Phylogenetic Relationship Between the Endosymbiont “candidatus *Riesia Pediculicola*” and Its Human Lice Host

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Research

Keywords: Human lice, Candidatus *Riesia pediculicola*, co-evolution, mitochondrial genes, housekeeping genes, phylogenetic analysis

Posted Date: September 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-895691/v1>

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Abstract

Background: The human louse is one of the most ancient haematophagous ectoparasites that is related intimately to its host and has been of great concern to public health throughout human history. Previously, *Pediculus humanus* was classified within six divergent mitochondrial clades (A, D, B, F, C and E). Like all haematophagous lice, *P. humanus* directly depends on the presence of bacterial symbionts, known as “*Candidatus Riesia pediculicola*”, to complement their unbalanced diet. In this study, we evaluated the coevolution of human lice around the world and their endosymbiotic bacteria. Using molecular approaches, we targeted lice mitochondrial genes from the six diverged clades and *Candidatus R. pediculicola* housekeeping genes.

Methods: A total of 126 lice were selected for molecular analysis of the *cytb* gene for lice clade determination. In parallel, four PCR primer pairs were developed targeting three housekeeping genes of *Candidatus R. pediculicola*: *ftsZ*, *groEL* and two regions of the *rpoB* gene (*rpoB*-1 and *rpoB*-2).

Results: The endosymbiont phylogeny perfectly mirrored the host insect phylogeny, using the *ftsZ* and *rpoB*-2 genes, suggesting a strict vertical transmission and a host-symbiont co-speciation following the evolutionary course of the human louse.

Conclusion: Our results unequivocally indicate that lice endosymbionts have experienced a similar co-evolutionary history, and that the human louse clade can be determined by their endosymbiotic bacteria.

1. Background

The human louse, *Pediculus humanus* (*Phthiraptera: Anoplura*), has been of great concern to public health throughout human history. It is one of the most ancient haematophagous ectoparasites which are intimately related to its host [1]. Two ecotypes can infest *Homo sapiens*: *Pediculus humanus corporis* and *Pediculus humanus capitis*. *P. h. corporis*, known as the body louse, infests people living in poor hygienic conditions and is the principal vector of *Rickettsia prowazekii* (epidemic typhus agent), *Borrelia recurrentis* (relapsing fever agent), *Bartonella quintana* (trench fever agent) [2, 3] and probably *Yersinia pestis* (pandemic plague agent) [4]. *P.h. capitis*, known as the head louse, has a widespread infestation regardless of the hygiene conditions [5,6]. However, its capacity as a potential vector of disease remains misunderstood [7]. The genetic diversity of human lice has been extensively investigated using mitochondrial genes (cytochrome b [*cytb*], cytochrome oxidase subunit 1 [*cox1*] and 12S ribosomal RNA [12S]), allowing their classification into six divergent clades that may be grouped in three sister groups (A–D, B–F and C–E), each exhibiting a specific geographical distribution [8]. Clade A is the most prevalent with a worldwide distribution, while clade D is only found in central Africa. Clade B is reported in all continents, while genetically close clade F has a geographically restricted distribution and was recently reported in South America. In addition, clade C has been identified in lice from central Africa and Asia, whereas clade E is reported in central and west Africa [6–9].

Like all haematophagous lice, *P. humanus* directly depends on the presence of endosymbiotic bacteria to supplement their unbalanced diet and metabolic integration [9]. Symbiosis is a common widespread phenomenon, which strongly affects the biology of haematophagous arthropods. This interaction encompasses a whole range of possible symbiotic associations, from strict parasitism to obligate mutualism [10]. Body and head lice host the same primary endosymbiotic bacteria (*Candidatus RIESIA pediculicola*) [9], which is a member of the *Gamma-proteobacteria* class, *Enterobacteriaceae* family within the *Candidatus RIESIA* genus [11, 12]. The symbiont is housed in mycetome that is localized on the ventral side of the lice midgut. In adult females the developing ovum is also infected for transovarial transmission to progeny [9, 11]. Genomic study of the human body louse and its primary endosymbiont has provided new insights into *Candidatus R. pediculicola* [11]. This bacterium has a small genome (< 600 genes) containing a panel of genes encoding for the synthesis of essential B-group vitamins that are crucial for the host's diet [13]. Indeed, the symbiont supplements the host with thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin and folate (B1, B2, B3, B5, B6, B7 and B9 vitamins) [13, 14]. In addition, removal of the mycetome in *Pediculus* females leads to their death a few days later, as well as to the production of deformed eggs [10, 13]. Moreover, *Candidatus R. pediculicola* can be targeted for possible lice control strategies, especially with the absence of antibiotic resistance genes [10, 13].

