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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe permettant un meilleur rangement des thèses traditionnelles. Par ailleurs, la partie introduction et la bibliographie sont remplacées par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

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RESUME

L'objectif de la thèse était d'enrichir les connaissances sur les poux de tête et les poux de corps de l'homme. Les poux de tête vivent et pondent leurs œufs à la base des cheveux et sont très répandus chez les enfants dans les écoles. Les poux de corps vivent dans les vêtements et infestent les personnes de milieux sociaux très défavorisés ne permettant pas une hygiène vestimentaire adéquate. Les poux de corps sont vecteurs de trois grandes maladies : le typhus épidémique, la fièvre des tranchées et la fièvre récurrente à poux. Ces insectes hématophages sont étudiés depuis des décennies afin de déterminer si ils constituent deux écotypes de la même espèce ou deux espèces distinctes.

Avant l'avènement des techniques de biologie moléculaire, la taxonomie des poux était basée sur leur morphologie et leur biologie. Bien que les différences biologiques décrites entre les poux de tête et les poux de corps ne sont pas toujours cohérentes, il est légitime de penser que leur séparation physique puisse mener à une différenciation spécifique. Certaines études expérimentales ont cependant montré certains cas de migrations de poux de tête vers les vêtements et inversement. Puis, grâce au séquençage du génome du pou, beaucoup de progrès ont été réalisés ces dernières années sur leur phylogénie. Ainsi, sur base de l'ADN mitochondrial, les poux de l'homme sont séparés en trois Clades phylogénétiques : le Clade A qui comprend à fois des poux de tête et des poux de corps et les Clade B et C qui comprennent uniquement des poux de tête. Cette organisation phylogénétique en trois clades montre de façon surprenante que les poux de tête de Clade A sont plus proches des poux de corps, que des poux de tête de Clade B ou C. Les poux de corps étant vecteurs de maladies, il est important de mieux comprendre l'épidémiologie des poux de l'homme.

Durant cette thèse, nous avons tout d'abord réalisé une bibliographie exhaustive qui a mené à la rédaction d'une revue. Ensuite, nous avons voulu évaluer la présence de corrélations entre phénotypes et génotypes au sein des poux de l'homme. Finalement, aucune corrélation n'a pu être établie entre la phylogénie des poux et d'une part leur couleur et d'autre part leur écotype (pou de tête ou pou de corps). Cependant, la phylogénie des poux (basée sur l'espace intergénique PM2) et leur source géographique étaient corrélées, ce qui nous a mené à décrire au sein du Clade A, trois sous clusters : les poux d'Afrique Subsaharienne d'une part et d'autre part, les poux hors Afrique Subsaharienne qui comprennent un cluster distinct : les poux d'Amazonie. Aussi, le séquençage d'autres espaces intergéniques semble témoigner que des événements de recombinaisons ont

lieu entre des poux de différents continents. Ces événements peuvent être corrélés aux mouvements de populations humaines. Des travaux complémentaires devraient pouvoir confirmer ces hypothèses.

Ensuite, afin de mieux comprendre l'épidémiologie des deux écotypes, nous avons réalisé une étude de génétique des populations sur des poux de tête et des poux de corps provenant de cinq sans-abris infestés par les deux écotypes et vivant dans le même foyer. Nous avons ainsi pu montrer que les poux de tête et les poux de corps de Clade A pouvaient avoir les mêmes allèles et appartenir à une seule population. Cependant, nous pensons que cela ne se produit que dans des cas d'infestations massives favorisant la migration de poux d'une zone à l'autre du corps. De plus, nos résultats montrent qu'il y a un très fort taux d'échange de poux entre individus habitant un même foyer et mettent en évidence l'urgence d'améliorer les conditions sanitaires dans les foyers pour limiter au maximum la transmission de poux d'une personne à une autre.

Enfin, nos travaux sur des échantillons du Sénégal confirment la présence d'ADN de *Bartonella quintana* dans les poux de tête. Cependant, leur rôle dans l'épidémiologie de la maladie reste à être déterminé. En effet, nous pensons que l'infection des poux de tête n'a lieu que dans des situations particulières : soit en association avec des infestations de poux de corps ou dans des conditions sociales très pauvres.

En conclusion, cette thèse nous a permis de montrer l'utilité d'utiliser les espaces intergéniques pour l'étude de la phylogénie des poux. Leur phylogénie étant corrélée à leur provenance géographique, il serait intéressant de poursuivre les travaux sur de plus larges échantillons. De plus, à plus petite échelle, notre étude de génétique des populations chez les sans-abris de Marseille a démontré que les poux récoltés sur la tête et sur les vêtements appartiennent à une même population. Il est donc important de continuer à tester l'infection des poux de tête par des bactéries surtout dans les environnement défavorisés où ils pourraient également jouer un rôle épidémiologique notamment en association avec les poux de corps.

Mots clefs : poux de tête, poux de corps, génotypage, phylogénie, épidémiologie

ABSTRACT

The objective of this thesis work was to increase the knowledge of human head lice and body lice. Head lice live and lay their eggs at the base of hair shaft and are found frequently in school going children. Body lice live in clothes and are usually associated with low income persons that do not have adequate clothes hygiene. Body lice are the vector of three major diseases: epidemic typhus, trench fever and relapsing fever. These blood feeding insects have been studied over the past decades to determine whether they are two ecotypes of the same species or two distinct species.

Before the advent of molecular biology, taxonomical classification of lice was based on their morphology and biological activities. Although described biological differences between head lice and body lice are not always consistent, their physical separation could lead to species differentiation. However, some experimental studies have shown that in certain cases head lice could migrate to the clothes and vice versa. In the recent years many progress were made in the body louse genome sequencing, and further their phylogenetic classification. Thus, on the basis of mitochondrial DNA, human lice are classified into three phylogenetic clades: Clade A that comprise both head lice and body lice and the Clades B and C that comprise only head lice. This phylogenetic organization clustered into three clades surprisingly shows that head lice of Clade A are closer to body lice than to head lice of Clade B or C. Since body lice serve as vectors of several diseases, in view of this, it is crucial to understand human lice epidemiology.

During the thesis work, extensive literature survey was done to write a review. Then, we aimed to estimate the correlation between phenotypes and genotypes among human lice. Finally, no correlation could be inferred between lice phylogeny and their color or the ecotype (head lice or body lice). However, the lice phylogeny (based on intergenic spacer PM2) and their geographic source were correlated. Hence, we described that, within the Clade A, Sub-Saharan lice, Non-Sub-Saharan lice and Amazonian lice form three distinct subclusters. Moreover, the sequencing of other intergenic spacers showed that interbreeding events occur between lice from different continents. These events could be correlated to migration of human populations. However, further work is needed to confirm this hypothesis.

Furthermore, in order to gain better insight of these two ecotypes epidemiology, we have done population genetic studies on head lice and body lice collected from five homeless persons infested by both ecotypes

and living in the same shelter. The obtained results showed that Clade A head lice and body lice may have the same alleles and belong to the same population. This probably happens only in the course of massive infestations enhancing the migration of lice from one place of the body to the other. Moreover, our results showed that there is a very high exchange of lice among individuals living in the same shelter and highlight the urgency to improve sanitary conditions in shelters to minimize as much as possible lice transmission from one person to the other.

In addition, our work on Senegal samples confirmed the presence of genomic DNA of *Bartonella quitana* in head lice. However, their role in the disease epidemiology remains to be determined. Indeed, we think that head lice infection happens only in particular situations: either in association with body lice infestations or in very poor social conditions.

In conclusion, the undertaken PhD thesis work showed the utility to use intergenic spacers to study lice phylogeny. Their phylogeny being correlated to their geographic origin, this would be interesting to pursue the work on larger samples. Moreover, at a smaller scale, our study of population genetic in homeless persons in Marseille has shown that lice collected from the head and clothes belong to the same population. Thus, it is important to continue to test head lice infection by bacteria above all in deprived environments where they could also play an epidemiological role notably in association with body lice.

Key words: head lice, body lice, genotyping, phylogeny, epidemiology

INTRODUCTION

Les poux sont des insectes hématophages qui font partie de l'Ordre des Phthirapterae. Quatre sous-ordres de Phthirapterae sont reconnus : trois sous-ordres de poux mâcheurs ou broyeurs (« chewing lice » en anglais) : les Amblycera, Ischnocera and Rhynchophthirina et un sous-ordre de poux piqueur-suceurs (« sucking lice » en anglais) : les Anoplura [1]. Les premiers sont des poux qui, pour prendre leur repas de sang déchiquettent la peau de l'hôte pour faire un lac de fluides dont ils se nourrissent. Ils parasitent les oiseaux et les mammifères. Les Anoplura, ont des pièces buccales de type piqueur-suceur qui leur permettent de prélever le sang directement dans les vaisseaux. Ils parasitent uniquement les mammifères euthériens et ce sont parmi eux que l'on retrouve les deux familles de poux qui parasitent l'homme: les Pthiridés et les Pédiculidés [2]. Au sein des Pthiridés, *Pthirus pubis* (ou pou du pubis) est un parasite qui se transmet essentiellement par contact sexuel. Il vit accroché aux poils du pubis et a une forme qui fait penser au crabe d'où son nom en anglais « crab louse » [3]. Au sein des Pédiculidés, on trouve le pou de tête et le pou de corps de l'homme qui ont fait l'objet de notre étude tout au long de cette thèse.

D'un point de vue morphologique, ces deux types de poux sont très similaires mais ils vivent dans deux écosystèmes distincts. Les poux de tête (*Pediculus humanus capitis*) vivent sur le cuir chevelu et pondent leurs œufs à la base des cheveux. Ils provoquent des épidémies dans les écoles et sont à priori inoffensifs mis à part l'apparition de réactions allergiques ou de surinfections provoquées par un prurit intense [4]. Les poux de corps ou « pou du vagabond » (*Pediculus humanus corporis*), vivent et pondent leur œufs dans les vêtements au plus proche de la peau. Ils infestent les personnes vivant dans des conditions d'hygiène vestimentaire précaire. Le pou de corps est vecteur de trois grandes maladies : le typhus épidémique

(*Rickettsia prowazekii*), la fièvre des tranchées (*Bartonella quintana*) et la fièvre récurrente à poux (*Borrelia recurrentis*) [5]. Il a ainsi laissé de tristes traces de son passage dans l'Histoire notamment en décimant des centaines de milliers de personnes dans le monde principalement dans des contextes d'instabilité politique, de guerres ou dans des environnements clos et peu hygiéniques comme les prisons. Malheureusement, ces parasites sont toujours répandus de nos jours dans les pays en voie de développement et sont également émergents dans les pays industrialisés, chez les populations de sans-abris des grandes villes [6].

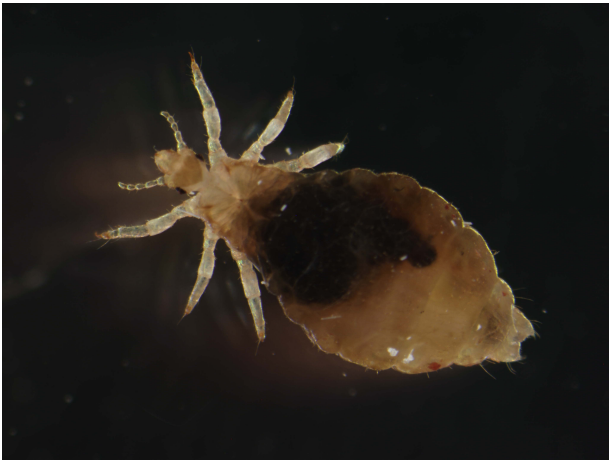
Les poux de tête et les poux de corps sont étudiés depuis des décennies. Au départ, leur morphologie et leur biologie ont été les critères utilisés afin de déterminer s'ils représentent deux espèces distinctes ou bien deux écotypes de la même espèce [7]. Ensuite, grâce à l'avènement des techniques de biologie moléculaire et au séquençage du génome du pou de corps [8], énormément de progrès ont été réalisés ces dix dernières années sur la taxonomie des poux [9–17]. Aussi, étant donné que le pou de corps est vecteur de maladies sévères et que des données suggèrent que le pou de tête pourrait servir de réservoir potentiel [12], il est crucial de déterminer si les épidémies de poux de tête et de poux de corps sont indépendantes ou si dans certains cas, les deux types de poux vivent en sympatrie.

Jusqu'à présent, aucune revue sur les poux de tête et les poux de corps n'a tenté d'associer à la fois les données « biologiques » et « génétiques ». Il est pourtant crucial d'intégrer les deux types d'information afin de bien comprendre l'épidémiologie des poux de l'homme. Ainsi, au cours de cette thèse, j'ai rédigé une revue reprenant l'ensemble des travaux pré et post-génétiques qui étudient les poux de tête et des poux de corps. Elle est présentée à la fin de la présente INTRODUCTION. Ensuite, suivent les CHAPITRES 1 à 4 dans lesquels sont présentés les Articles qui sont le fruit des travaux de recherche réalisés dans le cadre de ma thèse. Les articles 1 à

3 sont des travaux sur la génétique des poux de tête et des poux de corps. L'article 4 traite des infections des poux par les bactéries. Enfin, ce manuscrit finit par la partie CONCLUSIONS GENERALES ET PERSPECTIVES dans laquelle l'ensemble de nos résultats sont discutés tout en proposant de nouvelles pistes de recherche qui pourraient mener à une meilleure compréhension de l'histoire et de l'épidémiologie de ces parasites.

Revue

Current knowledge of the biology and genetics of human head and body lice



Préambule

Cette revue bibliographique permet à un lecteur non spécialiste des poux de mieux appréhender la lecture de ma thèse. Elle permet de situer mon travail dans un contexte global et de comprendre ce qui nous a poussé à entreprendre les travaux développés dans les articles 1 à 4.

Tout d'abord, un premier chapitre est dédié aux études menées sur les poux de tête et les poux de corps avant l'avènement de la génétique. Ces travaux étaient entièrement réalisés sur bases de critères morphologiques et éthologiques.

Le deuxième chapitre est consacré au génome du pou [8]. La structure du chromosome, le contenu génique et les fonctions géniques sont dans un premier temps développés. Ensuite, des données sur l'unique génome mitochondrial du pou sont mentionnées [18]. En effet, chez les poux, les 37 gènes mitochondriaux sont localisés sur 18 mini-chromosomes circulaires à la place d'un seul chromosome. Ce type de chromosome mitochondrial circulaire, présent chez les autres poux piqueurs-suceurs mais pas chez les poux mâcheurs, semble avoir co-évolué avec le fait de se nourrir exclusivement de sang [18].

Ensuite, des données sur le symbionte du pou [19], son génome [8] et le génome des bactéries transmises par le pou ont également été incluses car ces génomes sont particulièrement petits étant donné la très grande spéciation de leur hôte [20–22]. Les poux sont paradigmatiques de la coévolution avec leur hôte et sont probablement les seuls insectes se nourrissant uniquement sur l'homme.

La dernière partie a été dédiée aux études génétiques réalisées ces dix dernières années sur les poux de tête et de corps. Elle cite notamment des travaux que j'ai réalisés lors de ma thèse et qui seront développés dans ce manuscrit. Aucune autre étude n'avait jusqu'à présent réalisé une synthèse

bibliographique exhaustive comprenant à la fois des études morphologiques, éthologiques et génétiques des poux de tête et des poux de corps.

Revue

Current knowledge of the biology and genetics of human head and body lice

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In revision

Unité de recherche sur les Maladies infectieuses tropicales émergentes,

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Abstract

Human head and body lice have long been studied. Originally, their morphology and biology were used to assess whether they represented distinct species. As a result of body louse genome sequencing, much progress has been made over the last ten years regarding lice taxonomy.

Here, we review pre- and post-genetic studies on lice taxonomy, discuss the results of those studies and conclude that the two ecotypes have identical genomic content and differ only in gene expression, resulting in different phenotypes. Data on the human louse and its P-endosymbiont genome, as well as the genomes of louse-transmitted bacteria, are also included.

1. Introduction

Human lice

The order of *Phthirapterae* (lice) is divided into two main groups: the sucking lice that comprise the Anoplura suborder and the chewing lice that comprise three other suborders: Amblycera, Ishnocera and Rhynchophthirina (Figure 1) [1]. Lice are obligate ectoparasites, with each host species having its own type of louse [2]. Indeed, the parasite speciation often occurs at approximately the same time as its host's speciation (cospeciation). The two genera of sucking lice that parasitize humans are *Pthirus* and *Pediculus* (Figure 1), which includes two species of medical importance, *Phtirus pubis* (pubic louse) and *Pediculus humanus*. The latter is of great public health importance and consists of two ecotypes: head lice and body lice. Both ecotypes have the same life cycle, beginning with an egg stage of approximately 7 days, followed by three instars of approximately 3 days each before becoming adults that are capable of reproducing. Both lice need to take regular blood meals (approximately 5 times a day) on human skin to survive. However, they live in different ecological niches. Head lice live in human hair and are very commonly found among children. They are responsible for allergic reactions with very intense pruritus that may lead to high irritation and even wound infection. Body lice live in clothes and are associated with a lack of clothing hygiene and cold weather. They are often found in jails and unstable countries but are also currently re-emerging among homeless populations in industrialized countries [3].

Bacteria found in lice and louse-transmitted diseases

Body lice are responsible for the transmission of at least three bacterial diseases (Figure 1). Among them, two belong to the α subgroup of Proteobacteria (*Rickettsia prowazekii* and *Bartonella quintana*) and one is a

spirochete (*Borrelia recurrentis*). *R. prowazekii* is the etiologic agent of epidemic typhus, *B. recurrentis* causes louse-borne relapsing fever, and *B. quintana* causes trench fever [4]. Two other bacteria were found in body lice, *Acinetobacter* spp. and *Serratia marcescens* [5] but it is not known if they can be transmitted to humans by lice biting. Head lice have not been considered vectors of human diseases. However, recently, they have also been found to be infected by *Bartonella quintana* [6–9]. However, their role in trench fever transmission remains undetermined. Head lice were also found to be infected with *Acinetobacter baumanii*, but the clinical significance of this finding is unknown [10].

Body lice and head lice harbor the same endosymbiotic microorganisms (*Candidatus* Riesia pediculicola) that seem to be essential for the production of nutritional components, such as B vitamins, that are lacking in the host blood [11,12]. The primary endosymbiont and the bacterial pathogens harbored by body lice all possess genomes that are reduced in size compared to their free-living close relatives [13]. Thus, lice offer an appropriate model for understanding the coevolution of vectors, symbionts and pathogens in a specific niche in allopatry [13].

The aim of this review

Here, we propose the first exhaustive review of data on human head lice and body lice. First, we focus on relevant comparative studies on human head and body lice based on their morphology and biology before the advent of molecular biology tools. Second, we present information on the body louse genome, the genome of its symbiont and some data on the genome of the pathogens transmitted by body lice. Finally, the main genetic studies on human lice performed during the last ten years are reviewed and discussed, and some assumptions on human lice evolution are made.

2. Human lice taxonomy before molecular biology

The morphology and biology of these two types of lice, as reported over several decades, were used to assess the taxonomic status of head and body lice (Table 1).

2.1. Early classification

The genus *Pediculus* was established by Linnaeus in 1758. This genus was applied to both head and body lice, which he called *Pediculus humanus* varieties 1 and 2 in 1767. In 1778, De Geer proposed naming these varieties *Pediculus humanus capitis* and *Pediculus humanus corporis*, without determining whether these varieties should be considered separate species [14].

2.2. One or two species debate

2.2.1. Morphological characteristics

Among the numerous authors that described lice morphologically, Fahrenholz considered head and body lice to be two distinct species and wrote a detailed classification of these species that included their measurements, shape and pigmentation [15]. However, these descriptions were criticized by Nuttall in 1919 and 1920, because these morphological characteristics were proved to be inconsistent with the description of many intermediate forms [15]. Nevertheless, in 1985, Busvine observed from six double infestations in Ethiopia, under conditions where interbreeding could theoretically occur, that head lice and body lice possessed distinct nonoverlapping sizes, thus supporting the distinction of the two species [16].

2.2.2. Feeding

According to Nuttall [17], the *capitis* and the *corporis* forms feed the same way if they are reared under the same conditions. Nuttall explained that the greater blood intake by the *corporis* form resulted from the more difficult access to blood for lice in clothes as they deal with the host body movement. Moreover, this greater blood intake (increased internal pressure) of the *corporis* form explains its larger average size, loss of angularity in the abdominal segments and the more widely separated hairs upon the abdominal surface, compared to the *capitis* form.

