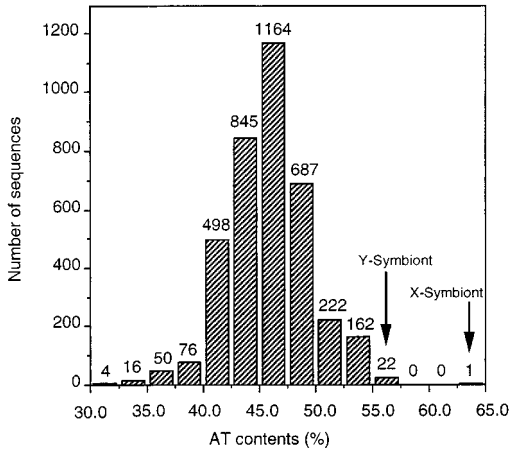

FIG. 5. Histogram of the AT contents of 16S rRNA genes deposited in the RDP database. In addition to the sequences of the X- and Y-symbionts, 3,745 sequences longer than 500 bases in the database were examined. Notably, the sequence of the X-symbiont was the most AT-rich sequence reported to date.

biont formed a monophyletic group with the symbionts of whiteflies, which was supported by a bootstrap value of 74.0%. The Y-symbiont formed a cluster with the intracellular symbionts of aphids and ants, although the level of bootstrap support was very low. The maximum-likelihood analysis gave essentially the same results (data not shown).

AT-biased nucleotide composition. The 16S rDNA sequences of the X- and Y-symbionts were extremely AT rich. The AT contents were 63.6% for the X-symbiont and 55.0% for the Y-symbiont. Figure 5 shows a histogram of the AT contents of 3,745 prokaryotic 16S rDNA sequences longer than 500 bases that have been deposited in the RDP database along with the AT contents of the X- and Y-symbionts. Notably, the sequence of the X-symbiont was the most AT-biased 16S rDNA reported so far.

DISCUSSION

Because multiple endosymbiotic bacteria coexist in the psyllid *A. mori*, simple PCR amplification and sequencing procedures were not sufficient to distinguish and characterize them. We identified two types of bacterial 16S rDNA sequences from the total DNA of the insect (Fig. 1 and 2) and performed in situ hybridization with highly specific oligonucleotide probes under stringent conditions. The 18-mer probes DIG-Kiji16SA and DIG-Kiji16SB were designed for a specific region of the A-type and B-type sequences, respectively. The high level of specificity of these probes was confirmed when they were subjected to a database homology search. When the RDP database was examined, no sequence exhibited 100% identity to DIG-Kiji16SA. Only three sequences exhibited 100% identity to DIG-Kiji16SB; one of these was from a Euglenozoa chloroplast (accession no. X14386), and two were from unidentified eubacteria (accession no. U05662 and X84607). These matches can be regarded as matches that occurred by chance. Under stringent hybridization and wash conditions that did not permit even a single base mismatch, both probes revealed specific localization in tissue sections of the insect. The signal obtained with DIG-Kiji16SA was coincident with the localization of the X-symbiont in the mycetocytes, whereas the signal obtained with DIG-Kiji16SB was coincident with the location of the Y-symbiont in the syncytium (Fig. 3). From these lines of evidence, taken together, we concluded that we successfully cloned and sequenced the 16S rDNAs of the X- and Y-symbionts of *A. mori*. This is the first report of molecular characterization of the endosymbionts of a psyllid.

When preparations were probed with EUB338 under stringent conditions, the X- and Y-symbionts were not detected. This result was expected from the 16S rDNA sequences, because both the X-symbiont and the Y-symbiont contained nucleotide substitutions in the EUB338 target region. Considering that not only the mycetome but also the other tissues were not stained with EUB338 and that RFLP analysis revealed only two 16S rDNA sequences, it is likely that there are no major bacterial endosymbionts in the psyllid other than the X- and Y-symbionts, although the possible presence of minor microbial associates cannot be ruled out. In fact, when some 50 inserted 16S rDNA clones were subjected to *Rsa*I digestion, we found two clones that were neither A-type nor B-type clones (data not shown).

Based on the 16S rDNA phylogeny data, both the X- and Y-symbionts are members of the γ subdivision of the *Proteobacteria*, although they belong to distinct lineages. No 16S rDNA sequence in the database was closely related to the 16S rDNA sequences of the psyllid endosymbionts. However, it should be noted that the sequences that were relatively closely related to the sequences of the psyllid endosymbionts were sequences of endosymbionts from homopteran and other insects. The X-symbiont constituted a monophyletic group along with endosymbionts of whiteflies, whereas the Y-symbiont formed a clade with endosymbionts of aphids and ants (Fig. 4).