Human and chimpanzee lice (*Pediculus schaeffi*) diverged from a common ancestor, as did their human (*Homo sapiens*) and chimpanzees hosts (*Pan troglodytes*), somewhere between approximately five and seven million years ago. Interestingly, *Candidatus R. pediculicola* shared a common ancestor with the *P. schaeffi* endosymbiont (*Candidatus R. pediculischaeffi*), roughly 5.4 million years ago [14, 15]. The evaluation of this co-evolutionary association between louse and their endosymbionts might provide new insights into human evolution [15]. Furthermore, phylogenetic studies have shown a higher sequence similarity between clade A-head and body lice endosymbionts compared to a lower similarity in clade A and B-head lice endosymbionts. These results suggest that the endosymbionts co-evolved with their hosts' clades [15].

In this study, we aimed to establish a co-evolutionary relationship between the endosymbiotic bacteria and their human lice hosts from different clades using molecular approaches. We investigated mitochondrial genes from the six divergent clades of human lice and the housekeeping genes of *Candidatus R. pediculicola*, in order to determine the lice clade using its endosymbiont bacteria.

2. Methods

2.1 Lice selection and DNA extraction

Among our human lice collection in the IHU Méditerranée Infection laboratory, we selected 126 head and body lice collected from around the world to perform the molecular study "Additional file 1: Table S1". Prior to DNA extraction, external decontamination of the lice was performed as previously reported [16]. Each specimen was cut longitudinally, and one half was frozen for subsequent analysis. DNA extraction

was performed using the DNA extraction kit, QIAamp Tissue Kit (Qiagen, Courtaboeuf, France) using the EZ1 instrument following the manufacturer's protocol.

2.2 Lice genotypic status

2.2.1. Haplogroup identification using qPCR assays

To identify the lice mitochondrial clades, DNA samples were subjected to clade-specific quantitative duplex real-time PCR (qPCR) reaction targeting a portion of the *cytb* gene [17]. Each duplex is specific to clades A–D and B–C, noting that the B–C duplex also amplifies clade E lice, classified as sub-sister clade within clade C lice. DNA amplification was performed as described previously [18]. Lice with a known clade were used as a positive control, while negative controls consisted of the master mixtures.

2.2.2. Haplotype identification using standard PCR and sequencing

Based on qPCR results, 46 lice specimens encompassing the full range of clade diversity were selected for phylogenetic analysis. DNA samples were subjected to standard PCR targeting a 347-bp fragment of the *cytb* gene [19]. The final reaction consisted of a 25µl volume, including 12.5µL Amplitaq gold master mixes, 0.5µM of each primer, 5µL DNA template, and water. The *cytb* amplification was performed using the Applied Biosystems 2720 Thermal Cycler model, using the following thermal cycling profile: one incubation step at 95°C for 15 minutes, 40 cycles of one minute at 95°C, 30 seconds at 56°C and one minute at 72°C, followed by a final extension step for five minutes at 72°C. Successful amplification was validated by electrophoresis via 1.5% agarose gel. Amplicons were then purified using NucleoFast 96 PCR plates (Macherey-Nagel EURL, Hoerd, France) according to the manufacturer's instructions and sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems).

2.3. *Candidatus R. pediculicola* housekeeping gene analysis

2.3.1. Primer design

In order to investigate the genotypic profile of the endosymbiotic bacteria, four standard PCR systems were designed targeting three *Candidatus R. pediculicola* housekeeping genes: *ftsZ*, *groEL*, and two regions of the *rpoB*. Four genomes of *Candidatus R. pediculicola* belonging to clade A and B-lice deposited in the GenBank database (accession numbers CP012841, CP012843, CP012845, CP001085) [14, 15] were aligned using Muscle in MEGA7 software [20] and screened for conserved and discriminative genes.