2.2.3. Rearing observations

The typical *capitis*, which are raised on humans under conditions that are favorable for *corporis*, gradually become morphologically indistinguishable from *corporis* after 4 to 5 generations [18,19]. Hence, Nuttall concluded that the typical *capitis* and *corporis* represent the extremes in the variation of the species *Pediculus humanus* [17]. However, a similar work by Busvine in 1948 was unable to confirm these observations [20].

2.2.4. Intermingling of *capitis* and *corporis* in nature

Many cases support the intermingling of the two forms of lice when they invade each other's feeding grounds [19,21,22]. Bacot observed that *capitis* and *corporis* pair freely and that their offspring are fertile [21]. However, Keilin and Nuttall observed the occurrence of an abnormal sex-ratio in the progeny of crosses with a great decrease in the proportion of females to males and the appearance of hermaphrodites [19]. Interestingly, in our lab, we found lice eggs on a cap from a homeless person, confirming that head lice may lay eggs on clothes [23]. Finally, a study undertaken in 2003 further confirmed that head lice may be established on the body [24].

2.3. Several subspecies or varieties debate

Fahrenholz classified lice into 6 subspecies on the basis of lice morphology and pigmentation: 3 subspecies of *capitis* (*P. capitis angustus*, *P. capitis maculatus* and *P. capitis capitis*) and 3 subspecies of *corporis* (*P. nigritarum*, *P. chinensis* and *P. humanus humanus*). Each of these species occur on what Fahrenholz called different "human races" [15]. Nuttall stated that using pigmentation as a criterion to describe and differentiate lice may lead to errors in differentiation, since unpigmented structures that may not be seen may be reported as absent even though they are effectively present [15]. Moreover, according to Shipley [25] and Nuttall [26], A. Murray reported that lice imitate the color of the skin upon which they live. He described a series of color gradations according to lice origin, extending from the black louse to the light grey louse. However, the accuracy of these results has been doubted by several authors who stated that the color difference was inconsistent, since a great variety of lice colors may be found on a single host [26]. Moreover, additional experimentations implemented by Nuttall showed that the pigmentation was entirely dependent on the color of the background and was not a genetically transmitted characteristic. He explained that the variability in lice colors on a single host was affected not only by the color of the skin but also by the color of the hair and clothing [26]. These results were confirmed in 1946 by Busvine [27]. Finally, H. E. Ewing used morphological characteristics to propose an identification key for American lice that included five varieties of human lice: *P. humanus nigritarum* Fabricius (also called *P. humanus corporis* De Geer), *P. humanus marginatus* Fahrenholz, *P. humanus americanus*, new variety and *P. humanus humanus* Linnaeus [28–30]. Ewing worked on both contemporary and mummy lice, because he was aware that America, a melting pot of human races, had also become a melting pot of hybrid lice from different origins.

2.4. First assumptions about the evolution of head and body lice

At the time, the predominant opinion was that *corporis* descended from *capitis* in nature [17,28]. Indeed, it was thought that when primitive humans lost the hair that covered their bodies and began to wear clothes, lice living in hair evolved to adapt to this new ecologic niche. Nuttall also noted a variation in the time required for the adaptation of the *capitis* typical form to evolve into the *corporis* typical form, illustrating that some races of *capitis* are more labile than others [17]. This finding was also stated later by Ewing (1926): "in certain races of humans a distinct variety of clothes louse developed from the head-louse type for that race, while in other races, no clothes-louse type distinctive from head louse developed". We will discuss this topic later in this review.

3. The louse genome

3.1. The chromosome

3.1.1. Structure

The genome sequencing of the human body louse [13] confirmed that body lice and head lice have the smallest genomes of any insect reported to date (108 Mb for females and 109 Mb for males), as previously estimated by flow cytometry data in 2007 [31]. Lice are diploid organisms that have 6 chromosomes (five metacentric chromosomes and one telocentric chromosome) [32]. The average GC content of the *Pediculus humanus* genome is 28% making this genome unusually AT rich. Transposable elements represent only 1% of the genome, which is markedly lower than any sequenced insect genome. Both class I and class II mobile elements are present [13]. No genes of prokaryotic origin have been found in the louse

genome, suggesting the absence of DNA transfer from *Candidatus* Riesia pediculicola to its host [13].

3.1.2. Gene content and function

The expectations for the reproductive evolution of obligate parasites would be a reduced genome with a reduced basal insect repertoire. However, despite its small size, the body louse genome is functionally complete [13]; 90% of the predicted body louse genes share homology and 80% of the genes show orthology to other sequenced insect genes [33]. The genome contains 10,773 protein-coding genes and 57 microRNAs. This genome composition is interesting because lice could constitute a robust outgroup to *Holometabolous*, as they share more orthologous genes with this group than the well-studied *Drosophila melanogaster* model does [13]. The genome contains significantly fewer genes associated with environmental sensing and response. First, odorant and gustatory receptors, as well as odorant-binding proteins and chemosensory proteins, do not seem to be necessary for host location and selection, as their respective genes are dramatically fewer in number than in other insects [13]. Second, the genome encodes the smallest number of detoxifying enzymes compared with other insect genomes [33]. Its obligate parasitism of a single host species and its simple life history may be indicative of an evolutionary process that ended with a smaller number of certain gene families. Moreover, the louse has a single insulin-like peptide (ilp) gene, which may be due to its restricted and homogeneous diet [13].

3.2. The mitochondrial genome

In eukaryotes, the mitochondrial chromosomes are typically circular, approximately 16 kb long and have 37 genes [34]. However, in lice, the 37 mitochondrial genes are located on 18 minicircular chromosomes

instead of one single chromosome. Each of the minicircular chromosomes is 3-4 kb long and has one to three genes [35]. The circular chromosomes also contain 3 blocks of highly-conserved regions that may form a stable stem loop to initiate replication and transcription. The coding regions show single nucleotide polymorphisms. There is evidence of recombination between minichromosomes that is likely facilitated by the identical sequences present on different minichromosomes, thus explaining the extreme sequence variation in the noncoding regions [35]. The recombination of these minichromosomes may be either homologous or nonhomologous. There are also different types of chimeric mt minichromosomes, in addition to the 18 mt minichromosomes [36]. This novel type of circular mitochondrial chromosome is also present in the other sucking lice but not in chewing lice or the *Psocoptera*. Blood feeding appears to have coevolved with minicircular mt chromosomes in sucking lice [35].

Moreover, a recent paper investigated the gene content of various eukaryotic mitochondrial genomes (including *Pediculus humanus*) in order to determine the origin of each mitochondrial gene and reconstitute the origin of mitochondria. This work showed that mitochondria do not have a stable or unique form and that mitochondria of different organisms do not have the same evolutionary history or the same number of genes [37].

4. The louse endosymbiont and its genome

4.1. Generalities about the endosymbiont

The human lice endosymbiont is a new bacterium belonging to the family *Enterobacteriaceae* in the *γ-Proteobacteria* class. Its closest relatives are species in the genus *Arsenophonus,* and it was called *Candidatus* Riesia pediculicola [12]. Many studies were undertaken during the past five years on lice endosymbionts [38–40]. The microorganism is primarily located in a

disc-shaped organ located on the ventral side of the midgut (the mycetome) and is transmitted from the female louse to its progeny after its migration to the ovaries [39,41,42].

4.2. The endosymbiont genome

The genome of the obligatory louse endosymbiont encodes less than 600 genes on a short, linear chromosome and a circular plasmid. When compared with other endosymbionts genomes, only 24 genes are unique to *Candidatus* Riesia pediculicola, including genes coding for transport and binding proteins and enzymes involved in lipopolysaccharide biosynthesis that may be essential for cell wall stability during extracellular migration [13]. There are 30 genes present in all bacteria studied but absent in *Candidatus* Riesia pediculicola. These genes are mainly exonucleases that are required for conjugation and enzymes that are involved in energy metabolism, thus reflecting the dependence of the symbiont on its louse host for nutrients. In return, the bacterium is thought to be required by the louse for the production of pantothenic acid (vitamin B5) [43]. The genes encoding this function are situated together on the plasmid, not on the linear chromosome of the bacteria. The reduction in genome size and the high AT bias suggest an old association between the louse and its primary endosymbiont [13]. However, *Candidatus* Riesia pediculicola is the youngest known insect primary endosymbiont (P-endosymbiont) as it has been associated with the louse for only 13-25 million years. Moreover, this bacterium was described as the fastest evolving insect P-endosymbiont, leading to the conclusion that nucleotide substitution rates decrease as the age of the endosymbiosis increases to slow the overall rate of endosymbiont extinction [44].

5. Genomic data on louse infesting bacteria

As mentioned above, three main intracellular bacteria are transmitted by lice: *Rickettsia prowazekii*, *Bartonella quintana* and *Borrelia recurrentis* [4]. Interestingly, in addition to being highly pathogenic, all of these bacteria share another common characteristic: an unusually reduced genome compared to close relatives. Hence, *Borrelia recurrentis* appears to be a degraded subset of the tick-borne relapsing fever-causing agent *Borrelia duttonii* [45]. Additionally, *Bartonella quintana* is described as a genomic derivative of the zoonotic agent *Bartonella henselae*, which is transmitted among cats by the cat flea and to humans by cat scratches or cat bites [46]. Finally, *Rickettsia prowazekii* is also known to have a reduced genome and to contain hundreds of degraded genes [47].

In fact, as bacteria are in relation with their environment, their genetic content vary through gene gain and gene lost. When a bacterium becomes intracellular, the possibilities of gene exchanges are reduced, leading to gene loss and a reduction in genome size. However, intracellular bacteria of amoebae are in sympatry with many other bacteria and viruses, leading to a very large genome [48]. In cases of intracellular bacteria living in allopatry, new characteristics may not be acquired, and the bacteria may become specialized to their environment without the possessing capability to adapt to a changing environment. A greater reduction in the genome will lead to deregulation and a higher level of pathogenicity [49–51]. It explains why the bacteria of the genera *Borrelia*, *Bartonella* and *Rickettsia* comprise both highly pathogenic bacteria with small genomes that are transmitted by a very specific vector (the louse) and less pathogenic bacteria with a larger genomes that are transmitted by ticks or fleas that feed on a larger variety of hosts (Figure 2) [45].

6. Genetic studies of human head and body lice

Genetic tools questioned the division of human lice into head lice and body lice (Table 2). The first study was based on the 18S rDNA gene [52], and subsequent studies focused on mitochondrial genes [53–56] and intergenic spacers [23,57]. Finally, there are three clades of head lice, one of which may also be body lice (Clade A). Recently, a transcriptome study of human head and body lice revealed that only one gene is present in body lice and not in head lice. Otherwise, the main differences that have been identified between head lice and body lice concerned gene expression [58]. Indeed, fourteen putatively differentially expressed genes were identified by comparing head louse and body louse data. Nine head louse genes were more highly expressed: genes coding for a putative enzymatic polyprotein, a putative cuticule protein, the cytochrome P-450, a putative triadia, a putative glucose dehydrogenase precursor, a putative trypsin-4 precursor, a putative parathyroid precursor and two hypothetical proteins. Five other genes were expressed at lower levels and encode for an agglutinin isolectin 2 precursor, a putative bardet-biedl syndrome 4 bbs4, a histone H2B.3, as well as a predicted protein and a hypothetical protein of unknown function. Thus, head lice and body lice have almost the same genomic content but are phenotypically different (different ecotypes) as a result of differential gene expression.

7. Perspective and conclusions

Body lice are found from only one lineage (Clade A). The theory that body lice evolved from head lice when humans began to wear clothes [56] is not compatible with genetic studies. The data suggest that evolution of body lice from head lice and vice versa happens every day among Clade A and that this evolution is facilitated by mass infestations (Figures 3 and 4). This finding is strengthened by the identification of body lice nits in a

homeless person's cap that may have originated from a head louse [23]. We now know that among Clade A lice, head lice and body lice are two ecotypes of the same species that, other than one gene, differ only in gene expression and not in gene content. The reported morphological and behavioral differences between head and body lice [20] could be the result of epigenetics. Epigenetics is the study of inherited changes in phenotype or gene expression that are caused by mechanisms other than changes in the underlying DNA sequence (see: http://en.wikipedia.org/wiki/Epigenetic). Such phenotypic modification in insects has been reported to occur in termites and migratory locusts. In termites, the descendants of a female "queen" may develop into different phenotypic forms such as "workers", "soldiers" or two sexual forms under genetic influences, as well as environmental and social factors [59]. In locusts, when the population increases to a certain level, the locust phenotype changes, and the population starts to migrate [60–62]. These changes also accumulate across generations through a maternal effect [61]. It is possible that something equivalent is happening in body lice when they are proliferating at high levels, maybe because of the influence of physical contact or pheromones that play the role of quorum sensing [63].

In conclusion, studying phenotypic characteristics of lice and their genetic data provides crucial information for understanding lice epidemiology. Obtaining data on these parasites is essential for preventing re-emerging diseases, since body lice are vectors for very severe diseases and head lice can serve as potential reservoirs for disease. In conclusion, there are three major clades of head lice, one of which can also generate a body louse that is phenotypically but not genotypically different from the head lice form.

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Table 1: Chronology of the main biology-based studies on the taxonomic status of human head and body lice			
Date	**Author**	**Main observations**	**Rfs**
1758	Linné	The genus *Pediculus* was established	
1767	Linné	Description of *Pediculus humanus* varieties 1 and 2	
1778	De Geer	Description of *Pediculus humanus capitis* and *Pediculus humanus corporis*	[14]
1861	Murray (in Nuttall 1919)	Lice imitate the color of the support upon which they live	[26]
1915 1917	Fahrenholz (in Nuttall 1919)	Human lice description and classification based on various morphological characteristics, including size, shape and pigmentation	[15]
1917	Sikora Bacot	Evidence that *capitis* raised under *corporis* conditions become gradually indistinguishable from *corporis*	[12,21]
1917	Bacot	In the laboratory, head lice sometimes lay eggs on clothes, but body lice rarely lay eggs on hairs and eggs are badly attached Body lice have a homing instinct, but head lice do not Head and body lice pair freely and their offspring are fertile	[21]
1917	Howlett	When head lice are put on the body, they have a tendency to go back to the head, but this tendency is less marked in the next generations	[22]
1918	Nuttall	Feeding habits of *capitis* and *corporis* They represent extremes in the variation of the species *Pediculus humanus*	[17]
1919	Nuttall	Pigmentation is entirely dependent on the color of the background and is not a genetically transmitted characteristic	[26]
1919	Nuttall	*Corporis* is a descended from *capitis* in nature and some races of *capitis* are more labile than others	[17]

1919	Keilin and Nuttall	Occurrence of an abnormal sex-ratio in the progeny of crosses and appearance of hermaphrodites. Review of many cases supporting evidence of intermingling of the two forms of lice	[19]
1920	Nuttall	Fahrenholz: description of human lice criticized Pigmentation is a poor criterion for differentiating lice	[15]
1924 1926 1929	Ewing	Description of American lice and observation that human lice are hybrids Description of mummy lice and comparison with contemporary lice conducted to develop an identification key for American lice In some races of humans, a distinct variety of clothes louse developed from the head louse, while this is not the case in other human races	[28–30]
1946	Busvine	Confirmation that lice pigmentation depends on the background color	[27]
1948	Busvine	Head lice reared in captivity without any signs of acquiring *corporis* characteristics	[20]
1955	Alpatov	Head lice may become body lice under body lice laboratory conditions	[18]
1985	Busvine	Description of head and body lice of distinct sizes without an overlap in Ethiopia	[16]

Table 2: Summary of the main genetic studies on human head and body lice

DNA Type	Gene	Fragment length	Date	First Author	Title	Rfs
Mitochondrial DNA	Cytochrome oxidase subunit 1 (COI)	524 bp	2002	Leo	Evidence from mitochondrial DNA that head and body lice of humans are conspecific	[55]
		610 bp	2003	Kittler	Molecular evolution of *Pediculus humanus* and the origin of clothing	[56]
		524 bp	2003	Yong	The geographic segregation of human lice preceded that of *Pediculus humanus capitis* and *Pediculus humanus humanus*	[52]
		854 bp	2004	Reed	Genetic analysis of lice supports direct contact between modern and archaic humans	[54]
		383 bp	2008	Raoult	Molecular identification of lice from Pre-Columbian mummies	[53]
		827 bp	2008	Light	Geographic distributions and origins of human head lice based on mitochondrial data	[64]
	Cytochrome b (Cyt b)	440 bp	2003	Kittler	Molecular evolution of *Pediculus humanus* and the origin of clothing	[56]

		671 bp	2004	Reed	Genetic analysis of lice supports direct contact between modern and archaic humans	[54]
		356 bp	2008	Raoult	Molecular identification of lice from Pre-Colombian mummies	[53]
		316 bp	2010	Li	Genotyping of human lice suggest multiple emergences of body lice from local head louse populations	[23]
	NADH dehydrogenase 4 (ND4)	579 bp	2003	Kittler	Molecular evolution of *Pediculus humanus* and the origin of clothing	[56]
Nuclear DNA	Elongation factor 1α (EF-1α)	485 bp	2003	Kittler	Molecular evolution of *Pediculus humanus* and the origin of clothing	[56]
		348 bp	2003	Yong*	The geographic segregation of human lice preceded that of *Pediculus humanus capitis* and *Pediculus humanus humanus*	[52]
	RNA Polymerase II (RPII)	601 bp	2003	Kittler	Molecular evolution of *Pediculus humanus* and the origin of clothing	[56]
	18S rRNA gene, ssu rRNA	1474 bp to 1493 bp	2003	Yong	The geographic segregation of human lice preceded that of *Pediculus humanus capitis* and *Pediculus humanus humanus*	[52]

		1195 bp	2005	Leo and Barker	Unraveling the evolution of the head and body lice of humans	[65]
	Microsatellites	130 to 180 bp	2005	Leo	The head and body lice of humans are genetically distinct; evidence from double infestations	[66]
	Intergenic spacers	133 to 155 bp 323 to 328 bp 165 to 185 bp 156 to 189 bp	2010	Li	Genotyping of human lice suggests multiple emergences of body lice from local head louse populations	[23]
cDNA	Transcripts prediction		2012	Olds	Comparison of the transcriptional profiles of head and body lice	[58]
Analysis based on data available in GenBank	Comparison of phylogenetic and population genetic approaches		2008	Light	What's in a name: The taxonomic status of human head and body lice	[67]
	Bayesian coalescent modeling approach for estimation of effective migration rates		2011	Toups	Origin of clothing lice indicates early clothing use by anatomically modern humans in Africa	[68]

*The EF-1α sequences of this study are contaminated by fungi [67]

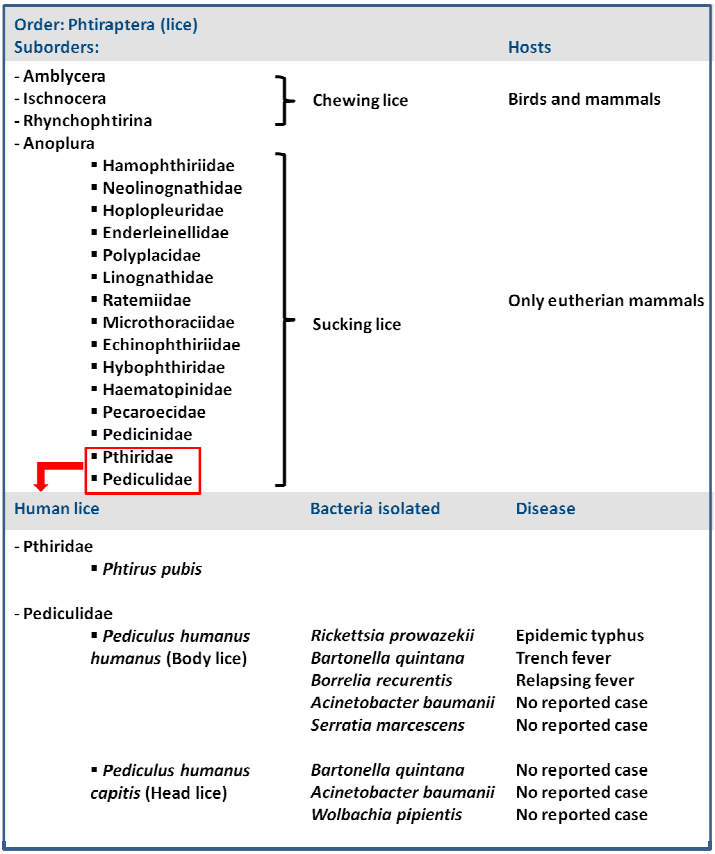


Figure 1: Classification of the *Phthiraptera*. List of the main suborders of chewing and sucking lice, list of the main families of sucking lice and details on the two families of human lice and the diseases that they can transmit