At a glance, these results suggest interesting phylogenetic inferences. Since psyllids (Psylloidea), whiteflies (Aleyrodoidea), and aphids (Aphidoidea) constitute the well-defined group Sternorrhyncha and all of these insects have mycetocyte endosymbiotic bacteria (6, 7, 35), it is tempting to assume that their common ancestor possessed two types of intracellular symbiotic bacteria, one of which descended to whiteflies and psyllids and the other of which passed to aphids and psyllids in the evolutionary course of the Sternorrhyncha. However, we should be careful in interpreting the molecular phylogenetic results. For instance, the statistical support for the phylogenetic affinities was far from satisfactory. In addition, the highly AT-biased nucleotide compositions of the 16S rDNAs of the psyllid symbionts (Fig. 5) might lead to misinterpretation (21). Although the X-symbiont of the psyllid formed a monophyletic group with the endosymbionts of whiteflies, with a bootstrap probability value of 74%, the 16S rDNA sequences of these organisms are among the most AT-biased 16S rDNA sequences reported so far (63.6% for the X-symbiont, 52.3% for the symbiont of *B. tabaci*, and 50.9% for the symbiont of *T. vaporariorum*) (Fig. 5), which could be responsible for the relatively high statistical support for the clade. Thus, all that we can suggest here is that the X- and Y-symbionts of *A. mori* belong to distinct lineages in the γ subdivision of the *Proteobacteria*. To clarify their phylogenetic relationships in detail, more data and additional molecular phylogenetic analyses are needed. At least, however, the endosymbionts of mealybugs (Coccoidea) are evolutionarily independent of the endosymbionts of the psyllids, because they belong to the β subdivision of the *Proteobacteria* (27).

The 16S rDNAs of the X- and Y-symbionts were both very AT rich (Fig. 5). Surprisingly, the sequence of the X-symbiont was the most AT-rich 16S rDNA ever reported. Considering that in situ hybridization that targeted rRNA was successful (Fig. 3), these genes are not pseudogenes but are transcribed to form functional ribosomes. It should also be noted that on

the phylogenetic tree, the branch lengths appear to be elongated in the lineages of the X- and Y-symbionts (Fig. 4), which could reflect accelerated nucleotide substitution rates in these lines. It has been suggested that a small population size and a lack of effective recombination in vertically transmitted endosymbiotic microorganisms result in the accumulation of mildly deleterious mutations, which could be detected as faster sequence evolution and a shift in base composition that reflects mutational bias (25). The aphid symbiont *Buchnera* sp. has only a single copy of the 16S rRNA gene, which might reflect slow growth of the endosymbiotic bacterium (5). Although it is not known how many copies of rRNA genes there are in the psyllid symbionts, it seems possible that these organisms also have only one, considering that the growth and reproduction of psyllids are much slower than the growth and reproduction of aphids (22). If this is true, mutations cannot be corrected by gene conversion. The extraordinary molecular features of the 16S rDNAs of psyllid endosymbionts might be explained in this context.

The biological functions of the mycetome endosymbionts of psyllids have not been investigated. However, the highly developed mycetome is conserved in all of the psyllids that have been examined (6, 8, 30, 36), suggesting that the endosymbionts may play an essential physiological and nutritional role in the host psyllids, as has been demonstrated in aphids, planthoppers, cockroaches, and other insects (5, 10–12, 32).

Profft (30) examined the endosymbiotic systems of 18 species of psyllids histologically. He found that the majority of these psyllids possess two types of intracellular symbiotic bacteria, one in round, uninucleated mycetocytes and the other in the syncytium. Later, Chang and Musgrave (8) arbitrarily designated the former X-symbionts and the latter Y-symbionts in their electron microscopic study of the pear psyllid *Psylla piricola*. Waku and Endo (36) confirmed that the same endosymbiotic organization occurs in the mulberry psyllid *A. mori*. Profft (30) reported that in contrast to the typical endosymbiotic system, 2 of the 18 species which he examined lacked Y-symbionts. *Strophingia ericae*, whose syncytium was limited to a small area and was free of symbionts, was apparently monosymbiotic. In *Trioza* sp., whose syncytium was atrophied and sterile, fat body cells adjacent to the mycetome were populated by a different type of bacterium. In addition, the morphology of the Y-symbionts is much more varied from species to species than the morphology of the X-symbionts (6, 30). Generally, the biomass of the X-symbionts in the mycetome is greater than the biomass of the Y-symbionts (6, 30). On the basis of these observations, it is conceivable that the X-symbionts may be more essential for the host psyllids, be more stable in the course of evolution, and have a more ancient origin than the Y-symbionts. Of course, these ideas have to be confirmed by further phylogenetic analyses of various psyllids and their symbionts.

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