AEM Accepts, published online ahead of print on 1 September 2006
Appl. Environ. Microbiol. doi:10.1128/AEM.01429-06


Submitted to *Applied and Environmental Microbiology*

# Symbiotic bacteria associated with stomach disc of human lice

Kayoko Sasaki-Fukatsu,[1] Ryuichi Koga,[1] Naruo Nikoh,[2] Kazunori Yoshizawa,[3] Shinji Kasai,[4] Minoru Mihara,[5] Mutsuo Kobayashi,[4] Takashi Tomita,[4] and Takema Fukatsu[1]*

*Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566,[1] Division of Natural Sciences, University of the Air, Chiba 261-8586,[2] Department of Ecology and Systematics, Hokkaido University, Sapporo 060-8589,[3] Department of Medical Entomology, National Institute of Infectious Diseases, Tokyo 162-8640,[4] and Department of Environmental Biology, Japan Environmental Sanitation Center, Kawasaki 210-0828,[5] Japan*

* Corresponding author. Mailing address: National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba 305-8566, Japan. Phone: 81-29-861-6087. Fax: 81-29-861-6080. E-mail: t-fukatsu@aist.go.jp.

Running head: Symbiotic bacteria of human lice

The symbiotic bacteria associated with stomach disc, a large aggregate of bacteriocytes on the ventral side of midgut, of the human body and head lice were characterized. Molecular phylogenetic analysis of 16S rRNA gene sequences showed that the symbionts formed a distinct and well-defined clade in the γ-*Proteobacteria*. The sequences exhibited AT-biased nucleotide composition and accelerated molecular evolution. In situ hybridization revealed that in nymphs and adult males the symbiont was localized in the stomach disc, while in adult females the symbiont was not in the stomach disc but in the lateral oviducts and the posterior pole of the oocytes due to female-specific symbiont migration. We propose the designation "*Candidatus* Riesia pediculicola" for the lice symbionts.

Sucking lice (Insecta: Phthiraptera: Anoplura), embracing over 500 described species from the world, are ectoparasitic insects exclusively feeding on mammalian blood (9). There are two closely related species of human lice: the body louse *Pediculus humanus* lives in clothes and feeds from body, and the head louse *Pediculus capitis* lives in hair and feeds from scalp. The head louse and the body louse are morphologically and genetically very similar, and some researchers regard them as subspecies or ecotypes of the same species (7, 16, 22).

Vertebrate blood is certainly nutritious but deficient in some nutritional components such as B vitamins, which is probably the reason why insects exclusively living on vertebrate blood all through their life, including tsetse flies, louse flies, bedbugs, assassin bugs, lice, etc., are generally in close association with endosymbiotic microorganisms (6). It was back to the 1920's when the human lice were first reported to possess a large aggregate of bacteriocytes, called stomach disc, on the ventral side of midgut, wherein rod-shaped symbiotic bacteria are harbored (5, 23). Since then, a number of histological (11, 20), embryonic (4, 20), experimental (1, 2, 3, 10) and nutritional (18, 19) studies have been conducted on the endosymbiotic system of the human lice. These studies demonstrated that the symbiont is vertically transmitted from maternal stomach disc to developing oocytes through a peculiar passage (11, 20), is essential for survival and growth of the host (3), and provides the host with B vitamins that are lacking in the blood meal (19). Despite the substantial body of early works on the lice endosymbiosis, microbial nature of the symbionts has been unknown to date. Hence, we characterized the symbiotic bacteria of the human lice by using molecular phylogenetic and histological approaches.

We mainly used a long-established inbred line of the human body louse, the strain NIID, which has been maintained in the laboratory since 1954 (26). The insects were kept in an evaporating dish with pieces of felt sealed in a plastic container with silica gel, reared at 30$^{o}$C in constant darkness, and fed with human blood once a day on the arm of one of the authors (M. M.). The insects at different developmental stages were sampled and preserved in acetone until molecular and histological analyses (13). Samples of body lice from 12 different sources and head lice from 4 different sources were also collected in either Japan or Nepal.

DNA was individually extracted from adult females of the strain NIID by using a QIAamp DNA mini kit (QIAGEN). A 1.5 kb segment of the eubacterial 16S rRNA gene was amplified with the primers 16SA1 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16SB1 (5'-TACGGYTACCTTGTTACGACTT-3'), and subjected to cloning, restriction fragment length polymorphism (RFLP) genotyping, and DNA sequencing as previously described (14).