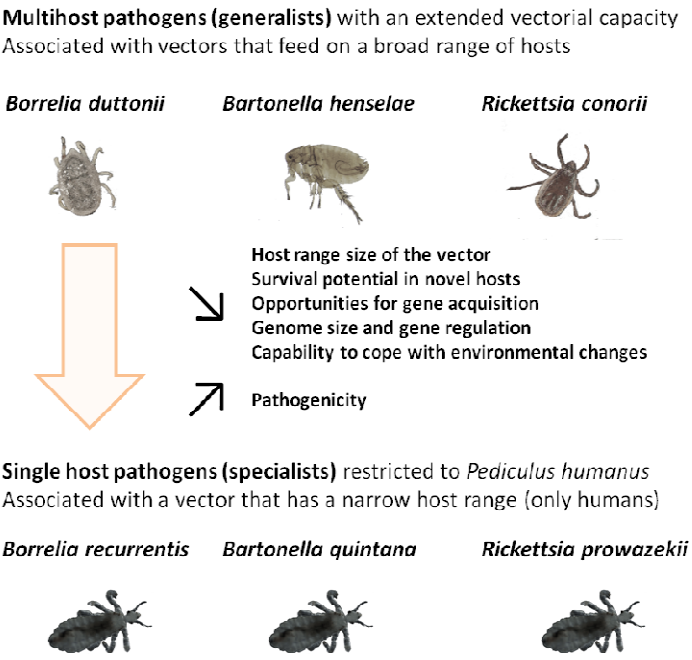


Figure 2: Reductive evolution of the highly pathogenic bacteria associated with *Pediculus humanus*, a narrow host range vector compared to less virulent closely related bacteria associated with broad-range-hosts vectors

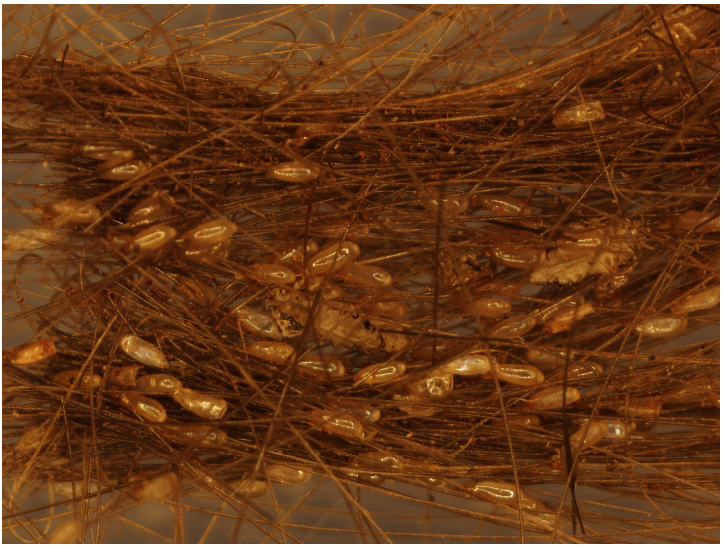


Figure 3: Head lice nits attached to hairs from a highly infested homeless person

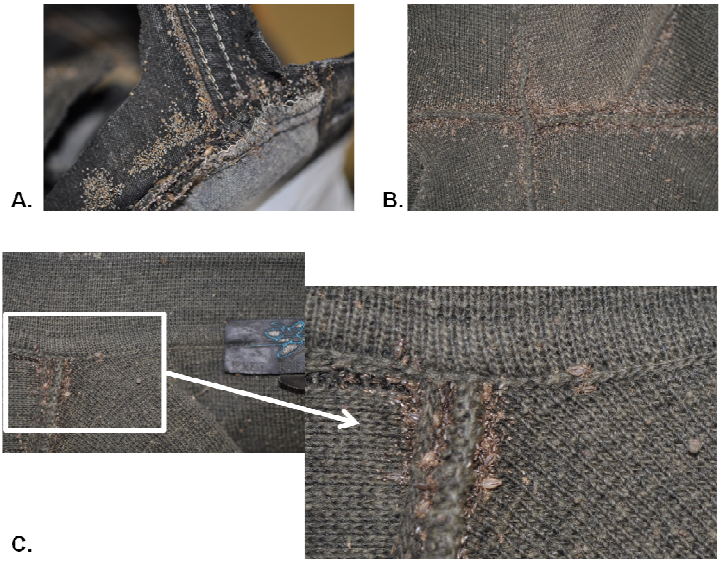
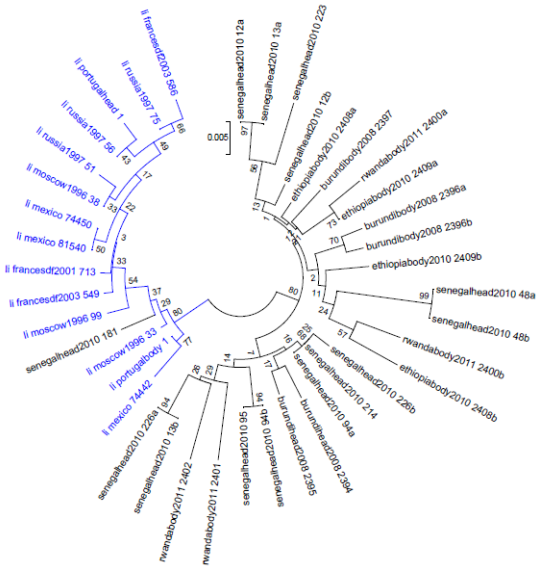


Figure 4: Pictures taken from clothes of a highly infested homeless person A. a piece of its pants B. the armpick of the pullover C. the collar of the pullover

CHAPITRE I

Evidence for an African Cluster of Human Head and Body Lice with Variable Colors and Interbreeding of Lice between Continents



Préambule

Ce travail avait pour but d'identifier des corrélations entre les données phénotypiques et génotypiques des poux de l'homme. En effet, les poux de tête et les poux de corps ont été classifiés sur base de caractéristiques phénotypiques et génotypiques mais aucune étude n'avait auparavant tenté de corréler ces deux types de données. Les principaux critères phénotypiques utilisés pour étudier la taxonomie des poux de l'homme sont: la source géographique, l'écotype (le pou pond ses œufs sur les cheveux ou sur les vêtements), la forme et la couleur [23–31]. Actuellement, d'un point de vue génétique et basé sur l'ADN mitochondrial, les poux de tête sont classifiés en trois clades phylogénétiques avec seulement un clade qui comprend aussi les poux de corps (le Clade A) [15,16].

Dans notre étude, nous utilisons une méthode de typage par séquençage d'espaces intergéniques (méthode MST) [12] de poux de Clade A collectés dans quatre pays africains : le Sénégal, le Burundi, le Rwanda et l'Ethiopie. Des données MST d'une étude précédente [12] ont également été utilisées dans nos analyses afin d'inclure des poux issus de pays non africains. Ensuite, les échantillons ont été photographiés et leur couleur comparées afin de voir si des corrélations entre la couleur du pou, la source géographique, l'écotype ou la phylogénie pouvaient être identifiées .

A la fin de l'étude, aucune congruence entre la couleur des poux et le génotype n'a pu être mise en évidence. Cependant, la phylogénie de l'espace intergénique PM2 montre une corrélation avec la source géographique des poux car les poux Africains et Non Africains clustérisent séparément. Par ailleurs, l'analyse d'autres espaces intergéniques suggère que des poux provenant d'aires géographiques très distinctes recombinent.

Article I

Evidence for an African Cluster of Human Head and Body Lice with Variable Colors and Interbreeding of Lice between Continents

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Evidence for an African Cluster of Human Head and Body Lice with Variable Colors and Interbreeding of Lice between Continents

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Abstract

Background: Human head lice and body lice have been classified based on phenotypic characteristics, including geographical source, ecotype (preferred egg laying site hair or clothes), shape and color. More recently, genotypic studies have been based on mitochondrial genes, nuclear genes and intergenic spacers. Mitochondrial genetic analysis reclassified lice into three genotypes (A, B and C). However, no previous study has attempted to correlate both genotypic and phenotypic data.

Materials and Methods: Lice were collected in four African countries: Senegal, Burundi, Rwanda and Ethiopia and were photographed to compare their colors. The Multi-Spacer-Typing (MST) method was used to genotype lice belonging to the worldwide Clade A, allowing a comparison of phenotypic and genotypic data.

Results: No congruence between louse color and genotype has been identified. Phylogenetic analysis of the spacer PM2, performed including lice from other sources, showed the existence of an African cluster of human lice. However, the analysis of other spacers suggested that lice from different areas are interbreeding.

Conclusions: We identified two geotypes of Clade A head and body lice including one that is specifically African, that can be either black or grey and can live on the head or in clothing. We also hypothesized that lice from different areas are interbreeding.

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Introduction

Humans are infested by two genera of lice: *Pthirus* and *Pediculus*. The *Pediculus* genus has been studied for decades and is classified based on its ecology, shape and color [1–14]. Two ecological forms of *Pediculus* lice may be distinguished: head lice and body lice. The head louse, *Pediculus humanus capitis*, lives and lays its eggs on hairs, whereas the body louse, *Pediculus humanus humanus*, lives and lays its eggs in clothing [7]. Differences in shape between head and body lice have also been described, but these criteria have not been shown to be relevant enough to divide the two into distinct species [12]. Also, louse coloration was described in the beginning of the 20th century, and it was noted that lice have different colors depending on their geographic region and the color of their host's skin [3,8,9,11]. A series of gradations between the black head or body louse of West Africa and the light dirty grey head or body louse of Europe was described [10,13,14].

Later, researchers began performing genetic studies on lice [15–23]. First, a study based on the 18S ribosomal RNA gene reported that head and body lice were not phylogenetically distinct. In fact, two phylogenetic groups were described: the Sub-Saharan African lice and other lice that are distributed elsewhere worldwide. Each of these groups contained two distinct subgroups: head and body lice [23]. The divergence of human head and body lice was considered to be a recent event occurring within each of the geographical groups. A second phylogenetic study was based on mitochondrial genes [21,22]. This allowed the description of three phylogenetically different clades of lice: the "most common worldwide clade", which comprises both head and body lice (called Clade A), the "head lice only clade", found in America, Europe and Australia (called Clade B), and "another head lice only clade", which was first found in Nepal and Ethiopia [21] but was also recently found in Senegal [24] (called Clade C). Finally, another phylogenetic study of Clade A lice based on intergenic spacers (Multi-Spacer-Typing method or MST) reported two clusters of lice: Non-African lice (which we will call A1) and African lice (which we will call A2) [20].

Since then, there have been no studies aiming to correlate phenotype and genotype in human lice. Therefore, we wanted to examine both aspects to determine whether there is any correlation among the color, geographical source, ecotype (head and body) and phylogeny of lice. For this approach, we used Clade

Table 1. Results of the Multi-Spacer-Typing of African lice.

Country	Type	Host ID	Louse ID	Spacer S2	Spacer S5	Spacer PM1	Spacer PM2
Senegal 2010	HEAD	1	12	**83**	36/42	13	**60/61**
			13	**83/84**	42	13	**60/62**
		2	14	NA	36/42	13	NA
		3	48	**85/86**	8/36	13	**63/64**
		4	94	**87**	8	13	**65/66**
			95	**87**	8/42	13	**66**
		5	181	**89**	**47**	13	43
		6	214	NA	35	13	**65**
		7	223	**90**	8/36	13	**67**
		8	226	**91/92**	**36/62**	13	**62/68**
Ethiopia 2010	BODY	9	2408	**100**	36	4/5	**85/87**
		10	2409	**96**	**54/59**	18	**83/92**
Burundi 2008	HEAD	11	2394	**97/101**	**35/53**	**25/34**	**82**
		12	2395	**93**	**51**	**35**	**76**
	BODY	13	2396	76	35	17	**80/74**
		14	2397	76	32/36	17	**69**
Rwanda 2011	BODY	15	2400	**103**	**36/54**	4	**69/70**
		16	2401	**108**	**50**	4	**92**
		17	2402	**104**	**60**	3/4	**86**

In case of heterozygocy, the numbers of the two genotypes were mentioned.
NA, not available.
New genotypes in bold.
doi:10.1371/journal.pone.0037804.t001

A lice collected in Senegal, Burundi, Rwanda and Ethiopia. They were photographed and then genotyped with the MST method [20]. To complete the phylogenetic analysis of our data, we also used previously genotyped Clade A lice from African and Non-African regions [20].

Materials and Methods

Ethics statement

Informed verbal consents were obtained from all the participants involved in our study. All participants of this study were illiterate. It is why a verbal informed consent procedure was preferred over written consent. The Ethic Committee of the Institute Fédératif de Recherche approves this verbal consent procedure as it is in accordance with the French Bioethics law N° 2004-800 60 (06/08/2004). Dr Oleg Mediannikov participated in the collection and was a witness of the participant's consents. Local authorities (village chief) approved, and were also present. This study was approved (Agreement #12-004), by the Ethic Committee of the Institute Fédératif de Recherche 48, Marseilles, France since this study was a non-interventional epidemiological research study as a part of the French Bioethics law N° 2004-800 (06/08/2004). The poorest districts of Dakar were chosen because of the highest possibility to find lice. Girls are more likely to have long hairs (boys are often shaved). Lice were anonymized before processing for genetic analysis.

The study

In this study, we had four parameters to compare: ecology, geographical origin, phylogeny based on 4 spacers and color. So we decided to sequence only Clade A lice because this is the only clade that comprise both head and body lice and therefore is a specific problem of public health as body lice are vectors of outbreaks. Lice from four countries were genotyped with the MST method including head lice from Senegal collected in October and November 2010 [24], body lice from Southern Ethiopia collected in 2010 [25], body lice from Rwanda collected in 2011 (three lice per individual) and both head and body lice from Burundi collected in 2008 (three lice of each ecotype per individual). Altogether, 19 Clade A lice were used in this study (Table 1). All

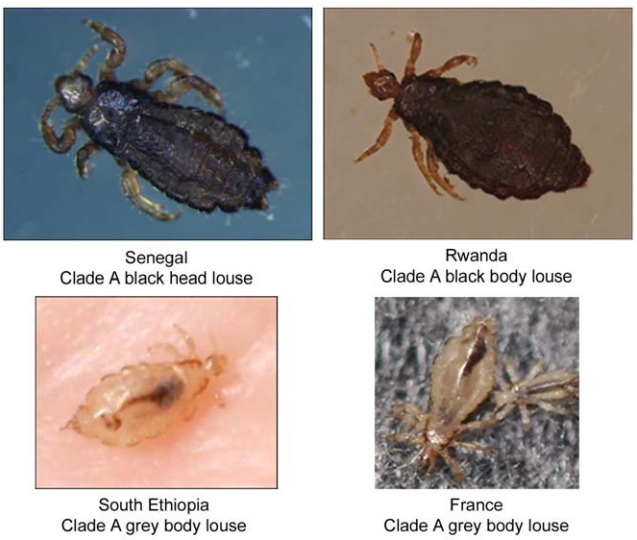

Figure 1. Pictures of lice.
doi:10.1371/journal.pone.0037804.g001

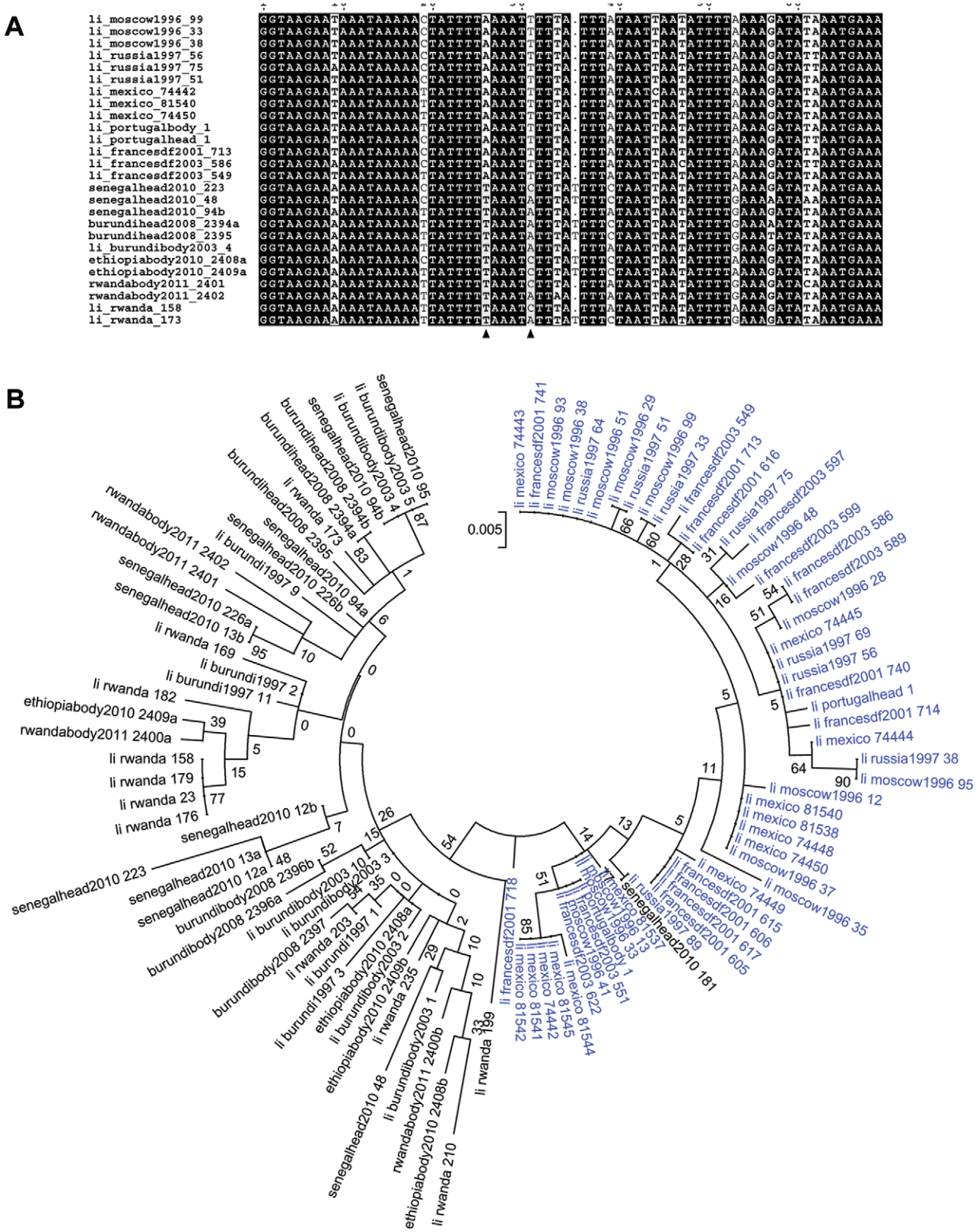

Figure 2. Spacer PM2 analysis. Phylogenetic analysis of African (black) and Non African lice (blue) (A) The first 69 bp of the alignment of a subset of PM2 spacer sequences. Two polymorphisms (shown with arrows) discriminate between African and Non-African lice (B) Phylogenetic tree based on PM2 sequences using Maximum likelihood method. For lice being heterozygote in PM2 spacer, the two alleles were included in the tree and they were called the same with one letter "a" or "b" to distinct them.
doi:10.1371/journal.pone.0037804.g002

lice were stored at −20°C before DNA extraction with the QiAamp Tissue kit (QIAGEN, Hilden, Germany). Senegalese and Rwandan lice were photographed with a camera (Olympus DP71) fixed on a low power stereo microscope (Olympus SZX16). The lice from Ethiopia and France were photographed in the field (with a Nikon D90) either in the hand of one of the authors (Ethiopia) or directly on the clothes of the infested person (France). Four intergenic spacers (S2, S5, PM1, PM2) known to be very polymorphic [20] were used in this study. The sequencing was performed following the protocol previously described [20] with some minor modifications. Briefly, the PCR reactions were prepared on ice and contained 3 µl of the DNA template, 4 µl of Phusion HF Buffer, 250 µM of each nucleotide, 0.5 µM of each primer, 0.2 µl of Phusion DNA Polymerase (Ozyme) and water to a final reaction mixture volume of 20 µl. The reactions were performed in a PTC-200 automated thermal cycler (MJ research, Waltham, MA, USA). The cycling conditions were 98°C for 30 sec; 35 cycles of 5 sec at 98°C, 30 sec at 56°C, 15 sec at 72°C; and a final extension time of 5 min at 72°C. The success of the PCR amplification was then verified by migration of the PCR product on an agarose gel. The NucleoFast 96 PCR Plates (Macherey-Nagel EURL, France) and BigDye Terminator version 1.1 cycle sequencing-ready reaction mix (Applied Biosystems, Foster City, CA) were then used to purify the PCR products before sequencing in both directions with the same primers used in the PCR amplification. The ABI 3100 automated sequencer (Applied