Based on the variability in the available *Riesia* genomes, three housekeeping genes were selected as candidates for primer design. In order to find a suitable and conserved region for primers, two sequence fragments (~ 100bp) separated by a minimum of 500bp for each gene were submitted to Primer3 software v. 0.4.0 (available online: <http://primer3.ut.ee/>). The melting temperature of each primer was

tested using the free online software programme Oligo Analyzer 3.1 (available online: <https://eu.idtdna.com/calc/analyzer>) [21]. Designed primers (Table 1) were then tested in silico, using the NCBI BLAST nucleotide sequence similarity tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1
Details of designed primers for *Candidatus R. pediculicola* gene amplification

<i>Candidatus R. pediculicola</i>	Target gene	Primer sequences (5'-3')	Tm	Fragment length
<i>ftsZ</i>	<i>ftsZ</i> -196F_GGGAATTTCTGATCTTCTTCTGCG	56°C	454-bp	
	<i>ftsZ</i> -650R_CTTTACTGGATGCTTTTGGYGC			
<i>groEL</i>	<i>groEL</i> -560F_GATAGAGGTTATCTGTCTCC	50°C	631-bp	
	<i>groEL</i> -1191R_GCAGCTCKAGTAGCATGTA			
<i>rpoB</i>	<i>rpoB</i> -865F_ACCTGGTGATAAATCGTCTCC	54°C	677-bp	
	<i>rpoB</i> -1542R_GAAAGAATCGTTCAGAAAGATCGG			
	<i>rpoB</i> -2619F_CAGCCATCTCTCCGATTGAACG	56°C	466-bp	
	<i>rpoB</i> -3085R_CGATGGAAAGCTAATTTGTTTCAGC			
Tm: Melting temperature, bp: base pair				

2.3.2. Housekeeping gene amplification

Prior to endosymbiotic DNA amplification, designed primers were tested against a panel of negative controls consisting of the DNA of various bacterial species and arthropods "Additional file 2: Table S2". Once validated, 74 samples were randomly selected from the 126 lice to amplify *ftsZ*, *groEL*, and two *rpoB* regions of the endosymbiont (Table 1). Standard PCRs were performed as described for *cytb* and amplicons were visualised on a 1.5% agarose gel.

2.4. Data analysis

2.4.1. Phylogenetic analysis

To assess clade diversity in human lice, the obtained *cytb* sequences were combined and compared with worldwide *cytb* data previously reported and deposited in the GenBank database [8, 22]. Alignments were performed using MEGA7.0.26 software and a Maximum-Likelihood (ML) tree was constructed using the Kimura2-parameter model under 1,000 bootstrap replicates [23]. *Candidatus R. pediculicola* sequences extracted from 21 lice specimens were combined with data reported previously by Boyd *et al.* [21] and were analysed as described for the *cytb* gene.

2.4.2. Bioinformatic analysis

To define the genomic profile of the *Pediculus humanus* endosymbiont within different lice haplogroups, we screened the *Candidatus R. pediculicola* sequences obtained for the presence of Single Nucleotide Polymorphisms (SNPs) using the free online tool SNP-Dists (<https://github.com/tseemann/snp-dists>). The free online Morpheus website (<https://software.broadinstitute.org/morpheus>) was used to visualise and analyse the resulting heatmap. In addition, to investigate our hypothesis more thoroughly, the degree of genomic similarity of deposited *Candidatus R. pediculicola* genomes (listed above) [15], was estimated using the Orthologous ANI Tool (OAT) [24].

3. Results

3.1. Lice clade identification

In this study, 110/126 (87%) head lice and 16/126 (13%) body lice were collected worldwide “Additional file 1:Table S1” and were selected for their clade determination. A qPCR assay revealed results for the 125/126 specimens tested. Overall, 76/125 (61%) of lice belonged to the most prevalent clade A with various origins including West Africa 17/76 (22.4%), Central Africa 10/76 (13.2%), North Africa 15/76 (19.7%), South Asia 24/76 (31.6%), Europe 2/76 (2.6%) and 8/76 (10.5%) were rearing lice used here as a reference. As for clade D, 8/125 (6.3%) specimens were genotyped and collected in the African continent (6/8 (75%) and 2/8 (25%) from Central and West Africa, respectively). Furthermore, 39/125 (31.2%) of the analysed specimens belonged to clades C/E and were distributed worldwide (21/39 (53.8%) and 11/39 (28.2%) collected respectively from West and Central Africa and 7/39 (18%) from Europe). Finally, only 2/125 (1.5%) samples belonged to clade B and were collected in South Asia (Pakistan).