More than ten clones of the 16S rRNA gene segment from each of the samples exhibited identical RFLP patterns (data not shown), suggesting that a single bacterial species is

dominant in the insects. Nucleotide sequences of some of these clones were determined, which were 1,482 bp in size and identical to each other. A BLAST search clearly showed that the sequence belongs to the Enterobacteriaceae in the γ-*Proteobacteria*. No closely related sequences were identified in the DNA databases: the highest hits were *Providencia stuartii* (AF008581; 89.6% [1,141/1,274] sequence identity) and *Enterobacter hormaechei* (AJ853889; 89.0% [1,119/1,258] sequence identity).

In order to confirm whether or not the 16S rRNA gene sequence is derived from the symbiotic bacteria in the stomach disc, we designed an oligonucleotide probe specific to the sequence, Cy3-Lice1255R (5'-Cy3-TTGGCTCGCTCTTACGAGT-3'), for whole-mount fluorescent in situ hybridization (wFISH). The insects preserved in acetone were, after their legs were removed to facilitate infiltration of reagents, fixed in Carnoy's solution (chloroform-ethanol-acetic acid [6:3:1]) overnight, and incubated with 6% $H_2O_2$ overnight for quenching the autofluorescence of insect tissues. The insects were thoroughly washed, and equilibrated with a hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide), and Cy3-Lice1255R probe and SYTOX green were added at final concentrations of 10 nM and 0.5 μM, respectively. After an overnight incubation, the samples were thoroughly washed in a washing buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate), and were observed under an epifluorescent microscope (Axiophot, Carl Zeiss) and a laser confocal microscope (PASCAL5; Carl Zeiss).

In nymphal insects, the probe specifically hybridized with a round stomach disc located in the ventral abdomen (Fig. 1A). In the stomach disc, the signals showed radial compartment-like structures (Fig. 1B), which had been described as location of the symbiotic bacteria (4, 11, 20). A 3-dimensional FISH movie image of the stomach disc is available in Supplemental Material. No probe control experiment and competitive suppression control experiment with excess unlabelled Lice1255R probe (14) did not identify these signals, confirming specificity of the FISH detection (data not shown). These results indicated that the 16S rRNA gene sequence was certainly derived from the symbiotic bacteria harbored in the stomach disc.

In adult males, the signals were localized in the stomach disc (Fig. 1C). In adult females, however, the signals were not detected in the stomach disc, but in the lateral oviducts and the posterior pole of oocytes in ovarioles (Fig. 1D-F). Ries (20) and Eberle and McLean (11) reported that in the last (= 3rd) nymphal instar, the symbiont cells escape from the stomach disc, migrate to the lateral oviducts, infect the ovarial epithelium, and are transmitted to the oocytes through a specialized tissue organization called ovarial ampullae. The results of wFISH were in accordance with these histological descriptions.

We also cloned and sequenced the eubacterial 16S rRNA gene segment from four body lice and two head lice of different sources. All the sequences were very similar to each other, 1,482 bp in size, with sequence identities ranging from 99.1% to 99.9%. Nucleotide composition of the sequences was remarkably AT-biased, ranging from 50.6% to 51.0%. These AT-content values were higher than those of related free-living bacteria such as *P. stuartii* (AF008581; 46.9%) and *E. hormaechei* (AJ853889; 44.9%), and were equivalent to those of obligate endosymbiotic bacteria of other insects such as *Buchnera* spp. of aphids (e.g. AJ296751; 51.3%) and *Wigglesworthia* spp. of tsetse flies (e.g. AB063521; 51.5%).

These 16S rRNA gene sequences were subjected to molecular phylogenetic analysis together with the sequences of related γ-proteobacteria that exhibited high BLAST scores in the DNA database search. A multiple alignment of the sequences was generated by using the program package Clustal W (24). Aligned nucleotide sites containing a gap were removed from the data set, and the final alignment was inspected and corrected manually. A neighbor-joining tree, with 1,000 bootstrap resamplings, was also constructed by the program package Clustal W (24).

