Evolutionary Relationships of “Candidatus Riesia spp.,” Endosymbiotic Enterobacteriaceae Living within Hematophagous Primate Lice

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The primary endosymbiotic bacteria from three species of parasitic primate lice were characterized molecularly. We have confirmed the characterization of the primary endosymbiont (P-endosymbiont) of the human head/body louse Pediculus humanus and provide new characterizations of the P-endosymbionts from Pediculus schaeffi from chimpanzees and Pthirus pubis, the pubic louse of humans. The endosymbionts show an average percent sequence divergence of 11 to 15% from the most closely related known bacterium “Candidatus Arsenophonus insecticola.” We propose that two additional species be added to the genus “Candidatus Riesia.” The new species proposed within “Candidatus Riesia” have sequence divergences of 3.4% and 10 to 12% based on uncorrected pairwise differences. Our Bayesian analysis shows that the branching pattern for the primary endosymbionts was the same as that for their louse hosts, suggesting a long coevolutionary history between primate lice and their primary endosymbionts. We used a calibration of 5.6 million years to date the divergence between endosymbionts from human and chimpanzee lice and estimated an evolutionary rate of nucleotide substitution of 0.67% per million years, which is 15 to 30 times faster than previous estimates calculated for Buchnera, the primary endosymbiont in aphids. Given the evidence for cospeciation with primate lice and the evidence for fast evolutionary rates, this lineage of endosymbiotic bacteria can be evaluated as a fast-evolving marker of both louse and primate evolutionary histories.

Insects in general are an incredibly successful and diverse group. Part of their success is undoubtedly due to mutualistic primary endosymbiotic bacteria, which have enabled insects to radiate into niches that include nutrient-poor diets (5, 9, 27), such as wood (e.g., termites), plant sap (e.g., aphids), and blood (e.g., sucking lice in the Anoplura). Primary endosymbiotic bacteria (as opposed to secondary endosymbionts [S-endosymbionts]) are normally maintained inside specialized host cells called mycetomes (9, 27), usually exhibit nucleotide A+T bias greater than 50%, and show elevated sequence evolution with respect to their free-living counterparts (4, 40, 41). These endosymbionts are required for host survival, and they provide nutrients that are not available in the insect’s specialized diet (5). Most primary endosymbionts (P-endosymbionts) leave their mycetomes to migrate to the ovaries so that they may be incorporated into developing eggs (transovarial transmission) and thus be passed onto the next host generation (9, 27), leading to long-term, shared coevolutionary histories between the insects and their symbionts.

It is estimated that there are 14,000 species of hematophagous insects (1, 20), but only a few P-endosymbionts have been described for these insects (e.g., Wigglesworthia). Because blood is a nutrient-poor diet, all sucking lice likely have some form of endosymbiont and many are reported to have obligate primary endosymbionts based on microscopic observation. The P-endosymbiont in the human head and body louse (Pediculus humanus) was first seen over 340 years ago (15) with some of the very first microscopes. It has a complex migration (5) that involves four different mycetomes and two extracellular migrations as it moves to the eggs in the adult female lice (28). This migration was first observed by Ries (35) and later shown by scanning and transmission electron microscopy by Eberle and McLean (11). Wolbachia is the only other bacterium that has been found among human lice (19, 29).

The complex migration associated with transovarial transmission stands as potential evidence of the importance of the relationship between the P-endosymbiont and the lice. If the mycetome is removed from a young female louse, she dies after only a few days and her eggs are deformed (3). Furthermore, if the bacteria are removed from the eggs directly, the larvae survive only a few days (2). Puchta (32) demonstrated that lice without P-endosymbionts were able to survive if their diet was supplemented with nicotinic acid, pantothenic acid, and betabiontin, suggesting a basis for a mutualistic long-term relationship.

Humans have three types of lice, head and body lice (Pediculus humanus), which are currently classified as two distinct subspecies (Pediculus humanis capitis and Pediculus humanis humanus), and pubic lice (Pthirus pubis) (10). Body lice are known to transmit three diseases: louse-borne epidemic typhus (LBET), relapsing fever, and trench fever (6). Although head lice can transmit LBET in a laboratory setting (14, 36), there has never been evidence of LBET transmission by head lice in nature. Currently, it is not known why one subspecies of P. humanus can transmit three deadly bacterial agents while the other subspecies, epidemic in schoolchildren, effectively cannot. Although the secondary endosymbiont of tsetse flies (Sodalis glossinidius) has been shown not to affect the ability of...
its host to transmit Trypanosoma congolense (13), it is possible that there are differences in the endosymbiotic bacteria of head and body lice and that these differences may reinforce patterns of disease transmission in human lice.