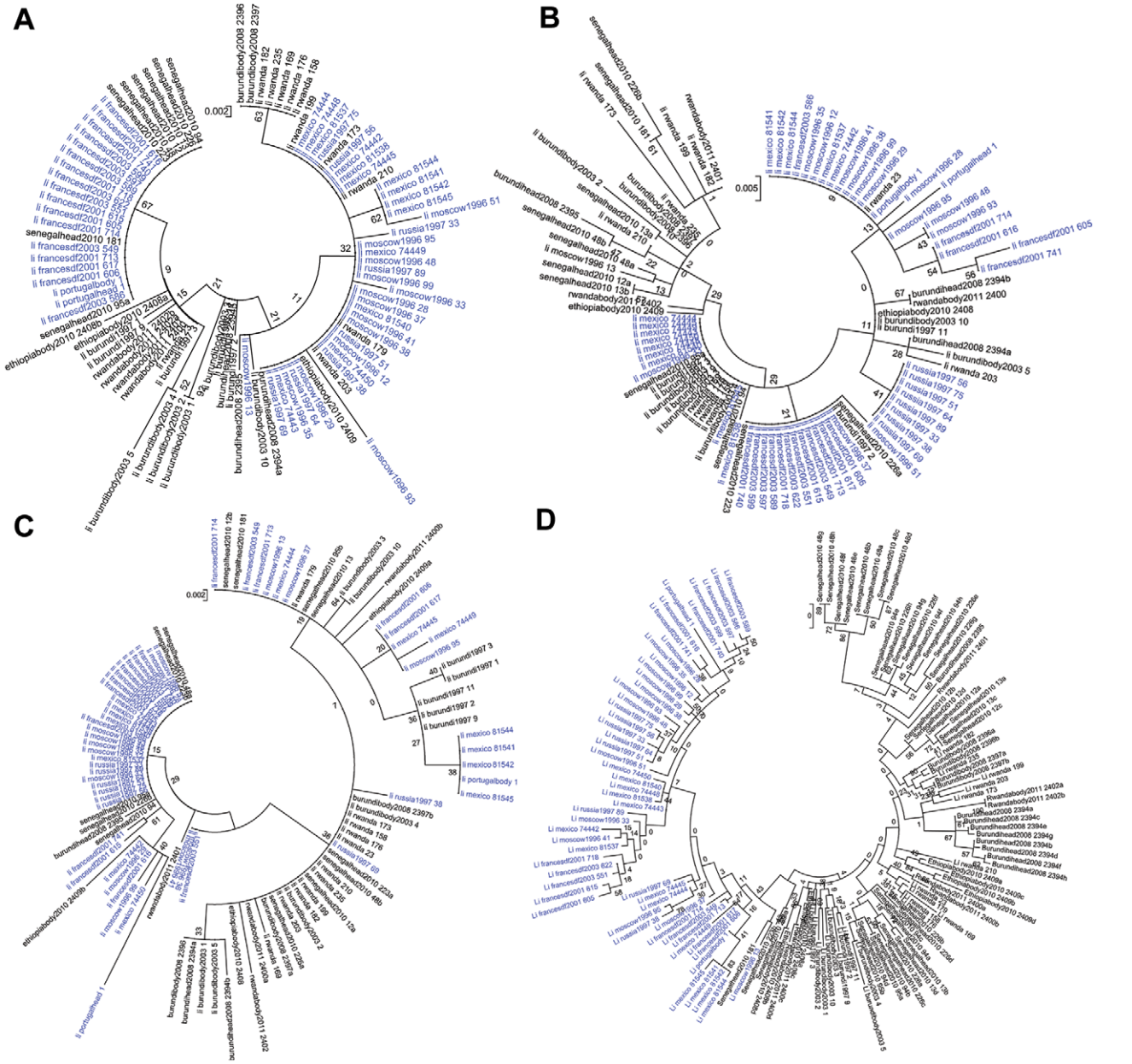

Figure 3. Analysis of spacer PM1, S2, S5 and concatenation of the four spacers. Phylogenetic analysis of African (black) and Non African lice (blue) using Maximum likelihood method based on spacer PM1 (A), spacer S2 (B), spacer S5 (C), the concatenation of the four spacers (D). For lice being heterozygote, the two alleles were included in the trees and they were called the same with one letter "a" or "b" to distinct them.
doi:10.1371/journal.pone.0037804.g003

Biosystems) resolved the sequenced products. The program ChromasPro was used to analyze, assemble and correct the sequences. When forward and reverse sequences could not be assembled, they were analyzed separately and resolved. Each sequence was aligned with genotypes published in Genbank [20]) for identification. When less than 100% homology was obtained, the new genotype was recorded, a new number was assigned to it and sequences were deposited in Genbank under accession numbers from JQ652371 and JQ652455. When the chromatogram indicated possible heterozygotic sequences, these samples were cloned to identify the different allelic sequences. The PCR products were cloned into pGEM-T-Easy vector (Promega, Madison, WI) following the manufacturer's instructions with some minor modifications. Before ligation, A-overhangs were added to the PCR product. This was performed by incubating 4.2 µl of purified PCR product with 1 U of DyNAzyme II DNA polymerase, 0.5 µl of Optimized DyNAzyme Buffer and 0.2 mM dATP with a final volume of 5 µl for 20 min at 72°C. Then, each reaction was ligated with 5 µl of 2X Rapid Ligation Buffer, 3 µl of purified A-overhangs-PCR product, 1 µl of T4 DNA ligase and 1 µl of pGEM®-T Easy Vector and incubated overnight at 15°C. Each ligation reaction was transformed into 50 µl of JM109 High Efficiency Competent Cells by letting them incubate together on ice for 20 min before a 1 min heat shock in a 42°C water bath. 950 µl of LB broth was then added to cells before incubation on a 37°C shaker for 1.5 hours. 300 µl of transformed cells was plated onto LBagar/ampicillin/IPTG/X-Gal plates and these were incubated overnight at 37°C. Eight white colonies per sample were then resuspended in 100 µl of RNase/DNase free water and subsequently PCR amplified and sequenced using the M13 universal and M13 reverse primers. Phylogenetic analysis was done using our data and data from a previous study performed in 2010, which included lice from France, Portugal, Mexico, Russia, Burundi and Rwanda (these lice were given names beginning with "li-" in the trees) [20]. Two phylogenetic methods, Maximum Parsimony (MP) and Maximum Likelihood (ML), were used to infer the trees for each individual spacer. For each spacer, the louse nucleic sequences were aligned with the genotype 1 found in Genbank (EU928781.1, EU928804.1, EU913096.1, EU913178.1 for PM1, PM2, S2 and S5, respectively) using the MUSCLE algorithm [26]. Then, trees were drawn within the MEGA 5 software with complete deletion [27]. A tree was also constructed with the concatenated sequences of the four spacers (S2, S5, PM1, PM2). Because the louse genome is diploid, in instances where there were two different alleles per locus, all possibilities of concatenation were made for each louse

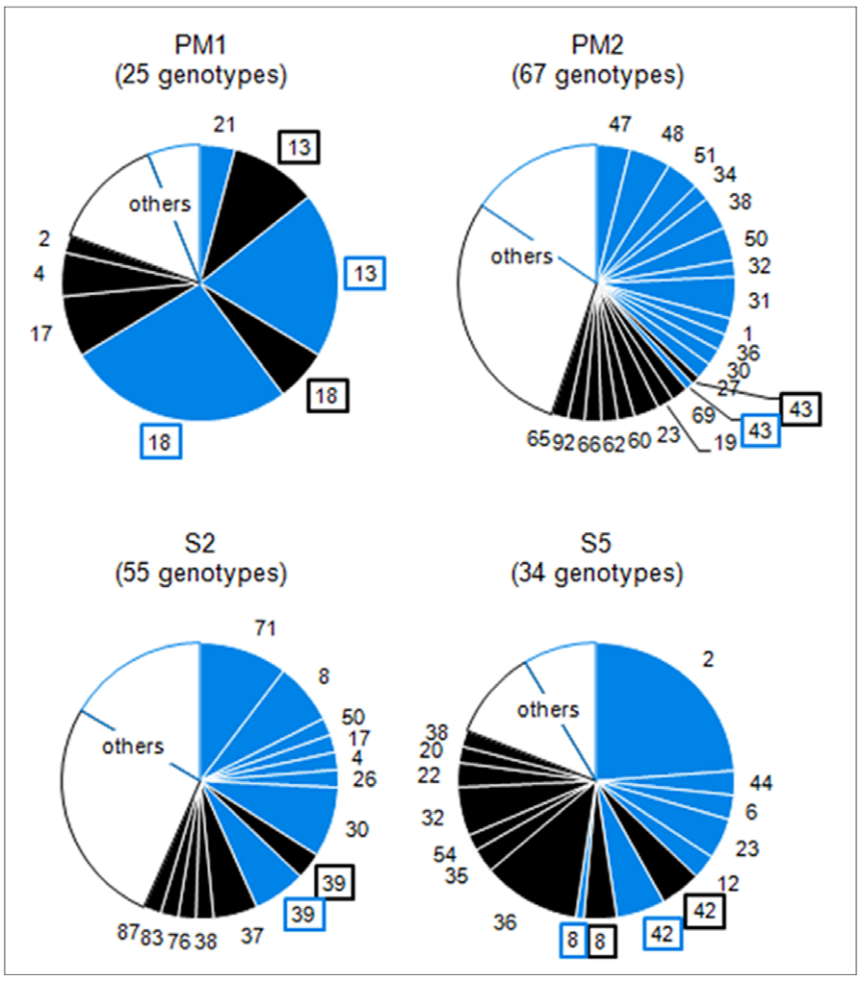

Figure 4. Proportion of each genotype among African (black) and Non-African (blue) lice. The name (ID numbers) of the genotypes found in both African and Non African countries were framed. Genotypes (either African and Non African) found in only one louse were not represented and were grouped as "others".
doi:10.1371/journal.pone.0037804.g004

and all of them were taken into account in the tree (the possibilities were labeled from a to h in the tree).

Results

Clade A lice collected from Senegal, Burundi and Rwanda (head or body) were all black. Clade A body lice collected in southern Ethiopia and lice collected in France were grey (Figure 1). Head lice collected in Ethiopia were black, belonged to genotype C and were not included here [25]. Photographs of genotyped lice from other Non-African regions (Russia, Portugal, Mexico) and from Burundi were not available. After sequencing (Table 1), many new genotypes were found, especially for S2 and PM2 spacers, but there were also new genotypes for spacers S5 and PM1. In Senegal, all 10 tested lice had genotype 13 for spacer PM1. The other spacers had higher variation. We found that some lice collected from the same human host have the same genotype (lice 12 and 13 and lice 94 and 95). There is a lack of data for lice 14 and 214 due to the failure of spacer S2 to amplify, despite three attempts and a good positive control. Moreover, for spacer PM2 of louse 14, the amplification was non-specific on three attempts.

Phylogenetic analysis was performed including lice from other sources [20], and trees were drawn for each of the four spacers. Altogether, 55 Non-African lice and 40 African lice were included in the analysis. The same tree topologies were found with Maximum Likelihood (ML) and Maximum Parsimony (MP) methods. However, the topologies of the trees constructed with the different spacers were not congruent and had low bootstrap support (Figures 2B and 3). The tree from spacer PM2 showed two distinct clusters: African lice separated from Non-African lice, which was supported by a 54% bootstrap (Figure 2B). The analysis of the sequence alignment of the spacer PM2 showed two positions at which single-base substitutions allowed us to distinguish African lice from lice coming from other regions: positions 26 (T in Africa and A out of Africa) and 31 (A or C in Africa and T out of Africa) and may have caused the observed clustering (Figure 2A). The only exception is the Senegalese louse 181 that clustered with Non-African lice in the tree (Figure 2B) and has the same signature than Non-African lice have at these positions (not shown). For spacers PM1, S2 and S5, no such clustering was observed (Figure 3, A to C). When concatenated sequences of the four intergenic spacers were used (Figure 3D), the resulting tree had the same topology as the PM2 spacer tree, but with a 43% bootstrap. Given that phylogenetic analysis of spacer PM2 sequences showed a correlation between geography and genotype, the proportion of each genotype found in African and/or Non-African countries (Geotyping) was compared for each spacer (Figure 4). We represented in the circles only genotypes found in two or more lice; the others genotypes were grouped and called "others." Spacers PM2 and S2 (67 and 55 different genotypes) were more variable than PM1 and S5 (25 and 34 different genotypes). In several cases, genotypes were shared between African and Non-African lice in each spacer. However, in the PM2 spacer, this was very rare, as only one louse from Senegal (louse 181) and one louse from Russia had the same genotype (genotype 43). All other genotypes were not shared by African and Non-African countries. In spacer S5, two genotypes were shared between the two geographic areas: genotype 8 (Senegal and Moscow) and 42 (Senegal, Rwanda, France, Mexico and Moscow). In spacer S2, one genotype was shared: genotype 39 (Burundi and Mexico). However, it was in spacer PM1 that the biggest proportion of lice were observed to have genotypes shared by the two areas. Indeed, genotypes 13 and 18 were the most prevalent genotypes (respectively 30 and 33% of genotyped lice) and were found both in African and non-African countries: genotype 13 was found in

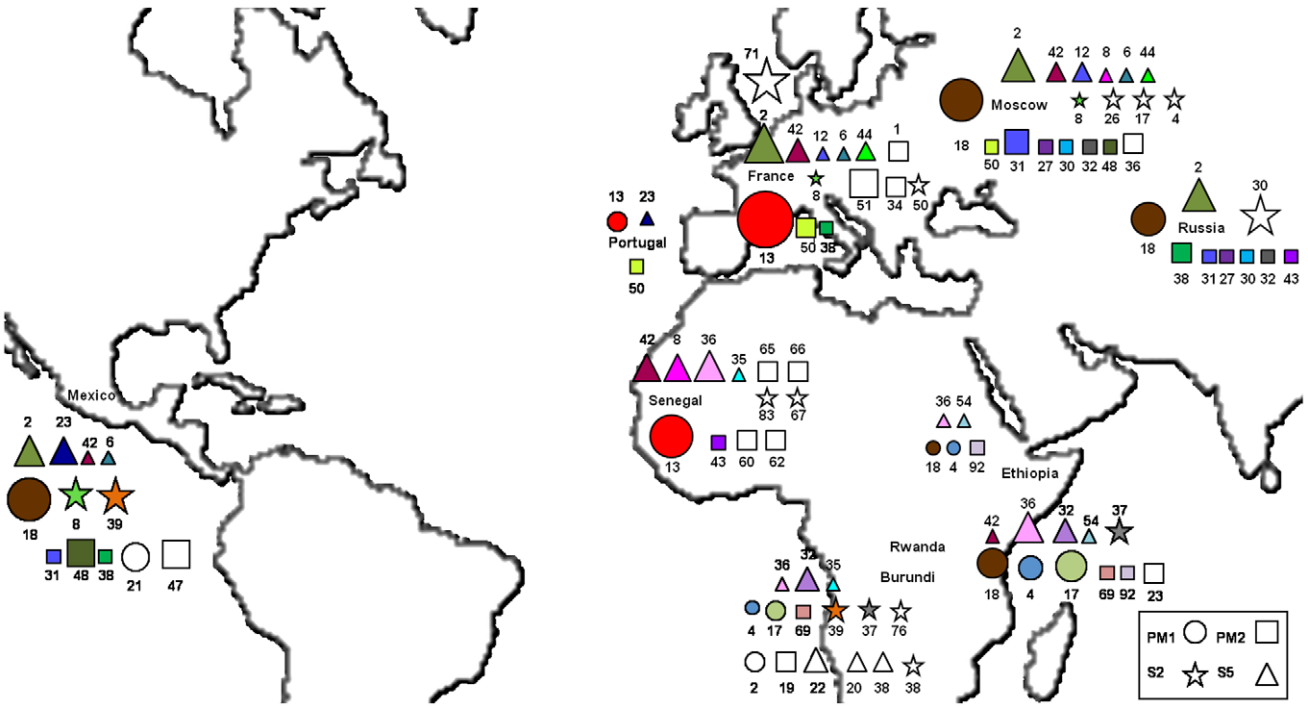

Figure 5. Geographic repartition of the four spacers genotypes. Genotypes found in at least two countries are colored (one color per genotype) and genotypes found in only one country are in white. The size of the symbols vary with the number of lice found for each genotype. Only genotypes found in at least two lice were represented.
doi:10.1371/journal.pone.0037804.g005

Senegal, France and Portugal and genotype 18 was found in Rwanda, Ethiopia, Mexico and Russia (Figure 5).

Altogether, these data show a correlation between geography and genotype but only in spacer PM2. Moreover, among the four spacers studied, none showed a correlation between the ecotype and the genotype, as head and body lice were not separated in the phylogenetic trees. Finally, no correlation has been observed between louse color and genotype as we did not find black and grey lice separately clustered in the trees.

Discussion

In this study, MST based on the four intergenic spacers S2, S5, PM1 and PM2 was confirmed to be a very sensitive method that is able to discriminate among individuals. The observation of identical genotypes from the sequences of two lice collected on the same person strengthens our faith in the reliability of the method. This method therefore appears to be well adapted for the study of human lice and able to address population-level questions.

Our first observation was that the PM1 genotype 13 appeared in all Senegalese lice, but not in lice from Burundi, Rwanda and Ethiopia. This genotype was also found in lice from France and Portugal [20]. This observation that lice from Senegal and France have the same genotype on spacer PM1 suggested that interbreeding has occurred between lice from these two countries. As the French have been in Senegal since the XVIII century, this hypothesis is historically logical. Genotype 18 is also prevalent in African and Non-African countries (Figure 4), supporting the possibility that lice from different continents are interbreeding.

When the frequency of the genotypes of the PM2 spacer were observed, it was noted that this spacer was the most variable among those tested and that only one louse from Senegal had the same genotype as a louse from Russia. In all other cases, African and Non-African lice harbored distinct genotypes, as confirmed by the phylogenetic tree that showed a cluster of African lice (54% extra support), regardless of the method used. This topology was similar to that of a previous study based on 18S rRNA [23]. However, with the MST method, head and body lice did not cluster separately inside their geographic cluster, as opposed to the results observed when using 18S-based phylogeny. Finally, when concatenated sequences of the four spacers were used, the resulting tree conserved the same topology as the PM2 tree but with lower bootstrap values. This showed the importance of first drawing separated trees for each spacer before drawing concatenated trees when using the MST method. This allows researchers to check if the different spacers tell the same story.

In conclusion, a clear correlation between genotype and phenotype could not be shown. First, a correlation between genotype and geographic origin was observed, but only with spacer PM2. Second, there was no correlation between color and genotype or ecotype (head and body). Moreover, contrary to results from previous studies [3,8,9,11], the color of lice may not be linked to the color of the host's skin as grey body lice were found on black hosts in Ethiopia.

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Author Contributions

Conceived and designed the experiments: DR. Performed the experiments: AV AB GD. Analyzed the data: AV VM DR. Wrote the paper: AV AB VM GD DR.