Standard PCR and sequencing of the *cytb* gene of the 46/126 (36.5%) samples revealed that 12/46 (26.1%) belonged to clade A, while 6/46 (13%) belonged to clade D, 2/46 (4.3%) specimens were clade B and only 1/46 (2.2%) belonged to the novel clade F. In addition, 4/46 (8.7%) specimens belonged to clade C, while 21/46 (45.7%) of lice belonged to clade E (Table 2). Phylogenetical analysis was performed on 21 representative samples of all lice clades and origins (Fig. 2-A).

Table 2
Results of mitochondrial analysis and *Candidatus R. pediculicola* housekeeping genes of human lice samples

Origin	Country	Nb of lice	Clade mitochondrial analysis		<i>R. pediculicola</i> housekeeping gene analysis			
			Nb lice-qPCR. Clade	Nb lice-PCR. Clade	ftsZ gene	groEL gene	rpoB-1 gene	rpoB-2 gene
West Africa	Senegal	10	3.A; 2.D; 5.C	2.A; 2.D; 4.E	2.A; 2.D; 4.E	2.A; 2.D; 3.E	2.D; 1.E	2.A 2.D
	Guinea	6	6.C	6.E	6.E	6.E	6.E	6.E
East Africa	Ethiopia	3	3.C	2.C	3.C	3.C	2.C	2.C
Central Africa	Gabon	1	1.C	1.C	1.C	1.C	1.C	1.C
	DRC	6	4.D; 2.C	4.D; 2.E	4.D; 2.E	3.D; 2.E	3.D; 1.E	3.D; 2.E
	Congo Brazzaville	3	3.C	NT	NT	NT	3.C	NT
North Africa	Algeria	3 ^b	3.A	3.A	2.A	2.A	1.A	2.A
	Morocco	6	6.A	2.A	6.A	6.A	6.A	6.A
South Asia	India	13	13.A	2.A	11.A	10.A	12.A	11.A
	Pakistan	12	11.A; 2.B	2.A; 2.B	10.A; 2.B	11.A; 2.B	11.A; 2.B	11.A; 2.B
Europe	France	3	2.C	1.C; 1.F	2.C; 1.F	2.C; 1.F	1.C; 1.F	1.F
USA	Orlando strain	7 ^b	7.A	2.A	6.A	6.A	6.A	6.A
^b : Body lice; Nb : number; NT : not tested								

3.2. Phylogenetic relationship between the endosymbiont and their lice host

In order to determine the phylogenetic profile of *Candidatus R. pediculicola*, 74/126 (58.7%) specimens were selected for DNA amplification and sequencing of three housekeeping genes (*ftsZ*, *groEL* and *rpoB*). In total we were able to generate all four fragments in 46/74 (62.2%) selected louse (65/73 (89%), 62/73 (84.9%), 60/73 (82.2%) and 57/73 (78.1%) sequences respectively for the *ftsZ*, *groEL*, and the first and second region of the *rpoB* genes) (Table 1). However, 27/46 (58.7%) of the endosymbionts were within

clade-A lice collected worldwide (7/27 (26%) North Africa, 16/27 (59.3%) South Asia and 4/27 (14.7%) were generated from Orlando strain). While 4/46 (8.6%) sequences belonged to clade D lice and originated from West and Central Africa. Furthermore, 2/46 (4.4%) specimens were clade-B endosymbionts collected in South Asia (Pakistan). Only 1/46 (2.2%) belonged to the novel clade F collected in Europe (France). Finally, 4/46 (8.7%) were clade-C endosymbionts collected in East Africa, while for its sister clade, 8/46 (17.4%) endosymbiont sequences belonged to clade E collected from West and Central Africa (Table 2).