Primate lice show a history of cospeciation with their hosts and have been used effectively to infer human evolutionary history (18, 21, 34, 42). Most of this work has relied upon mitochondrial DNA (mtDNA) from the lice and to a lesser extent on nuclear markers. If a single lineage of endosymbiont was found among primate lice, the endosymbiont could serve as still another marker of human and primate evolutionary history. In addition, this new three-tiered assemblage of primate, lice, and endosymbiotic bacteria would yield a new system in which to study relative and absolute rates of evolution in three disparate lineages (vertebrates, insects, and bacteria).

In this study, we describe the molecular characterization of the P-endosymbiont of primate lice, including head and body lice (Pediculus humanus) characterized by Sasaki-Fukatsu et al. (38), and present new data from chimpanzee lice (Pediculus schaeffi) and human pubic lice (Pthirus pubis). We used the full-cycle rRNA approach, including comparative 16S rRNA gene analysis and the detection of endosymbionts within the host cell by means of fluorescent in situ hybridization using specific 16S rRNA-targeted oligonucleotide probes. By sampling across a phylogenetically diverse assemblage of louse species, we expected to capture a greater percentage of the diversity in this lineage of P-endosymbiont.

MATERIALS AND METHODS

Specimen preparation. Specimens of human head lice (Pediculus humanus capitis) were collected from patients in West Palm Beach, FL, and from schoolchildren in La Rioja, Argentina (29). Specimens of body lice (Pediculus humanus humanus) were acquired from a rabbit-adapted strain held at insect control and research labs in Maryland and in Cambridge, United Kingdom. Chimpanzee lice were collected in Uganda, and samples of Pthirus pubis were collected in Utah. All lice were surface sterilized either with a lysis-sodium dodecyl sulfate buffer as described by Reed and Hafner (33) or with 0.7% sodium chloride-0.05% Triton X-100 for 20 s in an ultrasonic bath and then rinsed with sterile, distilled water to remove any surface contamination. The lice were rehydrated in phosphate buffer with Triton X-100 plus sodium azide (PBTA) in three consecutive steps: 30, 60, and 100% PBTA. Whole mycetomes were dissected manually under a Leica stereo microscope with a magnification of ×100 by use of tungsten tips and special carbon steel blades. A maximum dissecting resolution of 10 to 15 μm was obtained. The dissected bodies were fixed inside 1.5-mL tubes for DNA extraction using the DNA extraction methods described by Reed and Hafner (33).

Bacterial diversity in Pediculus humanus capitis, determined using molecular methods. Universal bacterial 16S rRNA gene primer 27F (5′-GAG TTT GAT CCT GGC TCA G-3′) was used with either 1492R (5′-CAG GAA TCC TAC GGG TAC CTT AGT ACC T-3′) or 1525R (5′-AGA AAG GTG ATC ACC CGG-3′) to amplify 16S rRNA gene sequences. PCR was performed on the isolated DNA by using standard reaction conditions with 10 ng of template DNA, 300 nM of each primer, 200 μM of each dNTPs (Tri/Phosphor), 2.5 mM MgCl2, and 0.02 U of Taq DNA polymerase per μL of reaction mix. Cycling conditions consisted of an initial denaturation step (95°C, 10 min) followed by 30 cycles of amplification involving denaturation (95°C, 1 min), annealing (50°C, 1 min), and extension (65°C, 1 min), followed by a final extension step at 65°C for 10 min. The PCR product was analyzed by gel electrophoresis. The 1.5-kbp 16S rRNA gene PCR product was purified with Exo-SAP-IT (USB Corp.) as prescribed by the manufacturer. The PCR product was then cloned into the pTOPO 4.0 vector (Invitrogen) according to the supplier’s instructions to generate clone libraries. Recombinant clones were sequenced to completion at the University of Florida sequencing facility and in the Bangor, United Kingdom, lab by use of vector-specific primers and internal sequencing primers that were designed as sequence information became available. The computer program Sequencher v. 4.1 (Gene Codes Corporation, Ann Arbor, MI) was used to join contiguous 16S rRNA gene fragments into a single consensus sequence.

Targeted sequencing of endosymbionts from additional taxa of lice. The 1,525-bp 16S rRNA gene sequence for the endosymbiont of P. humanus capitis was aligned to other 16S sequences in the ARB database (22). A new PCR primer specific to the endosymbiont sequence was then created with the intention of excluding contaminant sequences coamplified with the endosymbiont target. The single specific primer, in concert with the general eubacterial primer, preferentially amplifies the endosymbiont from whole-insect preparations. The 1525R primer was paired with the specific primer 461F (5′-ACA GAA GAA GCA CCG GCT GTA A-3′) to produce a 1,200-bp fragment of the 16S rRNA gene. These primers were used to amplify, clone, and sequence the endosymbionts from three additional taxa: body lice (P. humanus humanus), chimpanzee lice (Pediculus schaeffi), and human pubic lice (Pthirus pubis).