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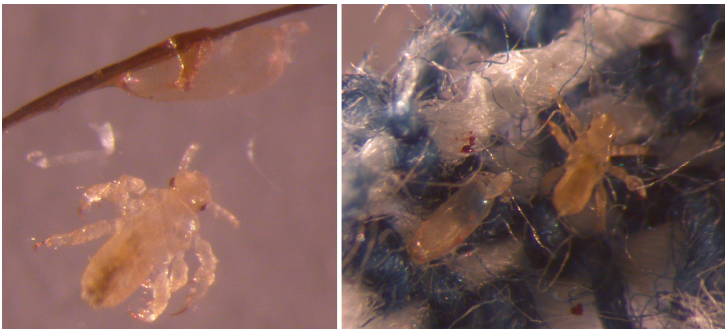
CHAPITRE II

Evidence that head and body lice on homeless persons have the same genotype



Préambule

Les poux de tête et les poux de corps de l'homme appartiennent à une même famille : les Pédiculidés. Ils appartiennent également à un même phylotype mitochondrial : le Clade A même si certains poux de tête appartiennent à l'un des deux autres phylotypes décris (les Clades B et C) [15]. Le but de ce papier était de tester si des poux de tête et des poux de corps de Clade A provenant de personnes sans-abris infestées par les deux écotypes appartiennent à la même population. Les sans-abris étant souvent très massivement infestés, on peut donc imaginer que les poux de tête et les poux de corps au stade larvaire et adultes sont susceptibles de migrer de la tête au corps ou inversement. C'est pourquoi, nous avons décidé de récolter les lentes pondues sur les cheveux ou les vêtements, de les incuber en laboratoire jusqu'à émergence du premier stade larvaire avant de procéder aux analyses génétiques et ce, afin d'être certains de l'origine (pou de tête ou pou de corps) de l'échantillon testé. Une technique de séquençage de quatre espaces intergéniques (méthode MST) [12] a été utilisée afin de réaliser des analyses de génétique des populations.

Nous avons trouvé deux paires de poux de tête et de poux de corps issus de la même personne qui ont les même génotypes au niveau des 4 espaces intergéniques. De plus, aucune différence significative entre les poux de tête et des poux de corps a pu être mise en évidence concernant leur diversité génétique ou nucléotidique. Enfin, de façon surprenante, aucune structure n'a également pu être observée entre les poux provenant de personnes différentes vivant dans le même foyer. Ces résultats confirment des travaux précédents qui concluent que les deux types de poux sont des écotypes de la même espèce et montrent l'importance d'améliorer les mesures d'hygiène dans les foyers d'accueil afin de limiter au maximum la transmission de poux d'une personne à une autre.

Article II

Evidence that head and body lice on homeless persons have the same genotype

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Abstract

Human head lice and body lice are morphologically and biologically similar but have distinct ecologies. They were shown to have almost the same basic genetic content (one gene is absent in head lice), but differentially express certain genes, presumably responsible for the vector competence. They are now believed to be ecotypes of the same species (Pediculus humanus) and based on mitochondrial studies, body lice have been included with head lice in one of three clades of human head lice (Clade A). Here, we tested whether head and body lice collected from the same host belong to the same population by examining highly polymorphic intergenic spacers. This study was performed on lice collected from five homeless persons living in the same shelter in which Clade A lice are prevalent. Lice were individually genotyped at four spacer loci. The genetic identity and diversity of lice from head and body populations were compared for each homeless person. Population genetic structure was tested between lice from the two body regions and between the lice from different host individuals.

We found two pairs of head and body lice on the same homeless person with identical multi locus genotypes. No difference in genetic diversity was found between head and body louse populations and no evidence of significant structure between the louse populations was found, even after controlling for a possible effect of the host individual. More surprisingly, no structure was obvious between lice of different homeless persons.

We believe that the head and body lice collected from our five subjects belong to the same population and are shared between people living in the same shelter. These findings confirm that head and body lice are two

ecotypes of the same species and show the importance of implementing measures to prevent lice transmission between homeless people in shelters.

Introduction

Human head lice (*Pediculus humanus capitis*) and body lice (*Pediculus humanus humanus*) are obligate parasites; head lice live on the scalp and lay their eggs at the base of hair shafts, and body lice live on the body surface and lay their eggs on clothing [1]. Head and body lice are considered to be sub-species and are generally thought to colonize their host in an independent manner [2]. However, in circumstances involving people heavily infested with lice, such as in homeless populations, head and body lice are often found on the same person. This finding raises the question of whether the lice can migrate between the different body areas. Although numerous studies have attempted to elucidate this issue, the species status of these two types of lice is still debated [3].

Body lice pose a serious public health problem as they are vectors of the pathogens *Rickettsia prowazekii*, *Bartonella quintana* and *Borrelia recurrentis*, which are responsible for epidemic typhus, trench fever and relapsing fever, respectively [4,5]. A comparison of the humoral and cellular immune responses of head and body lice following bacterial challenge showed reduced cellular (phagocytic) activity in body lice which may explain the higher level of vector competence that has been found in this subspecies [6]. The epidemiological role of head lice in the transmission of human pathogens has not yet been demonstrated, but several studies have reported the presence of *Bartonella quintana* in head lice [7-10]. It is therefore important to better understand the dynamics of human lice populations to minimize their propagation and the transmission of their associated pathogens in at-risk populations.

The first classifications of head and body lice were based on morphological characters. Some authors maintained that the morphological, behavioral and ecological differences between the two lice populations were

not sufficient to recognize them as distinct species [1,11]. Others, argued the reverse, that these differences required the recognition of these two groups as distinct taxonomic entities [12–15]. An analysis of primary endosymbionts indicated that these two types of lice are conspecific [16], but louse isoenzymes suggested that genetic differentiation may exist between the two forms [17]. After these phenotypic studies, numerous DNA-based molecular studies were performed, and again presented conflicting conclusions [3,18,19]. Currently, three deeply divergent clades (or phylotypes) of human lice with different geographic distributions are recognized: clades A, B and C. Phylotypes B and C contain only head lice, but phylotype A includes both head and body lice [20,21]. Clade A lice have been further subdivided into subclusters of non-Sub-Saharan African lice (called A1) and Sub-Saharan African lice (called A2), as reported by two independent studies. The first study was based on the 18S rRNA gene sequence [22] and reported the divergence of head and body lice as being a recent event that occurred independently in each geographical group. The second study was based on the PM2 spacer [23] and could not show head and body louse divergence within each of the two clusters. Head and body lice were also shown to be genetically indistinguishable in a worldwide study based on four intergenic spacers [24] and in a very recent study based on the louse transcriptome [25]. Finally, based on a Bayesian coalescent model, ancestral migration events between head and body lice were shown to happen in both directions [26].

As multispacer typing was shown to be useful in addressing population-level questions [24], we used this genotyping method to determine if homeless people were infested by head and body lice of the same population. We examined the genetic population structure between lice from two body regions of five human subjects. However, one of the critical problems associated with this experimental design is that lice can migrate

temporarily from one site to the other (with or without reproduction), making it difficult to determine their true origin (head or body). Consequently, to avoid any possible confusion regarding the origin of the tested lice, we collected eggs from the hair and clothing of homeless people from one shelter in Marseille, France. After hatching these eggs in the lab, we genotyped the first instar larvae and tested the genetic population structure of the lice from the two body areas.

Methods

Ethics statement

In Marseille, there are an estimated 1500 homeless people, and 600 of them sleep in one of two available shelters (A and B) [27]. Because these individuals live in poor sanitary conditions, homeless persons are exposed to a number of health problems and belong to the social class with the most limited access to healthcare. To implement appropriate preventive and curative interventions, a snapshot investigation of the two shelters of Marseille has been performed each year since 2000 by a multidisciplinary team [28,29]. The study protocol was reviewed and approved by the Institutional Review Board and Ethics Committee of Marseille No. 10.005 as it is in accordance with the French Bioethics law N° 2004-800 60 (06/08/2004). The study reported here was made based on samples collected in 2010. Homeless persons were informed of the purpose of the intervention and were asked if they would agree to participate by reading and signing an informed consent form . The document was divided into two parts: one for the patient with all information about the study and the other part including patient signature was kept by the investigators. The homeless persons were then interviewed and given a physical examination by a medical doctor. A nurse collected blood and other microbiological samples. One of the

researchers (AV) was assigned to this team to meet the homeless and to collect head and body lice. When an individual had a body louse infestation, we provided clean clothes and kept the lice-infested undergarments and T-shirts in a sealed container to later harvest the eggs. In the cases of head louse infestations, the hair was cut and used to harvest the eggs. The infested homeless person was then invited to take a shower and was offered treatment with ivermectin [30]. The investigations consisted in a clinical exam that was offered to any homeless who presented even if he would not agree to participate to our study. Every homeless including participating and non-participating persons were offered the same services and a prescription was given if needed. Moreover, depending on the results of the samples analysis, the patient was taken in charge in the hospital if needed. All homeless in France are eligible for a social security cover (free healthcare for people on low incomes), this permit us to include all potential participants.

Eggs incubation before hatching

In the laboratory, the infested clothes were cut to separate the collar from the rest of the clothes. Eggs situated on ball caps (Figure 1A), collars (Figure 1B) or beards were not included in the analysis to avoid using lice located on the “borders” between head and body lice. The fabric and hair that contained eggs were put in labeled and separated boxes with holes, and grouped by the homeless person that they were isolated from. All of the eggswere incubated at 29 degrees Celsius with 70 to 80 percent relative humidity until hatching. Each day for 8 days, the newly hatched larvae were collected and stored at -20°C until further processing. Figure 2 shows a head louse (Figure 2A) and a body louse (Figure 2B) with their respective nits (empty egg shells) one day after hatching in the laboratory.

Genotyping

Total genomic DNA extraction, PCR and sequencing of the intergenic spacers S2, S5, PM1 and PM2 were performed as described previously [23]. As lice are diploid, cloning was necessary to identify the different allelic sequences; therefore, PCR products were cloned using a previously described protocol [23]. The resulting sequences were aligned with genotypes published in GenBank [23, 24] for identification. When less than 100% homology was obtained, the new genotype was recorded, a new number was assigned to it and it was published in GenBank (JX041640-JX041654). This was done according to a new set up of GenBank submissions providing the gene names that this is the intergenic spacer between: PHUM005704-PHUM006210 for intergenic spacer 2 , PHUM007351-PHUM002191 for intergenic spacer 5, PHUM007934-PHUM003340 for intergenic spacer PM1 and PHUM002215-PHUM002223 for intergenic spacer PM2

We also used high-throughput 454 sequencing of the amplicons using tagged libraries. Libraries were created by PCR using the same protocol as above and the same specific primers with the addition of the 454 adaptor and a Multiplex Identifier sequence (MID). The same 8 nucleotide barcode was used for all primer pairs (spacer S2, S5, and PM2). A total of 13 barcodes were designed using *Barcrawl* software [31]. We excluded barcodes with the same 5' base as 3' end of the upstream 454 adaptor, and we added a guanine to the 3' end of the barcode to avoid the presence of the same 3' barcode base at the 5' end of the downstream primer. Barcodes that were converted to other barcodes by deletion were excluded. The numbers of 454 GS-FLX nucleotide flows to sequence the barcodes were as low as possible and were used between 5 and 9 flows (Supplementary Tables 1 and 2). The preparation of the 163 libraries was performed, as described in the Amplicon Library Preparation Method Manual from Roche. Additionally, 8

pools of 20 to 21 libraries were created to perform the clonal amplification, as described in the emPCR Method Manual from the Lib-A SV GS FLX Titanium Series from Roche. We worked with two Small Volume Emulsions of capture beads A and two Small Volume Emulsions of capture beads B per pool of libraries at a ratio of 1.8 copies of library per bead. The sequencing was performed in accordance with Roche using a GS FLX Titanium sequencing Kit XLR70 and the PicoTiter plate that was divided into 8 medium-sized regions.

For each region, barcodes were associated with only one DNA sample. We used *mothur* software [32] to trim the sequences and identify the barcodes using the following parameters: *minlength=100, bdiffs=1, qwindowsize=50, qwindowaverage=25* (Supplementary Table 3). The trimmed sequences were mapped to the 3 reference genes using the program CLC Genomics Workbench. A probabilistic variant table was created for each mapped gene and every SNP (small nucleotide polymorphisms) and DIP (deletion and insertion polymorphisms) were verified and associated to extract the two alleles. The 454 sequencing results were blasted against the results obtained from the PCR and cloning method. Differences in numbers of A or T in homopolymers were not taken into account.

Population genetic structure

The genotypic data were analyzed using tests based on both the allelic identity and the allele sequence. For the tests based on allelic identity, each unique sequence was assigned an allele number and the genetic distance among the sequences was considered equal. Using these data, we first tested to see if Hardy-Weinberg proportions (HW) were found within the populations. To determine the correct level of a population, we tested for HW by using two different combinations of the sampled lice. First, we broke the lice into the smallest possible biological unit by grouping all lice from a

given body area on a given homeless person (body location data, n = 10 populations). Next, we considered all lice from the same homeless person as representing as single population, regardless of whether the lice were found on the head or body (homeless person data, n= 5 populations). If there was significant isolation between head and body lice, we expected to find higher deviations from HW in the latter case due to a Wahlund effect [33]. Deviations from the expected HW proportions for each population and locus were measured by Weir and Cockerham's estimator of Wright's FIS index and tested for significance using exact probability tests implemented in the software GENEPOP v4.1. Exact p-values were calculated using the Markov chain method, and tests across body locations, individuals and loci were combined using Fisher's procedure [34].

Gene diversity and nucleotide diversity was estimated for each locus and population using the body location dataset and the software *F-STAT v 2.9.3* [35] and Arlequin v.3.5.1.3 [36], respectively. Differences in diversity among head and body lice were tested using paired t-tests for each locus. Tests across loci were combined using Fisher's procedure [34].

We used the sequence-based genotypic data to carry out an Analysis of Molecular Variance (AMOVA) that considers the allelic content of the genotypes and their frequencies to measure the population structure at different hierarchical levels of organization (i.e., within populations, among populations within groups, among groups) [37]. This analysis was carried out using the software *Arlequin v.3.5.1.2* [36] and tests for the significance of the covariance components associated with each organizational level were performed using a non-parametric permutation procedure (20,000 permutations of the data where the type of permutation depends on the organizational level). This analysis also provided fixation index estimates for each level, ie, a measure of population structure [38].

Results

Collections

During our investigations, not all homeless people were willing to cooperate, either because the rooms of the shelters were cold and not comfortable enough to allow them to change clothing or because they preferred to have their meal and go directly to bed. Additionally, because of the regular head shaving of homeless diagnosed with head lice that had been previously offered, we had difficulty finding head lice on many of the individuals. The presence of head lice was most frequently noted on hair near the neck or above and behind the ears. For body lice, we noted that eggs and even motile forms were found much more often near seams, particularly in the armpit.

During our investigations, we met 210 homeless people. Among them, 29 subjects had lice with 2 who had only head lice, 14 had only body lice and 13 had both. In addition to the head lice, we collected 163 body eggs (with 44 attached on the collar) and 727 mobile forms (larvae or adults). The head lice that we collected included 116 head eggs (with 10 attached to the beard) and 340 mobile forms. However, genotyping was only performed on the first instar larvae that were hatched from the eggs collected from homeless persons that had both head and body eggs. These criteria left us with 38 body lice larvae and 27 head lice larvae from 5 homeless people that were all sleeping at shelter A. Interestingly, we never found body lice eggs without larvae or adult body lice on the same body. In contrast, some homeless people had head lice eggs without larvae or adults found in hair (among our 5 studied homeless subjects, this is the case of homeless person S).

Genotyping

The PM1 spacer region was monomorphic (genotype 13) for all of the genotyped lice and was therefore not included in the analyses. Many of the collected lice were heterozygous as multiple sequences were overlaid in the chromatograms. For these individuals, cloning was needed to assess the genotypes. This was the case for almost all of the S2 sequences, many of the PM2 sequences and some of the S5 sequences. To ensure that all genotypes had been correctly assessed, the results obtained from the PCR and cloning method were compared with the results obtained from the high-throughput 454 sequencing of the same samples. In general, our results were congruent. However, it happened very often that the number of Ts or As found in homopolymers varied. Indeed, the polymerase can easily make mistakes at these positions, resulting in differences between the two sequencing methods and even between different clones or reads generated by the same sequencing method. Differences in homopolymer length were therefore not taken into consideration in the analyses. Moreover, the cloning method is long and fastidious when sequencing diploid organisms. The 454 sequencing method offers many advantages, including the production of hundreds of clones in one step. However, in some cases the reads obtained were not long enough to cover the studied region, so some adjustments to the protocol or to the chosen primers might prove useful. Overall, the sequencing run produced 285,002 reads with an average length of 484.5 nt and a median length of 507 nt. The total number of bases sequenced was 138,103,874, and the average quality score was 26.98.

As shown in figure 3, we observed that the majority of genotypes, including the most common genotypes, were shared between head and body lice (in green). The most prevalent alleles in head and body lice were the same. For the PM2 spacer region, alleles 1, 38 and 33 were present in the majority of lice. In spacer S5, the more frequent alleles were 42 and 12.

Finally, in spacer S2, the most frequent alleles were 48 and 68 (Figure 3). The raw data are provided in Supplementary Table 4. The concatenated genotypes of the S2, S5 and PM2 spacers that occurred at least twice in our sample are presented in Figure 4. We found two pairs of head and body lice on the same patient (homeless person 33) that had a unique multi-locus genotype, indicated with green arrows in Figure 4 (genotype 68, 42, 33 and genotype 68, 42, 48). This suggests that related individuals can be found on both regions of the body.

Genotypic data analyses

Genetic diversity and nucleotide diversity were similar among the head and body lice populations sampled from the homeless individuals (Table 1; Fisher's combined test $Chi^2_{h} = 2.323$, df = 6, $P = 0.888$; $Chi^2_{\pi} = 4.616$, df = 6, $P = 0.594$). HW proportions were not found within lice populations. Indeed, the fixation index (F_{IS} value) was positive (indicating a deficit in heterozygotes), and significant in almost all cases regardless of the spatial organization of the data (Table 2). Only the estimate for locus S2 was non-significant when populations were considered at the level of the body location, but this change is most likely caused by the high standard error of this estimate associated with the reduced population sizes of lice populations when divided into two groups for each homeless person. Indeed, restricting a louse population to only those lice found on the respective head or body zones of a given homeless person did not significantly reduce the overall heterozygote deficits present in the dataset (Table 2) and suggests that deficits at the homeless person level are not due to a Wahlund effect, i.e., an artifact of mixing different isolated populations.

AMOVA analyses revealed no significant population structure between head and body lice after controlling for the sampled person at any of the three loci tested (Table 3). Indeed, the fixation index at this level of

population organization was low (F_{SC}) and non-significant. Furthermore, no structure was evident among lice of different homeless persons (F_{CT}). However, some population structure was evident among all populations (Table 3, F_{ST}), but detailed pairwize comparisons could not reveal any interpretable pattern to this structure (data not shown). This structure may therefore be due different colonization histories and drift among some of the head and body populations.

Discussion

In people infested with both head and body lice, the original ecosystem of the lice can be dubious. Indeed, we found numerous eggs on collars, beards and ball caps that could have been from either the head or body (Figure 1). The method used to sample the two types of lice is therefore critical. For this reason, eggs collected from hair (head lice) and eggs collected from clothes (body lice) were kept separate and incubated until hatching, and molecular analyses were performed on the newly hatched larvae. All precautions to avoid DNA contamination were taken, and negative controls were used at each step of the study. Moreover, the use of two sequencing approaches strengthened our results because both techniques were concordant. Based on this data, and despite the elimination of individual lice in the potential overlap zones, our results strongly suggest a genetic mixing of lice from head and body populations.

After genotyping lice, the genetic diversity and the nucleotide diversity was calculated for each spacer and used to compare head and body lice populations. This was not calculated for the spacer PM1 because it was monomorphic in the tested populations. The spacers S2, S5 and PM2 showed high heterogeneity in both the head and body lice (Table 1). However, these two groups of lice did not differ significantly in gene

diversity or nucleotide diversity at the studied loci. This contrasts with a previous study that reported a higher nucleotide diversity in head lice compared to body lice [39]. This may be caused by a sampling bias as this previous study was based on 40 lice collected from across 12 different countries. We also found two pairs of head and body lice with identical genotypes collected from the same homeless person (homeless 33) (Figure 4) suggesting that related individuals move between body areas on the host.

Strong heterozygote deficits were present in all populations, regardless of how a population was defined (all lice from a given homeless person, or only those lice from the respective head or body populations). This result is not surprising given that transmission ratio distortion, that is, a non-Mendelian inheritance pattern of alleles, is known to occur in *P. humanus* populations and may have caused the HW disequilibria found in this study [40]. However, regardless of the presence of this distorter, the deviation from HW equilibrium proportions at the level of homeless person was not reduced by dividing lice into the smallest possible population unit, that of the body zone within a host individual. Although it is possible to have a Wahlund effect due to mixing of different louse families within each body zone, no Wahlund effect seems to be related to mixing lice from head and body populations on single host. These results are further supported by those of the AMOVA analyses which showed little genetic variation attributable to between host and body lice populations and no significant structure between these populations.