Maximum likelihood phylogenetic trees were constructed for the housekeeping genes including 21 samples already analysed for the *cytb* gene. Interestingly, lice endosymbionts from each mitochondrial host clade clustered in a separate group (Fig. 1- a, d), while the phylogeny based on the endosymbiotic *ftsZ* and the second region of *rpoB* (*rpoB*-2) genes followed that of *cytb* lice, which is not the case for the *groEL* and first region of *rpoB* (*rpoB*-1) genes (Fig. 1- b, c). To further investigate the present co-evolutionary relationship, we proceeded to concatenate *ftsZ* and *rpoB*-2 sequences (848-bp of the final fragment), and a phylogenetic tree was generated and compared to that of *cytb*. We noted that the concatenated 848-bp fragment of different *Riesia* harboured a higher bootstrap value and grouped in clades which were almost perfect compared to the one gene-based tree. In addition, the clustering of sister clades was also visible for the B–F and C–E clades, mimicking the mitochondrial phylogeny of *P. humanus* (Fig. 2).

The concatenated fragments were tested for the presence of SNPs. Results were in concordance with those obtained through phylogenetic analysis. All endosymbionts from sister clade lice clustered together and harboured a low rate of SNPs. Lower rates of SNPs were obtained within the sister haplogroup B and F with only four SNPs. The highest number of SNPs was observed between clades A and B with 28 present SNPs (Fig. 3).

Degree of genomic similarity of the deposited endosymbiont genomes of clade A and B lice [14] strongly support our results. A higher similarity (99.97%) was observed between *Candidatus R. pediculicola* of clade A head and body lice, with a lower similarity (97.85%) observed between clade A and B *P. h. capitis* specimens "Additional file 3: Figure S1".

4. Discussion

In this study, we highlighted the coevolutionary relationship of *Candidatus R. pediculicola* endosymbionts and their *Pediculus humanus* host's mitochondrial clades. Lice endosymbionts were first reported in the 1920s, and their characterisation was subsequently successful through various histological, embryonic, experimental, and nutritional studies [25]. This obligate intracellular primary endosymbiont is fully and uniquely attached to its host [13]. Its major role resides in providing essential B vitamins that are crucial for survival of the louse and lacking in the delivered blood meals [12, 26]. Furthermore, this bacterium had never been isolated in pure axenic culture, but molecular approaches presumed a polyphyletic origin [27]. Endosymbiotic microorganisms are generally associated with diverse arthropods such as *Buchnera* of

aphids, *Carsonella* of psyllids, *Portiera* of whiteflies, *Sulcia* of many homopterans, *Baumannia* of sharpshooters, *Blochmannia* of carpenter ants, *Nardonella* of weevils, as well as for bloodsucking insects such as *Wigglesworthia* of tsetse flies [28, 29]. Their endosymbiont phylogeny generally mirrors the host phylogeny, indicating a stable and intimate host-symbiont association over time. This is also the case of the *Nycteribiidae* family of bat flies, involved in ectoparasitic blood-feeding on bats, its endosymbiont *Candidatus Aschnera chinzei* clades phylogeny co-speciates over the evolutionary course of the *Nycteribiidae* [24]. The adjacent clade to the endosymbiont of nycteribiid bat flies is the endosymbionts of primate lice, *Riesia* spp., together forming sister clades within the *Gammaproteobacteria* class [30, 31]. Allen *et al.* dated the divergence between the *Riesia* and *Arsenophonus* (Endosymbiont of *Lipoptena cervi*) clades at 13–25 million years ago, making the louse–*Riesia* association one of the youngest [32]. Furthermore, 16S rDNA sequences confirmed a strict coevolution between the endosymbionts of *Anoplura* (i.e., *Haematopinus* sp. of ungulates, *Solenoptes* sp. of cattle, *Pediculus* sp. of hominids and *Polyplax* sp. of rodents) and *Rhyncophthirina* (*Haematomyzus* sp. of Asian elephants) genera. The endosymbiont sequences formed five separate monophyletic branches, each composed from only one louse genus [33]. These findings suggest that every louse group has their own endosymbiont.