Fluorescent in situ hybridization. Specimens were washed as for dissections, selected under a microscope on a slide with a drop of water, and immediately fixed with ethanol-glacial acetic acid (3:1) for 2 h. Preparations were kept overnight in a mixture of xylene and ethanol (1:1) at 4°C and then transferred to xylene-ethanol (1:2) for 30 min and to ethanol for 30 min, washed with 20°C 80% acetone for 20 min, and dehydrated in ethanol. Specimens were rehydrated using an ethanol-PBTA mixture with ratios of 2:1, 1:1, and 1:2 for 20 min each, with a final rehybridization of only PBTA for 30 min, where specimens remained at 4°C. Before hybridization, PBTA samples were hybridized with PBTA hybridization buffer (HB) at a ratio of 1:1 (hybridization buffer was Tri-HCL, 0.02 M; sodium chloride, 0.09 M; sodium dodecyl sulfate, 0.01%; formamide, 35%; Denhardt’s solution, 15%) for 20 min, followed by only hybridization buffer (around 500 μL/microtube), and sonicated in an ultrasonic bath for 20 s; then, probes and corresponding helpers were added (final concentration of 100 pmol each). Samples were incubated at 47°C in total darkness for 16 h, washed for 1 h with the HB without probes and then in the PBTA-HB at 4°C, and then sonicated to PBTAB-HB (1:1) at room temperature and finally to PBTA. Samples were mounted with PBTA-polyacrylamide mounting medium.

In order to ensure that the 16S rRNA gene sequences that we retrieved were from the same mycetome-bound bacteria described by Ries (35), we created a species-specific probe by using the ARB database in combination with known probes and helpers of bright fluorescence (12). The endosymbiont-specific probe 2-Sd-Cy5 (5′-CY5-GAG ATT GTT GCC TAG GTG-3′), which does not match any published sequence, and the three helpers Helper 1-Sd (5′-ACC TCA CTT ACT AGC TTA CCT GGA ACT AGC TTA CTT ACT CTA-3′), Helper 2-Sd (5′-GTA TGG GCT CAT CTA AAG-3′), and Helper 3-Sd (5′-TGT TAG ATY CCC ATA TTA-3′) were based on the endosymbiont sequence obtained from human head louse endosymbiont. No-probe and competition suppression control experiments using excess unlabeled probes were performed. Fluorescent in situ hybridization was conducted on whole-mount specimens and on 14-μm-thick serial sections of lice. Individual cells by use of the endosymbiont-specific probe. Samples were analyzed with a Zeiss LSM510 confocal microscope with a coherent helium laser.

Phylogenetic analysis. Nearly complete 16S rRNA gene sequences were obtained and added to the ARB RNA sequence database, which contains over 16,000 homologous small-subunit rRNA primary structures. Multiple sequence alignment was achieved using the ARB automated alignment tool (program available at http://www.mikro.biologie.tu-muenchen.de), with modification by eye. Comparative sequence analysis revealed that the 16S rRNA genes of the endosymbionts were novel and showed highest sequence similarities with members of the Enterobacteriaceae. Therefore, we downloaded an aligned data set of additional taxa from the ARB database and GenBank in order to perform more-complete phylogenetic analyses (alignment available in TreeBase [www .treebase.org], no. SN3132). We used the computer program ModelTest (31) as a guide to determine the best-fit maximum-likelihood (ML) model as described by Camacho et al. (7) and ModelTest examines maximum-likelihood models ranging from simple to complex. This method increases the number of parameters in the ML model incrementally until the addition of a new parameter no longer increases significantly the fit between the model and the data. ModelTest calculated likelihood scores for 56 nested ML models and used hierarchical likelihood ratio tests to determine the best-fit model. We incorporated the best-fit model of nucleotide evolution in ML heuristic searches in PAUP* (39) and in Bayesian searches in MrBayes (16) by using the maximum-likelihood optimality criterion. Multiple outgroups were chosen from phylogenetic studies of Enterobacteriaceae, especially endosymbiotic taxa, and the phylogenetic tree was rooted on the most divergent outgroup taxon, the alpha-proteobacterium Wolbachia pipientis. McBayes was run for the 43-taxon data set for 10 million generations. Burn-in was achieved within the first 100,000 generations; therefore,
to be conservative, our posterior probabilities are based on the last 9.8 million generations (phylogenetic trees available in TreeBase, no. M3012).