These results validate previous assumptions that the clade A lice may evolve and colonize both the hair and clothing niches [24,25]. First, our results suggest that the lice collected from our five subjects belong to a single population and, thus, that lice are shared between people living in the same shelter. They further indicate that head and body lice likely move frequently from one part of the body to the other. These results support

recent data comparing the transcriptional profiles of head and body lice [25]. Fourteen putative differentially transcribed genes were identified between head and body lice that could explain phenotypic differences [25]. The presence of two clades of lice living on some host individuals may help explain previous reports of independent head and body lice population [9,19]. Indeed, a study on doubly infested persons in Ethiopia showed that all of the head lice were black and of clade C and all of the body lice were gray and of clade A [9]. Moreover, the only other study that reported independent head and body lice populations on individuals infested by the two forms was undertaken in Nepal where both Clade A and C lice are prevalent [19]. In the case of the clade A lice from our study, it seems that migration occurs between the two body zones and that it may increase in case of massive infestations. However, here we consider only lice that hatched from collected eggs. It remains to be shown whether these individuals could durably establish in the ecological niche where they were found. From our data, we can also not say whether migration is bidirectional between body zones (from both head to body and body to head) or whether one zone acts as a source for the other. However, a previous study showed that a single gene of an unknown function seems to be lost in all head lice [25]. This finding suggests that head lice may originate from body lice rather than the reverse. More complete phylogeographic studies are called for to test this hypothesis.

Our failure to find population structure among homeless persons living in the same shelter may indicate that louse transmission frequently occurs in shelters. Prevention measures should therefore focus on avoiding the sharing of items such as mattresses, blankets and other personal belongings through which lice transmission is likely to occur from one homeless person to another.

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Table 1: Diversity estimates for each locus and population of lice from the bodies and heads of the sampled homeless persons.

Locus	Diversity	Host S		Host 33		Host F		Host M		Host D	
	estimate	Body	Head	Body	Head	Body	Head	Body	Head	Body	Head
		(n=17)	(n=2)	(n=6)	(n=9)	(n=5)	(n=4)	(n=5)	(n=3)	(n=5)	(n=9)
S2	*h*	0.836	0.500	0.833	0.764	0.650	0.708	0.875	0.667	0.667	0.804
	π	0.025	0.011	0.029	0.033	0.029	0.012	0.037	0.009	0.008	0.030
S5	*h*	0.669	0.000	0.667	0.743	0.375	0.833	0.675	1.000	0.667	0.518
	π	0.024	0.000	0.024	0.026	0.006	0.024	0.024	0.033	0.028	0.009
PM2	*h*	0.836	1.000	1.000	0.722	0.700	1.000	1.000	0.833	0.875	0.736
	π	0.011	0.010	0.009	0.005	0.006	0.007	0.012	0.007	0.004	0.005

Gene diversity (h) and nucleotide diversity (π) are based on Nei's (1987) estimates.

n refers to the number of lice genotyped for each locus.

Table 2: Summary of Hardy-Weinberg tests when louse populations are defined at the level of the body location of each homeless person (head or body), or when combined across body locations for each homeless person.

Dataset	Locus	n	Fis (SE)	P-value
Body location	S2	10	0.2210 (0.1688)	0.1809
	S5	9	0.5843 (0.1328)	0.0003
	PM2	10	0.6782 (0.0862)	<0.0001
	Overall	29	0.4914 (0.0835)	<0.0001
Homeless person	S2	5	0.3306 (0.0609)	0.0032
	S5	5	0.5891 (0.1047)	<0.0001
	PM2	5	0.6222 (0.1138)	<0.0001
	Overall	15	0.5140 (0.0620)	<0.0001

P-values represent the combined value across populations (Fisher's procedure).

n refers to the number of combined values

Fis (± standard error) refers the average unweighted value across populations and measures the deviation from panmixia

Table 3: Analysis of molecular variance (AMOVA) of louse populations for each spacer locus

Locus	Level	df	% variation	Fixation index	P-value
S2	Among homeless persons	4	0.31	Fct = 0.0031	0.57283
	Between body locations (homeless)	5	9.25	Fsc = 0.093	0.06647
	Within body locations	114	**90.45**	**Fst = 0.096**	**0.00782**
S5	Among homeless persons	4	10.84	Fct = 0.11	0.14374
	Between body locations (homeless)	5	11.30	Fsc = 0.13	0.19129
	Within body locations	116	**77.86**	**Fst = 0.22**	**0.00759**
PM2	Among homelesspersons	4	5.11	Fct = 0.051	0.12401
	Between body locations (homeless)	5	4.48	Fsc = 0.047	0.58660
	Within body locations	116	90.41	Fst = 0.096	0.08566

% variation indicates the amount of overall variation in the data explained at a given level of organization.

The fixation indices refer to the amount of genetic structure attributed to each level.

It should be noted that at the Within Body locations level, the % variation refers the amount of variation found within populations, whereas Fst measures the structure among population

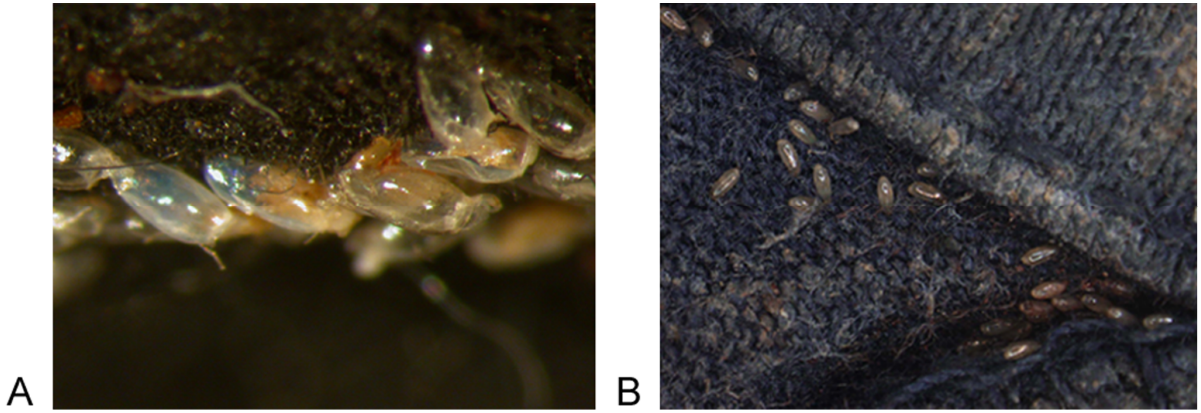


Figure 1: Lice eggs attached to a homeless cap (A) and a homeless collar (B)

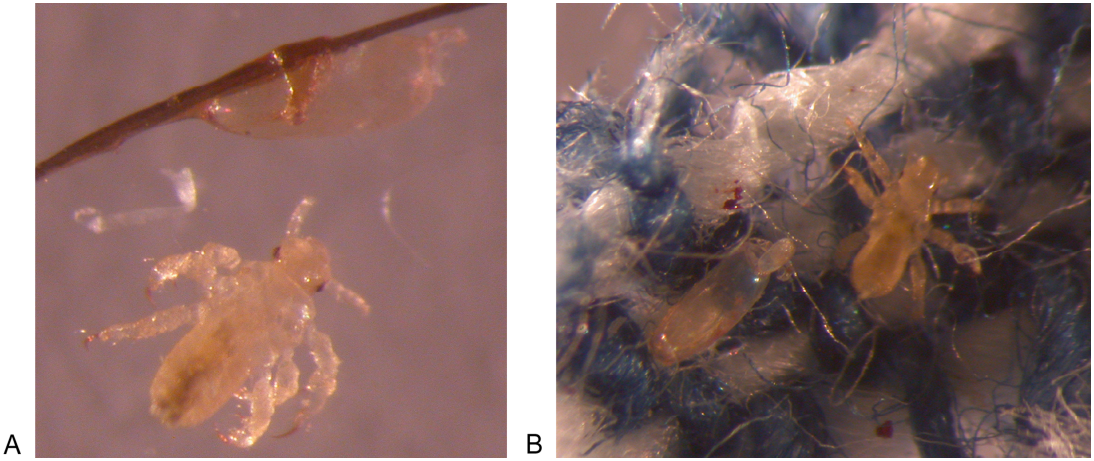


Figure 2 : Picture of a head louse (A) and a body louse (B) and their respective nits

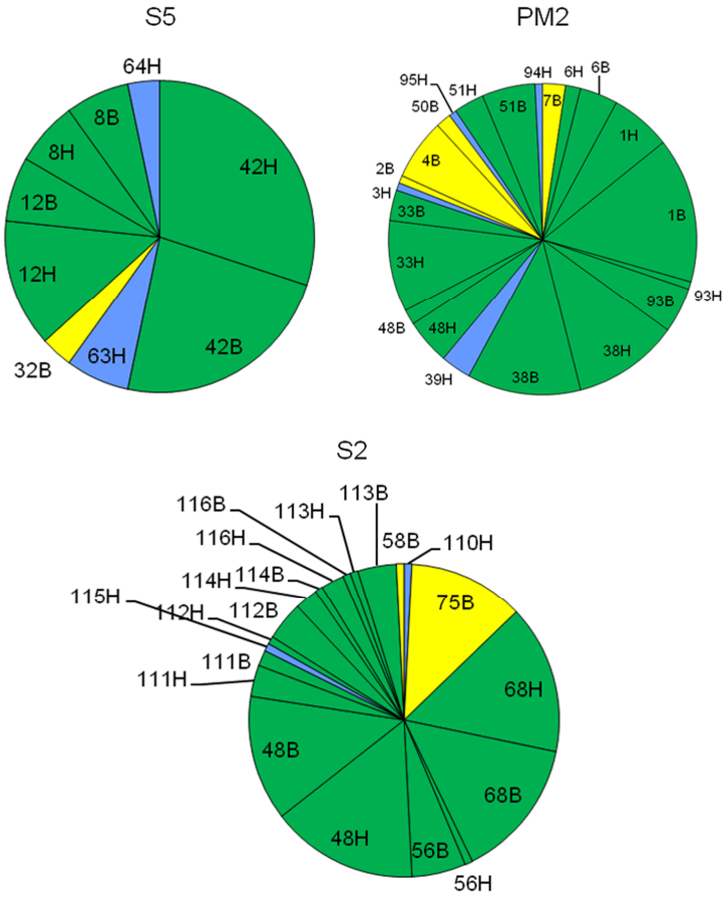


Figure 3 : Proportion of each allele among the head and body lice. The names (ID numbers) of the alleles are mentioned followed by the letter H for head lice and B for body lice. The alleles found in both the head and body lice are shown in green. The blue alleles were found only in the head lice, and the yellow alleles were found only in the body lice.

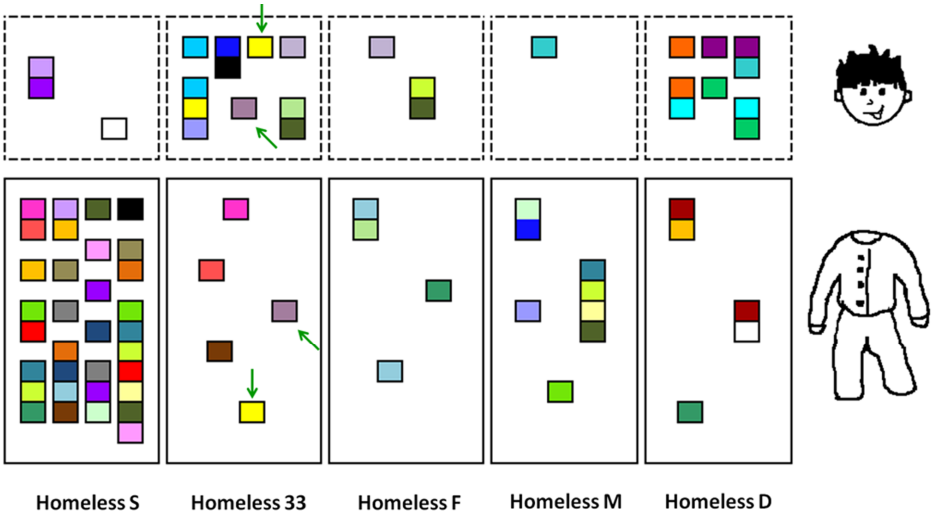


Figure 4: Repartition of head and body lice haplotypes found at least twice in our samples. The haplotypes are concatenated alleles of the S2, S5 and PM2 spacers. A single color was assigned to each unique haplotype. Each block represents one louse, but may be separated into several colored units if the alleles at the three spacers could be combined to generate multiple haplotype possibilities. The green arrows indicate identical head and body lice haplotypes collected from the same homeless person.

Supplementary Table 1 : Primer sequences

Spacer	Primer pairs					
	454 adaptor		Barcode	Last barcode base	Primer	Length
S2	Fw:	5'- CGTATCGCCTCCCTCGCGCCATCAG	-MID-	G	ATGATGTGCATTGCGAGTGT -3'	54
	Re:	5'- CTATGCGCCTTGCCAGCCCGCTCAG	-MID-	G	AAACTTAACCCGGGCCCTAT -3'	54
S5	Fw:	5'- CGTATCGCCTCCCTCGCGCCATCAG	-MID-	G	TCCAAATGAAACCCACACTTT -3'	55
	Re:	5'- CTATGCGCCTTGCCAGCCCGCTCAG	-MID-	G	TGGCAGACACTGCTTCCTTA -3'	54
PM2	Fw:	5'- CGTATCGCCTCCCTCGCGCCATCAG	-MID-	G	CCGAAGGAGCTGATTCTTTT -3'	54
	Re:	5'- CTATGCGCCTTGCCAGCCCGCTCAG	-MID-	G	CCACAACGAGTGATGTGAGC -3'	54

Supplementary Table 2 : Barcode sequences

Barecode ID	Barcode sequence	Number of 454 GS-FLX nucleotide flows
1	TTAACGGT	5
2	ACTTAACC	7
3	TAGTTACC	7
4	TTAACCAC	7
5	AACTTACG	8
6	AATTACCG	8
7	TGTTAACG	8
8	TTAACTCG	8
9	TTAAGGAG	8
10	TTACCAAG	8
11	TTATTCCG	8
12	TTCCTTAG	8
13	AACGTAGT	9

Supplementary Table 3 : Recovery statistics after *Mothur* processing

	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Region 7	Region 8
Number of analysed reads :	66238	36448	27596	43736	36284	27856	6967	39877
Barcode ID and **DNA ID** followed by the number of reads and the number of libraries associated	BC1_694 5879 3	BC1_693 4794 3	BC1_695 2411 3	BC1_696 3872 3	BC1_427 1174 1	BC1_428 1018 1	BC1_721 156 1	BC1_697 3457 3
	BC2_699 9486 3	BC2_698 4586 3	BC3_737 1093 1	BC2_700 5669 3	BC2_701 2630 3	BC2_702 3623 3	BC3_739 230 1	BC2_722 737 1
	BC3_703 9834 3	BC3_704 5534 3	BC7_720 2104 3	BC3_705 6506 3	BC3_738 2209 1	BC3_706 2236 3	BC4_708 363 3	BC3_707 3531 3
	BC4_433 7500 2	BC5_711 4341 3	BC8_427 2355 1	BC4_432 5190 2	BC8_723 2914 1	BC4_434 2233 2	BC6_717 269 3	BC4_431 3360 2
	BC5_709 9154 3	BC6_435 2953 2	BC9_429 1771 2	BC5_712 3612 3	BC9_430 3846 3	BC6_716 3467 3	BC8_721 38 1	BC8_722 2899 2
	BC6_714 8054 3	BC7_442 7936 3	BC10_727 4535 3	BC6_715 6199 3	BC10_728 4314 3	BC8_428 1187 1	BC9_725 942 3	BC9_726 7348 3
	BC7_719 10065 3	BC10_731 2520 3	BC11_732 2661 3	BC11_736 4070 3	BC11_733 5172 3	BC9_724 3943 3	BC11_734 566 3	BC10_730 3588 3
			BC12_737 2479 2		BC12_738 3874 2	BC10_729 3587 3	BC12_740 757 2	BC11_735 3896 3
			BC13_742 2256 3		BC13_743 3144 3	BC12_739 2307 2	BC13_746 856 3	BC13_744 1788 1
Total of all groups : number of reads (%) - number of libraries	59972 (91%) 20	32664 (90%) 20	21665 (79%) 21	35118 (80%) 20	29277 (81%) 20	23601 (85%) 21	4177 (60%) 20	30604 (77%) 21

Supplementary Table 4: Results of the Multi-Spacer-Typing of homeless lice

Homeless	Head or Body	Louse ID	PHUM005704-PHUM006210 intergenic spacer S2		PHUM007351-PHUM002191 intergenic spacer S5		PHUM002215-PHUM002223 intergenic spacer PM2		PHUM007934-PHUM003340 intergenic spacer PM1	
S	**H**	100431	48	68	8	8	51	51	13	13
		100432	48	68	8	8	6	6	13	13
	B	100693	**110**	75	8	32	**93**	51	13	13
		100694	68	68	42	8	38	51	13	13
		100695	68	68	8	8	1	38	13	13
		100696	56	48	32	8	4	4	13	13
		100697	48	48	8	8	1	**93**	13	13
		100698	75	**112**	8	42	1	38	13	13
		100699	56	68	32	42	1	**93**	13	13
		100700	48	68	8	8	7	7	13	13
		100701	48	56	32	42	1	4	13	13
		100702	**113**	**113**	42	42	38	51	13	13
		100703	48	75	8	8	1	4	13	13
		100704	48	48	8	**63**	38	51	13	13

		100705	75	75	8	12	38	38	13	13
		100706	**111**	48	12	8	7	51	13	13
		100707	48	**113**	42	42	1	1	13	13
		100433	48	**113**	42	42	4	4	13	13
		100434	56	56	8	8	38	38	13	13
33	**H**	100708	68	68	12	12	33	33	13	13
		100709	68	**112**	42	12	33	33	13	13
		100710	68	68	42	42	33	33	13	13
		100711	48	**113**	42	42	38	38	13	13
		100712	**115**	**111**	**63**	**63**	51	51	13	13
		100713	68	68	42	42	48	48	13	13
		100714	68	**111**	8	8	33	33	13	13
		100715	**111**	48	42	42	1	1	13	13
		100435	48	**111**	**64**	12	33	33	13	13
	B	100716	75	75	8	42	2	**93**	13	13
		100717	68	75	12	32	50	51	13	13
		100718	68	68	42	42	48	48	13	13
		100719	**116**	48	8	12	1	1	13	13

		100442	**114**	**112**	42	42	38	38	13	13
		100441	68	68	42	42	33	33	13	13
F	**H**	100720	48	48	42	42	39	39	13	13
		100721	68	68	8	8	33	33	13	13
		100427	48	56	42	42	1	1	13	13
		100428	48	68	44	44	48	48	13	13
	B	100722	**111**	75	32	42	1	1	13	13
		100723	68	75	42	42	50	1	13	13
		100724	75	75	42	**65**	1	1	13	13
		100430	68	**112**	42	42	6	6	13	13
		100429	75	75	42	42	6	6	13	13
M	**H**	100725	68	68	44	44	38	38	13	13
		100726	48	48	**66**	**66**	**94**	38	13	13
		100727	48	48	42	42	48	48	13	13
	B	100728	48	58	12	42	38	51	13	13
		100729	75	**112**	42	42	33	33	13	13
		100730	48	56	32	42	1	1	13	13
		100731	NA	NA	8	8	NA	NA	13	13

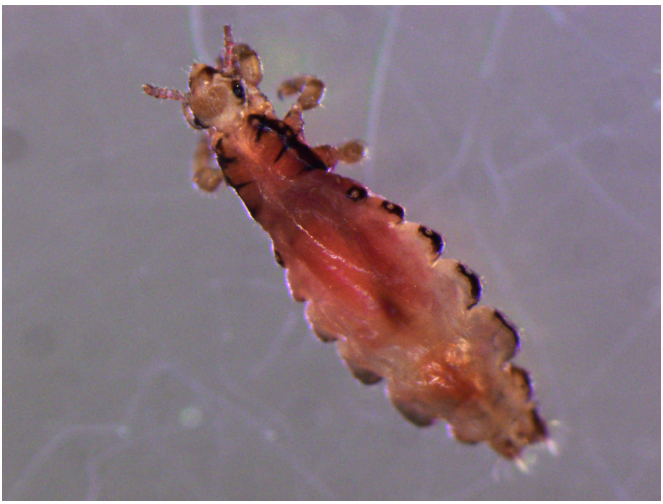
		100732	56	**112**	42	42	4	4	13	13
D	**H**	100733	68	68	44	44	1	**93**	13	13
		100734	**114**	**114**	8	44	38	38	13	13
		100735	48	68	44	44	1	1	13	13
		100736	48	48	44	44	38	38	13	13
		100737	48	48	46	46	38	38	13	13
		100738	**114**	68	44	44	38	**95**	13	13
		100739	48	**116**	44	44	1	38	13	13
		100740	**116**	**116**	46	46	3	38	13	13
		100741	NA	NA	NA	NA	39	39	13	13
	B	100742	68	68	8	8	38	**93**	13	13
		100743	68	68	42	42	1	1	13	13
		100744	48	48	42	42	NA	NA	13	13
		100745	NA	NA	NA	NA	38	38	13	13
		100746	68	**113**	8	8	6	**93**	13	13

NA, not available

new genotypes submitted to GenBank in bold

CHAPITRE III

Amazonian head lice-specific genotypes are putatively pre-Columbian



Préambule

Les Pédiculidés sont classés en trois grands clades dont un seul comprend à la fois des poux de tête et des poux de corps, le Clade A. Les études génotypiques réalisées précédemment sur des poux de Clade A ont montré une corrélation entre la phylogénie basée sur les espaces intergéniques (méthode MST) et la provenance géographique de l'échantillon [12,17]. En effet, sur base de l'espace intergénique PM2, les poux hors Afrique (sous-type A1) clustérisent séparément des poux d'Afrique (sous-type A2) [32]. Dans la présente étude, nous avons voulu travailler sur des poux collectés en Amazonie afin de savoir à quel clade mitochondrial ils appartiennent et où ils se situent dans un arbre phylogénétique MST parmi des poux du monde entier.