Figure 2 shows the neighbor-joining phylogeny on the basis of 1,408 unambiguously aligned nucleotide sites. The lice symbionts formed a distinct monophyletic group with 100% bootstrap support, constituting a basal lineage in the γ-subclass of the *Proteobacteria*. The symbionts of body lice could not be phylogenetically differentiated from the symbionts of head lice, probably reflecting the close morphological and genetic relatedness between the body louse and the head louse (7, 16, 22). No endosymbiotic bacteria of other insects showed phylogenetic affinity to the lice symbionts.

In comparison with the related free-living bacteria, the lice symbionts exhibited remarkably elongated branches on the phylogenetic tree (Fig. 2), which was suggestive of accelerated molecular evolution in the lineage of the lice symbionts. Thus, we performed a relative rate test based on genetic distances estimated under the Kimura's two parameter model (15). Statistical significance was evaluated by using the program package RRTree (21). The following 16S rRNA gene sequences were subjected to the analysis: AB263101 from symbiont of body louse, AB263105 from symbiont of head louse, AF008581 from *Providencia stuartii*, AM040495 from *Providencia* sp., and X74694 from *Vibrio cholerae* as outgroup. The evolutionary rate of 16S rRNA gene in the lineage of the lice symbionts was around 3.1 times faster than that in the lineage of related free-living γ-proteobacteria. The difference was highly significant statistically ($P = 10^{-7}$).

Recent molecular evolutionary analyses revealed that the lifestyle of obligate insect endosymbionts has strongly affected their genome evolution, causing AT-biased nucleotide

composition, accelerated rate of molecular evolution, and significant genome reduction. These peculiar genetic traits are hypothesized to be the consequence of attenuated purifying selection due to small population size and strong bottleneck associated with their endosymbiotic lifestyle (17, 25). The AT-bias and the accelerated evolution in the 16S rRNA gene sequences are also suggestive of a stable and intimate host-symbiont association in the human lice over evolutionary time.

How prevalent is the symbiont in the lice populations? We examined 57 body lice from 12 different sources and 9 head lice from 4 sources for their symbiont infection. For confirmation of successful DNA preparation from each of the samples, mitochondrial 16S rRNA gene of the host insect was amplified with the primers MtrA1 (5'-AAWAAACTAGGATTAGATACCCTA-3') and MtrB3 (5'-ACACTTTCCAGTACAYTTACTTTGT-3') under a temperature profile of 95°C for 4 min followed by 35 cycles of 95°C for 30 sec, 48°C for 30 sec and 65°C for 3 min. Diagnostic PCR of the symbiont was performed with the primers 16SA3 (5'-TGCATGGYTGTCGTCAGCTCG -3') and Lice1255R under a temperature profile of 95°C for 4 min followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 70°C for 1 min. The diagnostic PCR analysis identified the symbiont in all the lice samples examined. Thus, it was strongly suggested that the symbiont infection is fixed in the lice populations, corroborating the essential biological roles of the symbiont for the host insect (3, 19).

From all these results taken together, it was concluded that the symbiotic bacteria associated with the stomach disc of the human lice constitute a phylogenetically distinct and coherent bacterial group in the γ-*Proteobacteria*. Hence, we propose the designation "*Candidatus* Riesia pediculicola" for the symbiont of the human lice. The generic name honors Erich Ries who first comprehensively investigated the endosymbiotic system in lice (20). The specific name indicates the association with lice.

The human lice are notorious as medical and hygienic pests. The body louse is the vector of the epidemic typhus pathogen *Rickettsia prowasekii*, the relapsing fever pathogen *Borrelia recurrentis*, and the trench fever pathogen *Bartonella quintana* (12). The head louse is recently re-emerging even in advanced countries, particularly among school children, and is increasing its resistance to insecticides (8). Considering the essential roles of the symbiont for the host (3, 19), microbiological studies on the lice symbionts would lead to new means of control for the pests. Other than the human lice, a number of lice are known from a wide variety of mammals, and it was histologically described that the localization, morphology and life cycle of symbiotic bacteria in animal lice are distinct from those of the human lice in many respects (6, 20). Whether the symbionts of animal lice are phylogenetically related to

the symbionts of the human lice is of evolutionary interest.

We thank Noboru Yaguchi, Naomi Seki, Madan Shrestha, Harufumi Yui and Akio Kobayashi for lice samples.