**Nucleotide sequence accession numbers.** The nucleotide sequences for the endosymbionts from body lice (*P. humanus humanus*), chimpanzee lice (*Pediculus schaeffi*), and human pubic lice (*Pthirus pubis*) have been deposited in GenBank under accession no. EF110569 to EF110574.

**RESULTS**

**Phylogenetic analysis and taxonomic position of louse endosymbiont.** DNA amplification and sequencing with the eubacterial primers 27F, 1492R, and 1525R led to the nearly complete sequencing of the 16S rRNA genes for two head lice and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp). The design of species-specific primers led to the sequencing of shorter fragments, and one body louse (ca. 1,520 bp).

**DISCUSSION**

Bayesian phylogenetic analysis and BLAST searches demonstrated that this new lineage of *Enterobacteriaceae* is closely related to a lineage of *S*-endosymbionts ("Candidatus Arsenophonus spp."). "Candidatus Arsenophonus" endosymbionts have been found living within hippoboscid flies (8), commonly called louse flies. Louse flies parasitize a wide range of birds and mammals and are known to physically carry lice as hitchhikers among vertebrate host individuals (called phoresy). The lice, which are particularly bad dispersers, having no wings of their own, use the flies as a means of dispersal. While phoresy itself does not involve any humoral interaction between passenger (phoront) and carrier, in one case, a phoretic mite (*Macrocheles subbadius*) has been shown to feed on the hemolymph of its transporting host (*Drosophila nigrospiracula*) (30).

The close association between the endosymbionts of lice and louse flies might be explained by horizontal transmission, which appears to be more common among microorganisms than previously thought (23, 37). One might presume that the *P*-endosymbiont in lice is derived from the *S*-endosymbiont (i.e., increasing specialization over evolutionary time); however, this presents a hypothesis that can be tested directly in future studies. Our phylogenetic analysis shows that the endosymbionts of primate lice are distinctly different from each other and their closest relatives, and yet they represent a strongly supported monophyletic clade.

**Nomenclature.** These endosymbiotic bacteria have not been characterized completely or grown in pure culture, although they have been known and visualized since the early 1600s (28).

Our endosymbiont sequences from *Pediculus humanus* were nearly identical to sequences from "Candidatus Riesia pediculicola," but the additional primate louse species had closely related yet distinctly different endosymbionts. The endosymbionts from *P. humanus* were more than 5% divergent from

**TABLE 1. Pairwise sequence divergence**

<table>
<thead>
<tr>
<th>Organism (country of origin, %A+T)</th>
<th>Pairwise sequence divergence</th>
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<tbody>
<tr>
<td></td>
<td><em>P. h. humanus</em> (United States)</td>
</tr>
<tr>
<td><em>P. h. humanus</em> (United States, 50.9)</td>
<td>0.0000</td>
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<tr>
<td><em>P. h. humanus</em> (United Kingdom, 50.9)</td>
<td>0.0031</td>
</tr>
<tr>
<td><em>P. h. capitis</em> (United States, 50.7)</td>
<td>0.0032</td>
</tr>
<tr>
<td><em>P. h. capitis</em> (Argentina, 50.8)</td>
<td>0.0395</td>
</tr>
<tr>
<td><em>P. schaeffi</em> (50.4)</td>
<td>0.1239</td>
</tr>
<tr>
<td><em>Pthirus pubis</em> (52.5)</td>
<td>0.1315</td>
</tr>
<tr>
<td>&quot;Candidatus Arsenophonus insecticola&quot; (52.2)</td>
<td>0.1315</td>
</tr>
</tbody>
</table>

* a Shown are pairwise differences for newly sequenced endosymbionts from primate lice compared to the most closely related taxon from GenBank ("Candidatus Arsenophonus insecticola" [GenBank accession no. DQ115536]). The GTR+I+γ maximum-likelihood model was used.
those from the chimpanzee louse, *P. schaeffi*, and endosymbionts from the human pubic louse, *P. pubis*, were more than 10% divergent from those of both *P. humanus* and *P. schaeffi*. Therefore, following recommendations for uncultured microorganisms (25, 26), we suggest that the endosymbionts of *P. schaeffi* and *P. pubis* be recognized as distinct species within the genus "*Candidatus* Riesia."