We demonstrated the evolutionary phylogenetic relationship that connects *Candidatus R. pediculicola* to their host mitochondrial clades. Phylogenetic trees based on *ftsZ*, *rpoB-2* and their concatenated genes enabled an identical endosymbiotic clusterization depending on human lice clades. The endosymbiont phylogeny perfectly mirrored the host insect phylogeny, suggesting strict vertical transmission and a host–symbiont co-speciation during the evolutionary course of the human lice. These data will allow the classification of human lice clades through their endosymbiotic bacteria based on the *ftsZ* and *rpoB* genes. The lack of discrimination of endosymbiotic phylogenetic status targeting the *groEL* gene is probably due to the absence of a sufficient number of mutations within this gene. Our findings need further investigation by sequencing and analysing the endosymbionts' whole genomes within all human lice clades to better establish the evolutionary time courses within their hosts.

5. Conclusion

Based on phylogenetic and genomic analyses, this work highlights the co-evolutionary relationship between *Candidatus R. pediculicola* and their host mitochondrial clades. Our results unequivocally indicate that endosymbiont lice have experienced a similar co-evolutionary history and that human lice clades can be determined by their endosymbiotic bacteria based on their *ftsZ* and *rpoB* housekeeping genes. In the future, further robust phylogenetic examination of all endosymbiont genome clades will be fundamental to better understand the evolution of *Candidatus R. pediculicola* depending on the mitochondrial divergence of their hosts. However, it is crucial to isolate and identify this bacterium in order to evaluate the effectiveness of a drug treatment targeting the lice endosymbiont.

Declarations

Acknowledgements: Thanks to “IHU Méditerranée-Infection” for its support

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the “Investissements d’avenir” programme, reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d’Azur and European ERDF PRIMI funding.

Data Availability Statement: Sequences of the *Candidatus R. pediculicola* housekeeping genes referred to here with the attributed GenBank accession numbers: *ftsZ* gene: clade A (Orlando-MW588459, Senegal-MW588463), clade D (Congo-MW588466, Senegal-MW588464, clade B (Pakistan- MW588462), clade F (France-MW588460), clade C (France-MW58846) and clade E (Congo- MW588465, Guinea-MW588458). Second region of endosymbiont *rpoB* gene: clade A (Orlando-MW588485, India-MW588486, Algeria-MW588487), clade D (Senegal-MW588488, Congo- MW588489), clade B (Pakistan-MW588491), clade F (France-MW588490), clade C (Ethiopia- MW588492) and clade E (Congo-MW588494, Guinea-MW588493).

Author contributions: AH, ML and OM designed the study. AH performed the experiments. AH, ML and OM analysed the data. AH and ML wrote the manuscript. AH, ML, DM, FF and OM edited and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare that they have no competing interests. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Figures

Figure 1

Maximum Likelihood (ML) phylogenetic trees of the *P. humanus* endosymbiont housekeeping genes, distributed in six divergent clades. a: ML tree of a 454-bp fragment of the *ftsZ* gene; b: ML tree of a 631-bp fragment of the *groEL* gene; c & d: ML tree of the 677-bp and 466-bp fragment of the *rpoB* gene (*rpoB*-1 and *rpoB*-2 respectively). Phylogenetic inference was conducted in MEGA 7 using the maximum likelihood method under the Kimura 2-parameter with 1,000 bootstrap replicates.

Figure 2

Phylogenetic tree of the cytb mitochondrial gene of *P. humanus* (A) projected with the concatenated *ftsZ* and *rpoB* (*rpoB*-2) genes (848-bp) of *Candidatus R. pediculicola* (B), showing the relationship and coevolution of the endosymbiont dependently on the mitochondrial clades of their host. Phylogenetic inference was conducted in MEGA 7 using the maximum likelihood method under the Kimura 2-parameter with 1,000 bootstrap replicates.

Figure 3

Heatmap representing the numbers of SNPs present between *Candidatus R. pediculicola* isolated from *P. humanus* belonging to clades A to F. Matrix generated by performing a pairwise SNP distance from a FASTA sequence alignment using Dists software (<https://github.com/tseemann/snp-dists>). Matrix visualisation and heatmap analysis using Morpheus software, (<https://software.broadinstitute.org/Morpheus>). SNP values presented here are calculated from concatenated sequences of *Candidatus R. pediculicola* (*ftsZ* and *rpoB* genes).

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