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## FIGURE CAPTIONS

FIG. 1. Whole mount in situ hybridization of the symbiotic bacteria in the human body lice. (A) A 1st instar nymph with a large round stomach disc at the center of the ventral abdomen. The symbiont cells are specifically detected in the stomach disc. (B) An enlarged image of the stomach disc. The location of the symbiont cells exhibited radial compartment-like structures. (C) An adult male, in which the symbiont is localized in the stomach disc. (D) An adult female, in which the symbiont is not detected in the stomach disc but in the lateral oviducts and the posterior pole of oocytes. (E) An enlarged image of the female reproductive organs.

(F) An enlarged image of an oocyte. Arrows and arrowheads indicate the lateral oviducts and the posterior pole of oocytes, respectively.

FIG. 2. Molecular phylogenetic analysis of 16S rRNA gene sequences of the stomach disc symbionts from human lice. A neighbor-joining tree is shown, while maximum parsimony analysis gave essentially the same result (data not shown). Bootstrap values higher than 70% are shown at the nodes. In brackets are sequence accession numbers.

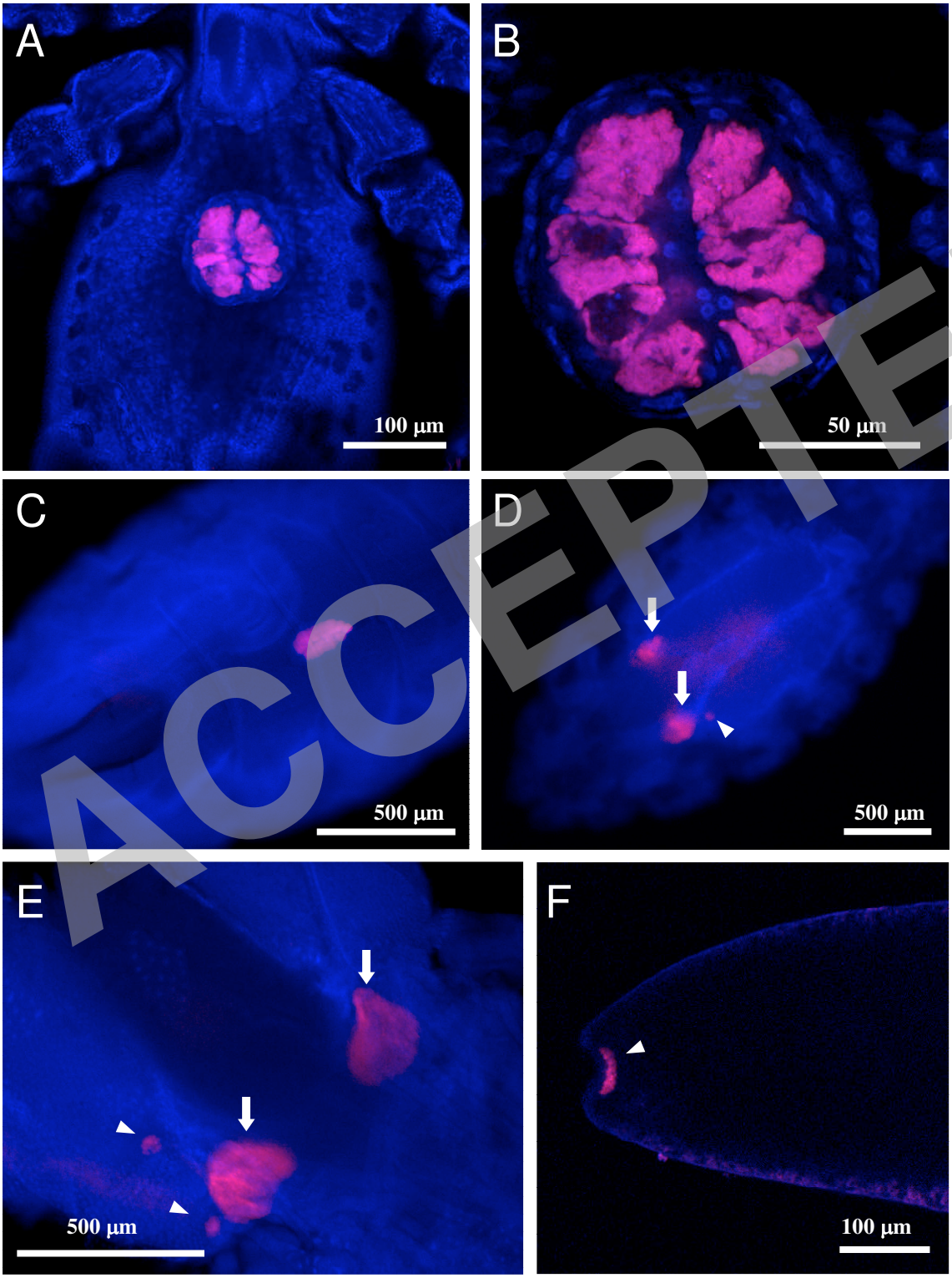




FIG. 1

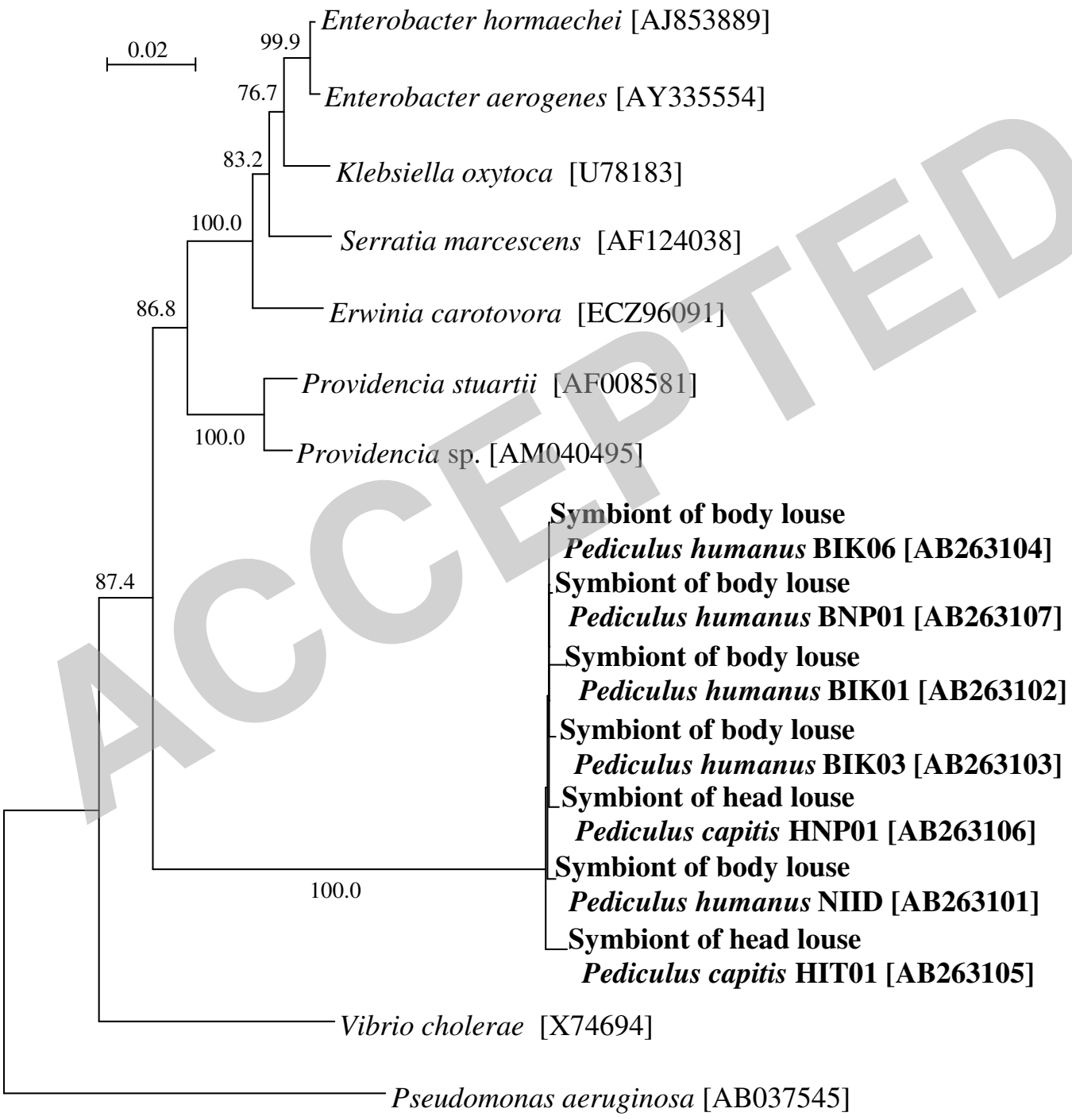




**FIG. 2**