The current taxonomic name "*Candidatus Riesia pediculicola*" is ambiguous as to the specific host species with which it is associated. The specific epithet of "*Candidatus Riesia pediculicola*" refers only to the genus of the louse host, and yet there are two species of *Pediculus* (*P. schaeffi* and *P. humanus*) which have closely related P-endosymbionts. Therefore, in order to reduce further confusion, we propose to use the concatenated scientific names of the louse host for the specific epithet of the endosymbiont. We propose the name "*Candidatus Riesia schaeffii*" for the primary endosymbiont of the chimpanzee louse (*Pediculus schaeffi*). Similarly, we pro-

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**FIG. 1.** Phylogenetic tree based on Bayesian phylogenetic analysis of 10 million generations, produced using MrBayes. Posterior probabilities above 0.50 are shown above nodes. Endosymbionts of primate lice are monophyletic, with a posterior probability of 1.00. GenBank accession numbers are given in parentheses.
pose the name “Candidatus Riesia pthiripubis” for the primary endosymbiont of the human pubic louse (*Pthirus pubis*). By latinizing the full binomial, we can accommodate additional species within a genus, such as the endosymbiont from the gorilla louse, *Pthirus gorillae*, when and if it is characterized. Although the names are verbose, we find this nomenclature appropriate and beneficial.

**Coevolution and rates of substitution.** The two 16S sequences from the endosymbionts of human head lice differ from the two endosymbiont sequences from body lice by five fixed differences (sites 218, 492, 877, 950, and 1203) in our aligned matrix (available in TreeBase [www.treebase.org]). Although this appears to suggest distinction between the endosymbionts of head and body lice, recent additional sequence data acquired in the lab of D. L. Reed suggest this is an artifact of sampling and is not indicative of real phylogenetic differences between the endosymbionts of head and body lice. The head and body lice of modern humans have been reasonably well sampled, and their mtDNA can be divided into three distantly related haplotypes (18, 21, 42). The first mtDNA haplotype (type A) is worldwide in distribution and is the most common in both the head and body louse morphotypes. The second mtDNA haplotype (type B) has been found in the New World, Europe, and Australia and exists only in head lice. The third mtDNA haplotype (type C) has been found only among head lice from Nepal and Ethiopia. Unpublished data from our lab suggest that the endosymbionts of *P. humanus* mirror the host mtDNA haplotypes for *P. humanus*. This is perhaps not surprising given that both the endosymbionts and the mtDNA are maternally inherited in these lice.

The phylogenetic relationships of the endosymbionts of primate lice (both species of *Pediculus* plus *Pthirus*) are identical in topology to the phylogenies of their hosts (34), suggesting a long-term coevolutionary history between primate lice and their endosymbionts. Reed et al. (34) demonstrated that *P. humanus* and *P. schaeffi* diverged from one another ca. 5.6 million years ago based on mtDNA, which coincides precisely with the estimated divergence of their human and chimpanzee hosts based on mtDNA. The average percent sequence divergence (model corrected) between the endosymbionts of *P. humanus* and *P. schaeffi* is 3.75% (Table 1). We can use the 5.6 million year divergence date as a calibration point to determine the rate of nucleotide substitution in these endosymbionts, which equates to an absolute rate of 0.67% per million years. This rate is 15 to 30 times faster than the rates of 16S rRNA evolution estimated previously for *Buchnera*, the P-endosymbiont in aphids (1 to 2% per 50 million years) (24). A more thorough comparison of the “Candidatus Riesia” lineage with *Buchnera* will determine whether our rate calculations are indeed correct. It is conceivable that the same mechanisms that cause more mtDNA substitutions in lice than in aphids (17) have the same effect on the endosymbionts as well.

Because endosymbionts evolve more quickly than their hosts (24), they may record more nucleotide substitutions during recent (or rapid) evolutionary events than do their hosts. Because lice have strictly coevolved with their primate hosts and provide clear resolution of both recent and rapid evolutionary events (e.g., the population expansion of humans out of Africa is also evident in louse mtDNA), they have been used as a fast-evolving marker to examine different events in human evolutionary history (18, 21, 34, 42). The evidence for coevolution between “Candidatus Riesia spp.” and their primate louse hosts, along with the estimate of higher evolutionary rates in “Candidatus Riesia,” leads us to conclude that this new lineage of endosymbiotic bacteria shows promise as another independent evolutionary marker of primate and human evo-
volutionary history. If faster-evolving markers can be found within the endosymbionts (markers much faster than the 16S rRNA gene), then very recent events in human evolutionary history (e.g., peopling of the Americas) might be studied from the perspective of the endosymbiont of a human parasite. This new three-tiered assemblage of host, parasite, and endosymbiont is among the first tripartite assemblages of this type and will permit many new tests relating to their shared coevolutionary history.

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