Ainsi, nous avons pu montrer que les poux d'Amazonie appartiennent tous au clade A et constituent au sein de ce clade un sous-type distinct (sous-type A3) des deux autres décrits précédemment. Etant donné que l'Amazonie fait partie des quelques endroits dans le monde qui furent les moins touchés par la globalisation, ces poux sont peut-être descendants directs de populations précolombiennes.

Article III

Amazonian head lice-specific genotypes are putatively pre-Columbian

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Submitted

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Abstract

Head and body lice are strict obligate human ectoparasites with three mitochondrial phylotypes (A, B and C).

Using molecular methods for genotyping lice (Cytochrome b and Multi-Spacer Typing), we assessed the presence of a specific American genotype that most likely predates the Columbian era in head lice collected from Amazonia.

Introduction

Three louse species specifically associated with humans have been described including the pubic louse and two species belonging to the Pediculidae family (Raoult et al. 1999). The head louse (*Pediculus humanus capitis* de Geer) is prevalent in all countries, it lives and lays eggs in the hair on the head and the body louse (*Pediculus humanus humanus* Linnaeus), which lives and lays its eggs in clothing and multiplies when such conditions as cold weather, lack of hygiene, or war are present (Kittler et al. 2003). It is responsible for the deaths of millions as a result of vectoring several deadly bacterial pathogens (Badiaga et al.2012).

Several phenotypic and genotypic studies have been conducted to assess whether these two species are distinct (Li et al. 2010; Yong et al. 2003). Furthermore, a partial *Cytb* gene sequence analysis classified the lice into three phylotypes of human head lice (clades A, B and C) and a single phylotype (clade A) of body lice. Each phylotype has a unique geographical distribution: clade A can be found worldwide, clade B can be found in north America, central America, Europe and Australia (Raoult et al. 2008) and clade C can be found in Nepal (Sasaki et al. 2006), Ethiopia (Angelakis et al. 2011), and Senegal (Boutellis et al. 2012). Li et al introduced a new genotyping technique involving the sequencing of four variable intergenic spacers for clade A and found a relatively specific geographic distribution for each genotype. This method identified two clusters in France, one cluster in Central Africa and one cluster in Russia (Li et al. 2010). Veracx et al confirmed the presence of an African cluster through a phylogenetic analysis of the spacer PM2 in lice from several different regions (Veracx et al. 2012). Here, we report the study of the genotypic distribution of Amazonian head lice.

Materials and methods

Informed verbal consent was obtained from all of the participants involved in our study. We collected 30 head lice samples from 3 different states in Brazil. A total of 26 lice were collected from Amazonas State (3°6’ S and 60°00’ N), 2 were collected from Pará State (1°52'30"S, 48°07'30"W) and 2 were collected from Amapá State (3°52'30"N, 51°52'30"W) (Figure 1). All of the lice were preserved dry under sterile conditions and transported to our laboratory in August 2011. All of the samples were photographed with a camera (Olympus DP71). Before the DNA isolation, each louse was rinsed twice in sterile water, and the total genomic DNA was extracted using a QIAamp Tissue kit (QIAGEN, Hilden, Germany), as described by the manufacturer.

The quantitative real-time PCR primers and probes targeting a portion of the Bartonella 16S–23S intergenic spacer region (ITS) (Angelakis et al. 2011) and one *Acinetobacter baumannii*-specific gene (*rpoB*) were performed as were previously described (Bouvresse et al. 2011). Negative and positive controls were included in each assay. The mitochondrial gene *CytB* and each intergenic spacer were amplified as described previously (Li et al. 2010; Veracx et al. 2012). Each DNA sequence was aligned with previous data (Veracx et al. 2012). The sequence similarities were determined using MEGA 5, and phylogenetic trees were obtained using the Maximum Likelihood (ML) method with 100 bootstrap replicates (Tamura et al. 2011).

Results

All of the Amazonian head lice are brown, distinct from French lice (gray) and African lice (black) (Figure 1). The qPCR assay showed that

all of the samples were negative for Bartonella spp. and *A. baumannii*, while the positive controls amplified normally.

After sequence correction and assembly of the partial *Cytb* gene sequence, all of the lice were found to belong to clade A (Genbank accession no. JX178718 - JX178747) and very little variation was observed among the collected specimens.

The intergenic spacer S2 was analyzed in 14 of the Amazonian head lice. These samples exhibited 6 different genotypes, 3 of which (genotype 117, 118 and 119) are novel (Genbank accession no. JX178748 - JX178750) (Table 1). Four of the genotypes are specific to the Amazonian lice (66%), and the other genotypes (39 and 40) are common with the other lice from America, Africa and Asia (Li et al. 2010) (Figure 2).

Spacer S5 was analyzed in 26 of the Amazonian lice, and four different genotypes were found. Two of these genotypes (68 and 69) (Genbank accession no. JX178751 - JX178752) are specific to the Amazonian head lice and were found in 50% of the samples. We found two lice specimens (1 and 22) that were heterozygous for genotype 23 and genotype 67 (Table 1). Genotype 23 was found in 39% of the Amazonian lice and is also found in Europe and America (Li et al. 2010).

The analysis of spacer PM1 showed that 72% of the 25 Amazonian lice had genotype 13; this genotype is prevalent in Europe and is also found in America and Africa (Veracx et al. 2012). A new genotype (36) (Genbank accession no. JX178753) was found to be specific to the Amazonian head lice and was present in 28% of the tested samples (Table 1).

All of the Amazonian head lice presented genotype 47 for spacer PM2 but 3 samples were not properly amplified. For the phylogenic tree constructed using the PM2 spacer, we included sequences from lice found in the USA and UK that were previously genotyped only for PM1 and PM2

(Figure 2). These lice exhibited genotype 47 for spacer PM2 and belonged to clade B based on *Cytb* analysis (Li et al. 2010).

Two distinct clusters are shown: one cluster containing all of the African lice and another containing all of the non-African lice. Among the non-African lice, the Amazonian lice cluster separately with some Mexican, British and American lice (Figure 2).

This clustering is correlated with the two bases specific to the genotype 47. Another tree was constructed using the concatenated sequences of the four intergenic spacers S2, S5, PM1 and PM2. The dendrogram showed the same topology as the PM2 tree.

Among the non-African lice, the Amazonian lice are clustered together with four Mexican lice (Figure 3). This tree supports the hypothesis that the head lice in Amazonia represent an independent cluster.

Discussion

In this study, we confirmed that the sequencing of the partial mitochondrial *Cytb* gene and MST are sensitive methods for evaluating the genotypic distribution of human lice. The genotyping of the *Cytb* gene showed that all of the Amazonian head lice belonged to clade A. The MST results indicate that genotype 47 of the PM2 spacer is found among all of the clade A Amazonian lice and is associated with the clade B American and British lice (Li et al. 2010). The phylogenic tree based on the PM2 spacer and the concatenated sequences show a net separation of the Amazonian lice.

Clade A includes three subtypes of lice. The first subtype, A1, is the ubiquitous gray louse (Raoult et al. 2008), the second subtype, A2, includes black African lice from Rwanda and Burundi (Veracx et al. 2012), and the third subtype, A3, is the American subtype, which grouped the Amazonian brown head lice with the lice from Mexico and the United States

(Li et al. 2010). The A3 subtype most likely dates from the pre-Columbian era. Our previous study of the head lice from Peruvian mummies confirmed that the clade A lice were prevalent in America before the arrival of Columbus (Raoult et al. 2008). These lice may date from the Clovis culture, which is presumed to be responsible for populating America (Curry 2012). Amazonia is one of the few places in the world that has not been strongly affected by globalization, and these lice may be the descendants of a pre-Columbian population.

In conclusion, we confirmed the presence of a specific, likely pre-Columbian American louse genotype. Although it was interesting to test the lice from ancient mummies, the DNA extracted was insufficient for phylogenetic analyses. Collecting more louse samples from a wide variety of origins, particularly lice from ancient American mummies, such as the Andean mummy of Arica in Chile (Arriaza et al. 2012), for MST and *Cytb* analyses will help us to clarify the origin and the distribution of human lice in the Americas.

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Table 1: Results of the multi-spacer typing of Amazonian head lice.

Locality	Subject	Age	Louse ID	S2 Type	S5 Type	PM1 Type	PM2 Type
Amazonas	F	12	1	NA	23 /67	13	47
Amazonas	F	11	2	NA	NA	13	47
Amazonas	M	15	3	43	**69**	NA	47
Amazonas	F	17	4	NA	NA	NA	47
Amazonas	F	19	5	NA	**69**	13	47
Amazonas	F	11	6	43	**69**	13	47
Amazonas	F	13	7	NA	**69**	13	47
Amazonas	F	14	8	NA	**69**	13	47
Amazonas	F	10	9	NA	23	13	(-)PCR
Amazonas	M	9	10	43	**69**	13	(-)PCR
Amazonas	M	5	11	**117**	**69**	13	(-)PCR
Pará	F	6	12	NA	67	13	(-)PCR
Pará	M	13	13	43	**69**	13	47
Amapá	F	12	14	NA	**69**	13	47
Amapá	F	12	15	39	67	13	47
Amazonas	F	6	16	NA	23	NA	47
Amazonas	F	6	17	43	23	**36**	47
Amazonas	F	30	18	NA	67	**36**	47
Amazonas	F	8	19	40	67	**36**	NA
Amazonas	F	30	20	NA	67	**36**	47
Amazonas	F	3	21	**118**	23	13	47
Amazonas	F	6	22	**119**	23/ 67	NA	47
Amazonas	F	3	23	NA	NA	13	47
Amazonas	F	3	24	40	**68**	13	47
Amazonas	F	3	25	40	NA	13	47
Amazonas	F	6	26	NA	23	**36**	47
Amazonas	F	6	27	NA	23	**36**	47
Amazonas	F	8	28	43	23	**36**	47
Amazonas	F	8	29	43	23	NA	NA
Amazonas	F	3	30	NA	23	13	47

NA, not available; **(-)**, negative; **PCR**, polymerase chain reaction.
Underlined and bold genotypes are
new.

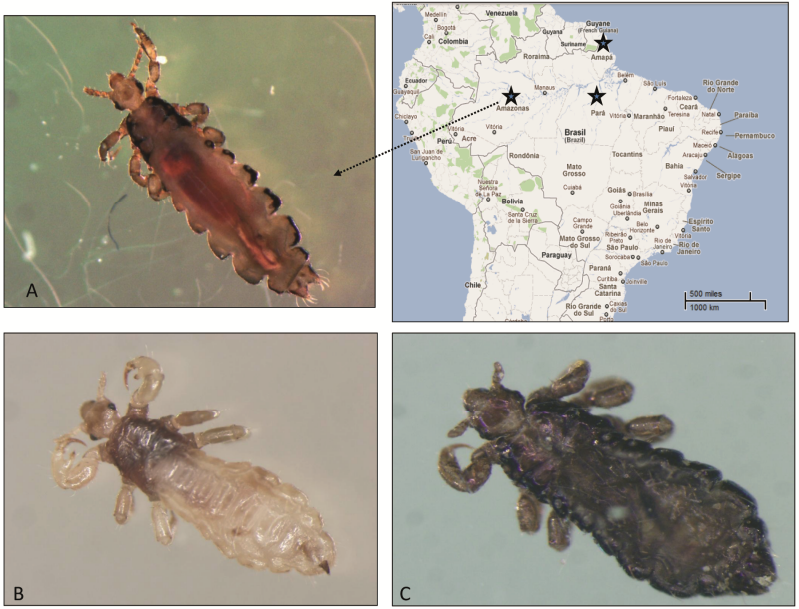

Figure 1: Photographs of the Amazonian brown head louse (A), French gray head louse (B) and Senegalese black head louse (C), showing the three different phenotypes (2011).

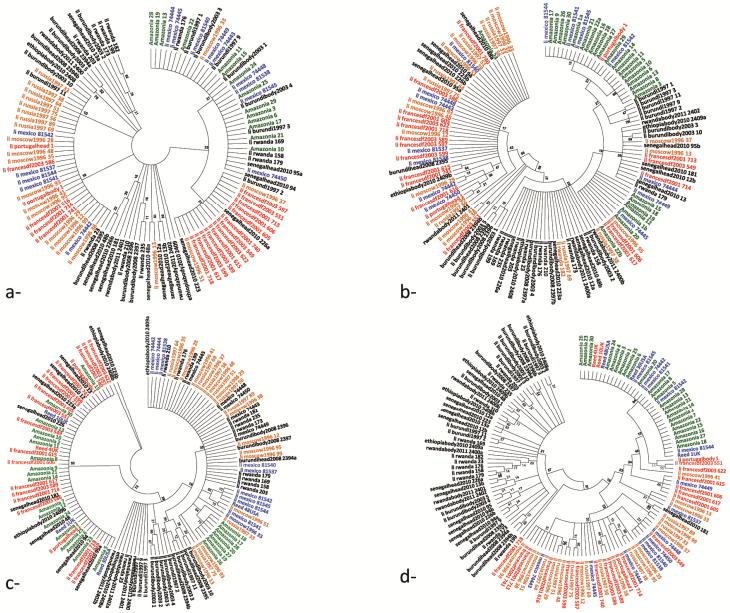

Figure 2: The phylogenic organization of Amazonian lice (green), American lice (blue), European lice (red), Asian lice (yellow) and African lice (black), constructed using the Maximum Likelihood method with each spacer region independently. (a) S2 spacer; (b) spacer S5; (c) spacer PM1; (d) spacer PM2.

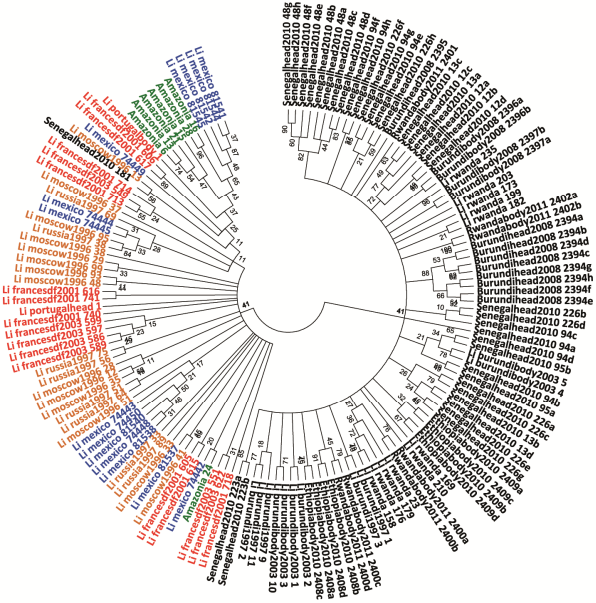

Figure 3: The phylogenic organization of Amazonian lice (green), American lice (blue), European lice (red), Asian lice (yellow) and African lice (in black), determined using the Maximum Likelihood method. The tree was constructed based on the concatenated sequences of the four nuclear intergenic spacer regions, S2, S5, PM1 and PM2.

CHAPITRE IV

Bartonella quintana in Head Lice from Senegal

Préambule

De façon générale, parmi les poux de l'homme, seuls les poux de corps ont été reportés comme vecteurs de maladies. En effet, ils peuvent transmettre la fièvre récurrente à poux (*Borrelia recurrentis*), le typhus épidémique (*Rickettsia prowazekii*) et la fièvre des tranchées (*Bartonella quintana*) [33]. Cependant, ces dernières années, il fut reporté quelques cas d'infections de poux de tête par *Bartonella quintana* [34–37]. Au Sénégal, les études épidémiologiques et cliniques sont assez rares sur le sujet.

Le but de cette étude était donc de savoir à quels groupes phylogénétiques appartiennent les poux du Sénégal et d'identifier si ces poux étaient infectés ou non par *Bartonella quintana*.

Parmi les poux de tête analysés, 19 (6.93%) provenant de 7 patients différents contenaient l'ADN de la bactérie. De plus, parmi ces 7 patients, trois portaient à la fois des poux appartenant au Clade A et des poux du Clade C.

Ces résultats confirment ceux d'études précédentes qui montrent que les poux de tête peuvent contenir de l'ADN de *Bartonella quintana* mais leur rôle dans l'épidémiologie de la maladie reste à être déterminé. De plus, cette étude démontre que des poux de tête appartenant à deux groupes phylogénétiques distincts (Clade A et C) peuvent cohabiter sur une même personne.

Article IV

Bartonella quintana in Head Lice from Senegal

Boutellis A, Veracx A, Angelakis E, Diatta G, Mediannikov O,

Trape JF, Raoult D.

Vector Borne Zoonotic Dis. 2012 May 18

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Bartonella quintana in Head Lice from Sénégal

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Abstract

Head and body lice are strict, obligate human ectoparasites with three mitochondrial clades (A, B, and C). Body lice have been implicated as vectors of human diseases, and as the principal vectors of epidemic typhus, relapsing fever, and *Bartonella quintata*-associated diseases (trench fever, bacillary angiomatosis, endocarditis, chronic bacteremia, and chronic lymphadenopathy). Using molecular methods (real-time and traditional PCR), we assessed the presence of *Bartonella quintana* DNA in black head lice collected from three locations in Sénégal. DNA from *B. quintana* was identified in 19 lice (6.93%) collected from 7 patients (7%) in Dakar. *B. quintana*-positive lice collected from three subjects were identified as clades C and A.

Key Words: *Bartonella quintana*—Head louse—*Pediculus humanus capitis*—Sénégal—Trench fever.

Introduction

Head and body lice (*Pediculus humanus capitis* de Geer and *Pediculus humanus humanus* L*innaeus*, respectively) are strict, obligate human ectoparasites that differ mainly in their habitat on the host, and have been parasites of humans for thousands of years (Light et al. 2008). The type A mtDNA phylotype is the most common among both head and body lice and is distributed worldwide (Ingman et al. 2000; Reed et al. 2004). The second mtDNA group (type B) occurs only in head lice, and has been found in the New World, Europe, and Australia. The third group (type C) has been found only among head lice from Nepal and Ethiopia (Kittler et al. 2003; Reed et al. 2004; Raoult et al. 2008). Traditionally, only body lice have been implicated as vectors of human diseases and as the principal vectors of epidemic typhus (*Rickettsia prowazekii*); louse-borne relapsing fever (*Borrelia recurrentis*); and trench fever, bacillary angiomatosis, endocarditis, chronic bacteremia, and chronic lymphadenopathy (*Bartonella Quintana*; Brouqui 2011). Body lice usually feed five times per day; proteins in their saliva provoke an allergic reaction and lead to pruritus and scratching that facilitates the fecal transmission of these agents (Foucault et al. 2006). Recently, DNA from *B. quintana* has been found in head lice collected from homeless individuals in Nepal (Sasaki et al. 2006), the U.S. (Bonilla et al. 2009), France (Angelakis et al. 2011a), and Ethiopia (Angelakis et al. 2011b). Cases of endocarditis due to *B. quintana* have been reported in a patient in Sénégal (Thiam et al. 2002), and in Sénégalese travelers to France (Monticrol et al. 2009) and to Switzerland (Barbe et al. 2000). The objective of our study was to use molecular methods to assess the presence of *B. quintana* DNA in head lice collected from Sénégal, where epidemiological and clinical studies of zoonoses are scarce, and to identify which phylotyping clade they belong to.

Materials and Methods

The study was conducted during October and December 2010 and January 2011. After ethical approval for the search for pathogens responsible for non-malarial fever, and after informed consent was obtained from parents for minors, we collected head lice from different locations in Sénégal: Kaolack City (14°14′N,16°08′W), Dakar City (14°34′N,17°29′W) and its suburbs, and two villages in the Fatick region, Dielmo and Ndiop (14°20′N,16°25′W). All lice were preserved dry in sterile conditions, except two that were kept in ethanol at room temperature and then sent to our reference center in Marseille in January 2011. Pictures of the ventral and dorsal sides of each louse were taken in the laboratory (Fig. 1). Before DNA isolation, each louse was rinsed twice in sterile water for 15 min. Then total genomic DNA was extracted from each louse using a QIAamp Tissue kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. The extracted genomic DNA was stored at −20°C under sterile conditions to avoid cross-contamination until the PCR assays were performed. DNA was used as a template in a previous study that utilized an RT-PCR assay targeting a portion of the *Bartonella* 16S-23S

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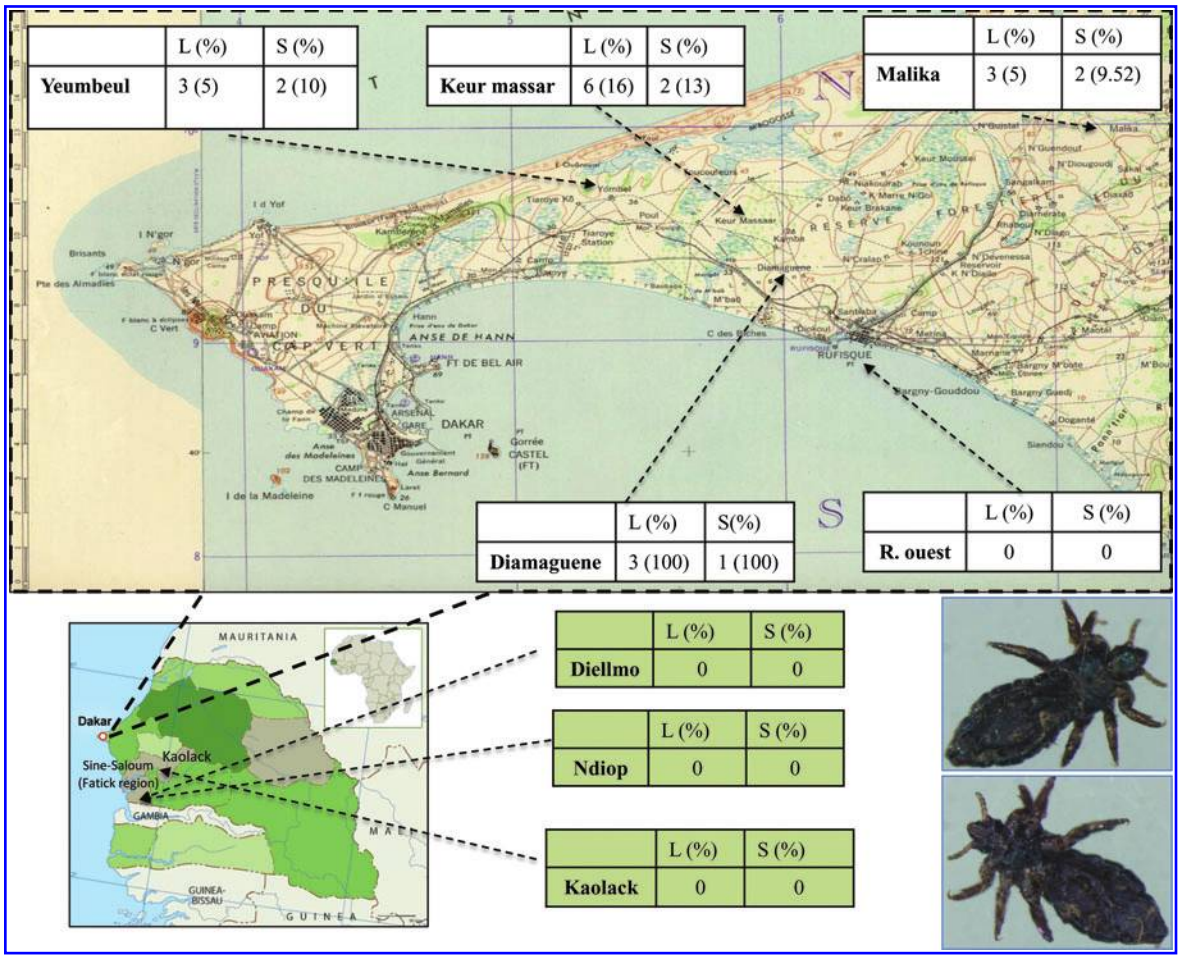

FIG. 1. Lice infected with DNA from *Bartonella quintana* and subjects infested with these lice in Sénégal [2010; L (%), number and percentage of infected lice; S (%), number and percentage of subjects infested with infected lice].

intergenic spacer region (ITS) (Angelakis et al. 2009), and a specific *B. quintana* gene, *fabF3*, encoding 3-oxoacyl-(acyl-carrier-protein) synthase II (Angelakis et al. 2011a). Negative controls (DNA from uninfected lice and sterile water) and positive controls (DNA from *Bartonella elizabethae*) were included in each assay. Nine head lice infected with *B. quintana* DNA collected from three subjects (three lice per person), and two non-infected lice collected from two other subjects, were randomly selected for amplification and sequencing of the mitochondrial gene cytochrome b (cytB), as previously described (Li et al. 2010). Lice with *B. quintana* DNA were collected from two subjects from Keur Massar and one from Diamaguene (Dakar). The non-infected lice were collected from one subject in Yeumbeul and one in Dielmo. For phylogenetic analysis, we used MEGA 4.1 (Molecular Evolution Genetic Analysis; The Biodesign Institute, Tempe, AZ), and for data comparison, we used Epi Info version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA). A p value < 0.05 was considered significant.

Results

Overall, we tested 274 adult head lice collected from 100 females (78 children, 14 youths, and 8 adults). From each person, we collected 1 to 10 adult head lice. *B. quintana* was identified in 19 lice (6.93%) collected from 7 patients (7%). Four children (5.12%), one youth (7.14%), and 2 adults (25%) were infested with head lice infected with *B. quintana* (Table 1). No significant difference was found according to age ($p = 0.52$ by the Kruskal-Wallis test). Positive and negative controls yielded the expected results in all tests. All the *B. quintana*-positive lice were found in Dakar and in its suburb. No positive lice were found in the villages Dielmo and Ndiop. Among *B. quintana*-positive lice from Dakar, 7 were from the Yeumbeul district, 6 were from the Keur Massar district, 3 were from the Diamaguene district, and 3 were from the Malika district (Fig. 1). All these positive lice were kept dry without ethanol.

A 316-bp fragment was obtained from each of 11 lice (GenBank numbers JN400090, JN400091, JN40009, JN969581, JN969582, JN969583, JN969584, JN969585, JN969586, JN969587, and JN969588). We found that 4 *B. quintana*-positive head lice belonged to clade A and 5 belonged to clade C (Fig. 2). The non-infected lice belonged to clade A.

Discussion

In this study, we confirmed the presence of DNA from *B. quintana* in head lice collected from Dakar (Sénégal). Clade C head lice are known to be prevalent in Ethiopia (Kittler et al. 2003; Reed et al. 2004; Raoult et al. 2008), and in Nepal (Sasaki et al. 2006), and here we found that they are also present in

Table 1. Presence of DNA from *Bartonella quintana* in Head Lice from 7 Different Locations in Sénégal

Region	*Locale*	*Subjects infested*	*No. lice tested*	B. quintana *subjects infested (% positive) DNA*			
				Lice (%)	Children (%)	Youth (%)	Adult (%)
Kaolack		1	10	0	0	1 (0%)	0
Dakar	Keur Massar	15	36	6 (16%)	9 (22%)	5 (0%)	1 (0%)
	Diamaguene	1	3	3 (100%)	1 (100%)	0	0
	Malika	21	56	3 (5.35%)	14 (0%)	4 (0%)	3 (66%)
	Rufisque ouest	21	53	0	20 (0%)	0	1 (0%)
	Yeumbeul	20	66	7 (10.60%)	18 (5%)	2 (50%)	0
Fatick	Dielmo	20	48	0	15 (0%)	2 (0%)	3 (0%)
	Ndiop	1	2	0	1 (0%)	0	0
Total		100	274	19 (6.93%)	78 (5.12%)	14 (7.14%)	8 (25%)

Sénégal. Co-infestation of the same subject with different louse clades has been described, but only between body and head lice; co-infestation with different head louse clades has never before been reported (Angelakis et al. 2011b). In addition, in Ethiopia, type C head lice were found on a subject with type A body lice, but we did not observe co-infestation by head lice of clades A and C on the same subject (Angelakis et al. 2011b). It appears that dual transmission cycles of lice are occurring, and that type C head lice do not inhibit the development of type A head louse outbreaks.

Poor living conditions and crowded shelters provide ideal conditions for the spread of lice. The role of the head louse in the maintenance and transmission of *B. quintana* remains to be determined because all attempts to cultivate *B. quintana* from nits or larvae have failed (Angelakis et al. 2011a). DNA from *B. quintana* has been detected exclusively in head lice collected from people living in poverty, and Sasaki and associates were the first to identify *B. quintana* in head lice collected from two heavily infested homeless children in Nepal (Sasaki et al. 2006).

In addition, DNA from *B. quintana* was detected in head lice collected from 3 alcoholic homeless adults in San Francisco (Bonilla et al. 2009; Schroff 2010), and in head louse nits collected from a homeless man in Marseille (Angelakis et al.

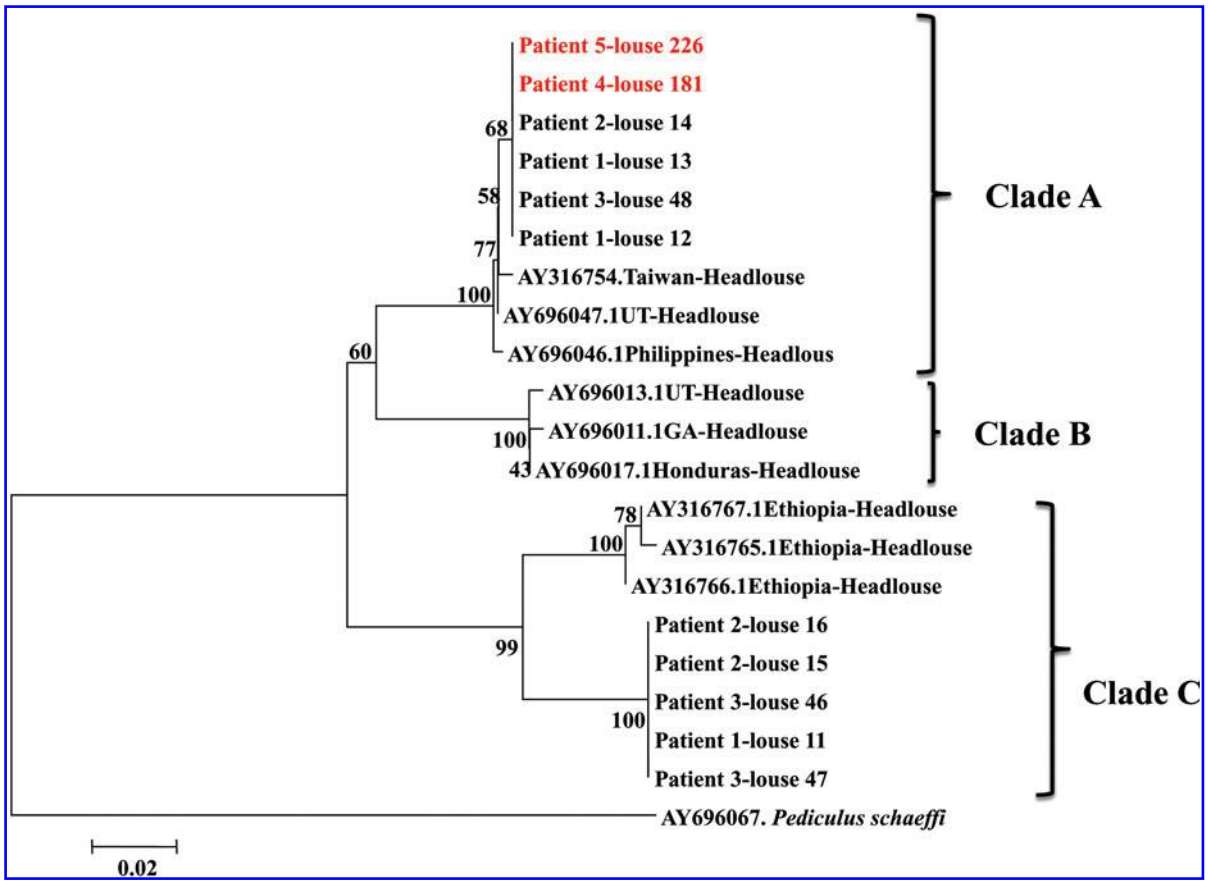

FIG. 2. Neighbor-joining tree of head lice collected from five subjects in Sénégal (2010) based on cytochrome b gene sequencing (black type, lice with DNA from *B. quintana*; red type, non-infected lice).

2011a). People living in more hygienic conditions were not infested with lice infected with *B. quintana*, and in a previous study all attempts to detect *B. quintana* DNA in head lice collected from schoolchildren failed (Fournier et al. 2002).

In this study, no homeless persons were included, but all subjects lived in poor conditions. Recently, DNA from *B. quintana* was also found in head lice and body lice collected from subjects in Ethiopia living in conditions of poverty (Angelakis et al. 2011b).

In conclusion, we identified the presence of *B. quintana* DNA in clade A and C head lice from Dakar, Sénégal. In Dielmo and Ndiop we tested a small number of lice and we did not find any lice with *B. quintana* DNA. As a result, we believe that a larger number of lice should be tested in the future in these areas. Moreover, further studies using head lice collected from more subjects, and from subjects in different countries, are needed to determine if head lice infected with *B. quintana* having phylotype C exist in African countries other than Ethiopia and Sénégal.

Author Disclosure Statement

No competing financial interests exist.

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CONCLUSIONS GENERALES ET PERSPECTIVES

L'Unité de Recherche sur les maladies infectieuses et tropicales émergentes (URMITE) est un acteur prépondérant dans l'étude des pathogènes émergents et ré-émergents. Elle est mondialement connue pour ses travaux sur les pathologies causées par les bactéries intracellulaires strictes et notamment les maladies vectorielles. Les nombreuses collaborations internationales de l'URMITE permettent de travailler sur des échantillons en provenance de multiples sources géographiques. Aussi, dans le cadre de cette thèse sur les poux de l'homme, la présence de deux foyers de sans-abris à Marseille fut propice à la collecte d'échantillons afin de mener à bien nos travaux [38].

L'objectif principal de cette thèse était de mieux comprendre l'épidémiologie des poux de tête et des poux de corps de l'homme. Les poux de l'homme sont étudiés depuis des décennies. A l'origine, les travaux étaient basés sur leur morphologie et leur biologie. Puis, avec l'avènement des techniques de biologie moléculaire, ces travaux furent oubliés et très peu cités malgré leur extrême importance. Nous avons donc, dans un premier temps, entrepris une étude bibliographique exhaustive qui mena à la rédaction d'une revue. Cela nous a permis de prendre connaissance de ces données peu connues ainsi que des travaux plus récents afin de déterminer les objectifs spécifiques de la thèse.

Le premier objectif spécifique était de déterminer si une corrélation entre phénotype et génotype pouvait être établie chez les poux de l'homme. Ce travail fit l'objet d'un premier article. Sur base d'échantillons issus du monde entier, nous avons ainsi pu conclure que ni la couleur des poux, ni leur écotype (pou de tête ou pou de corps) ne pouvait être corrélé à leur phylogénie. Seule l'origine géographique semble concorder avec la

génétique des poux. Nos données montrent également que la phylogénie des poux reflète les mouvements de populations humaines. Ces données sont intéressantes car les poux du genre *Pediculus*, étant probablement les seuls insectes se nourrissant uniquement sur l'homme, sont un très bon modèle de co-spéciation hôte-parasite. Notamment, les résultats de l'Article 3 décrivant un sous-type Amazonien distinct des autres régions géographiques peuvent s'expliquer par un faible impact de la globalisation dans cette zone. Des travaux ultérieurs à plus grande échelle devraient pouvoir confirmer ces hypothèses.

Le second objectif spécifique était d'étudier les populations de poux dans un foyer de sans-abris de Marseille. Nous voulions déterminer si les poux issus d'individus infestés par les deux écotypes appartenaient à une seule population ou si les poux de tête et les poux de corps évoluaient séparément. Pour cela, nous avons fait appel à une technique de séquençage d'espaces intergéniques (méthode MST). Le pou étant diploïde, le séquençage des amplicons nécessite une étape préalable de clonage afin de séparer les deux allèles en cas d'hétérozygotie. Pour cela, nous avons mis au point un protocole de séquençage à haut débit (High throughput 454 sequencing) et vérifié nos résultats avec ceux obtenus par amplification, clonage de l'amplicon et séquençage des clones. Nos résultats d'étude de génétique des populations montrent que les poux de tête et les poux de corps des cinq sans-abris étudiés appartiennent à une même population. Nous pensons que dans de tels cas d'infestations massives par des poux de Clade A (clade qui comprend à la fois les poux de tête et les poux de corps), des poux peuvent migrer d'une zone du corps à l'autre. Ceci expliquerait le fait qu'on ait trouvé deux fois un pou de tête et un pou de corps ayant les mêmes allèles au niveau des quatre espaces intergéniques pourtant très polymorphiques. En dehors de ces cas d'infestations massives, il est probable que les deux écotypes aient des épidémiologies distinctes. Par

ailleurs, nos analyses de génétique des populations suggèrent que l'échange de poux entre les résidents du foyer sont très importants. Nos données soulignent donc un besoin urgent d'adapter les mesures sanitaires prises dans les foyers en évitant le partage de fournitures telles que les matelas, couvertures et autres objets personnels par lesquels la transmission de poux est susceptible de se produire.

Le troisième objectif spécifique de cette thèse était d'étudier l'infection des poux par les bactéries. En effet, jusqu'à présent seuls les poux de corps furent reportés comme vecteurs de maladies. Cependant, des études controversées ont montré que les poux de tête pouvaient également être infectés par *Bartonella quintana* et donc servir de vecteurs de la fièvre des tranchées [34–37]. Les données épidémiologiques et cliniques étant rares sur le sujet au Sénégal, nous y avons entrepris l'étude des poux de tête. Nous avons pu montrer qu'ils appartenaient au Clade A et au Clade C et que environ 7% d'entre eux étaient infectés. Cependant, nous pensons que l'infection des poux de tête par *Bartonella quintana* a lieu uniquement dans des situations particulières : soit en association avec des infestations de poux de corps ou dans des conditions sociales très pauvres. Rien ne prouve que les poux de tête en dehors de ces conditions, transmettent la fièvre des tranchées.

A l'avenir, il serait intéressant de poursuivre les travaux réalisés lors de cette thèse en travaillant sur de plus larges échantillons. Cette thèse a permis de montrer l'utilité d'utiliser les espaces intergéniques pour l'étude de la phylogénie des poux. Cette technique est en effet utile autant lors de l'étude de petites populations comme celles des poux infestant les foyers de sans-abris que lors d'études à grande échelle sur des poux issus de divers continents. Il serait notamment intéressant, sur base de la MST, de poursuivre les travaux sur la phylogénie des poux, tant ces insectes semblent être le reflet des migrations de populations humaines. Aussi, des études ultérieures devraient permettre de mieux comprendre comment se régulent

les populations de poux de tête et de poux de corps issues d'individus infestés par les deux écotypes. Notamment, la récente découverte d'un gène présent uniquement chez le pou de corps (et non chez le pou de tête) [14] devrait pouvoir aider à mieux comprendre l'épidémiologie des poux de l'homme.

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