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Review

Pneumocystis Species Co-evolution: State-of-the-Art Review

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Abstract

Pneumocystis spp. are a group of fungi that are known for causing opportunistic infections in immunocompromised individuals. It was only at the end of the 20th century that the scientific community challenged the notion of a unique species in the genus *Pneumocystis* (i.e., *Pneumocystis carinii*) that drastically changed the understanding of the natural history of pneumocystosis. It is now accepted that the *Pneumocystis* genus comprises a group of heterogenous fungi having multiple stenoxenic biological entities. These are widely distributed in the ecosystems and closely adapt to the mammalian species they colonize. The infection is transmitted via airborne route, allowing them to successfully dwell in the lungs of infected individuals. This article reviews some of the atypical features of these fungal microorganisms, namely host specificity and their parallel history with the mammalian hosts in which they co-evolve. *Pneumocystis* organisms can serve as powerful tools for phylogenetic and phylogeographic studies in mammals. Finally, the review challenges the genetic markers used historically to study the genetic diversity of

Pneumocystis spp. to improve our understanding of *Pneumocystis* co-evolution with their hosts.

Keywords
Pneumocystis spp.; mammals; host specificity; co-evolution

1. *Pneumocystis* Organisms as Stenoxenic Organisms with A Long History Parallel to that of Their Mammalian Hosts

Advancements in the fields of phylogeny and genetics have drastically changed our understanding of the biology and evolutionary history of *Pneumocystis* organisms in the past 30 years. We now know that the genus *Pneumocystis* comprises highly diversified fungal organisms with a very high degree of host specificity [1]. *Pneumocystis* organisms have been detected in all mammalian species studied till date [1-6], owing to their unique mechanisms of adaptation to proliferate and survive exclusively in mammalian hosts [7-10]. Moreover, the genus consists of a group of parasitic microorganisms infecting a vast diversity of hosts in various ecosystems [2-6].

A high level of host specificity (stenoxenism) is a condition that allows cospeciation. Frenkel was the first one to describe in 1976 that rat- and human-derived *Pneumocystis* were different entities due to differences in their host specificity and antigenicity [11]. Therefore, he suggested a new taxonomic nomenclature for these organisms. However, his proposition to use a new specific name for human *Pneumocystis* organisms was not taken into consideration. An important observation that confirmed stenoxenism in these organisms was the systematic failure of cross-infection experiments [12-16]. Genomic and phenotypic divergence made possible the description of several *Pneumocystis* species [17-20] such that at the end of the 20th century, the unique taxonomically enigmatic entity called "*Pneumocystis carinii*" suddenly became a group of stenoxenic species. Another aspect of host specificity is that *Pneumocystis* species are mostly obligate biotrophs [9, 21-23]. Indeed, *Pneumocystis* organisms secrete low amounts of lytic proteases and cause little damage to their hosts. These are two hallmarks of biotrophy, where, in a parasitic relationship, the parasite obtains food from living host cells [21, 23]. Moreover, most biotrophs are obligate parasites, meaning they cannot survive without their hosts and cannot be cultured axenically in the laboratory [24]. In addition, data obtained from the genome analysis of *Pneumocystis* species revealed very compact genomes, suggesting that these organisms have lost several families of genes and metabolic pathways during the course of evolution, and whose products they scavenge from the lung environment of the host [7, 9, 22, 25, 26]. Consistently, *Pneumocystis* pneumonia seems to be rare in wild mammals and only low rates of *Pneumocystis* organisms are usually detected in their lungs [1-6].

Although several recent studies have described the existence of hybrid *Pneumocystis* using multiple genetic markers [27], isoenzymatic and first genetic data suggested that speciation in the *Pneumocystis* genus resulted from long genetic isolation and a potential co-speciation process [28, 29]. The approximate time of *Pneumocystis* speciation was estimated by Keely et al. [30], who examined the genetic variations at rRNA and DHFR loci between different *Pneumocystis* species. The speciation time was confirmed by a recent study by Cissé *et al*. in 2018 [10] using a different

methodology. Accordingly, rat-derived *Pneumocystis* organisms diverged from the human-derived *Pneumocystis* approximately 90 to 100 million years ago. Interestingly, this estimation matches well with the divergence time of rodents and primates [31].

Assessing the congruence between phylogenetic trees from two groups of organisms (*Pneumocystis* species and mammals in the present case) is a major step to check for co-speciation. This has been performed for the first time between primates and primate-related *Pneumocystis* [4, 32, 33]. A global study based on the analysis of lung tissues from different kinds of mammals confirmed the results obtained in primates, i.e., *Pneumocystis* phylogenetic trees resemble the phylogeny of corresponding mammalian host species or groups [6]. However, as the congruence in the time of divergence has not been formally tested in these studies, it is difficult to state with certainty that the congruence of phylogenetic host and parasite trees is the result of co-speciation [4, 33] or host shifts events, as recently suggested [10, 34]. Moreover, it is known that both co-speciation and host-shift speciation can result in congruent phylogenies and that co-phylogenetic methods often overestimate the occurrence of co-speciation events [35].

2. *Pneumocystis* Organisms as Powerful Tools for Phylogenetic and Phylogeographic Studies in Mammalian Hosts

A study conducted in primate-derived *Pneumocystis* reported specific DNA sequence divergence among *Pneumocystis* species to be clearly correlating with the phylogeny of their corresponding hosts [4, 33] (Figure 1). To collect original material, postmortem lung tissues from non-human primates were obtained from the French zoological parks and from the French Primate Research Center in Strasbourg, France. Additional lung tissues from wild monkeys were collected from French Guyana. The genetic diversity of *Pneumocystis* from primates was examined by analyzing mitochondrial large subunit (mtLSU) rRNA and *dihydropteroate synthase* (DHPS) gene sequences, both of which are highly conserved. Each of the 18 non-human primate species or sub-species that was proved to harbor *Pneumocystis* had its own type of organism with specific mtLSU rRNA or DHPS sequence (Figure 1). Furthermore, it is interesting to note that in the case of the red-handed tamarin (*Saguinus midas*), positive PCR amplifications were obtained from both captive and wild animals (three samples from a French zoological park, and one sample from French Guyana), and the corresponding mtLSU rRNA sequences were identical [4]. No human-derived *Pneumocystis* (*P. jirovecii*) was found in non-human primate lung tissues or air samples examined. A detailed comparison of *Pneumocystis* and primates phylogenies was performed using TreeMap 1.0b [36] (Figure 2). The molecular phylogeny of *Pneumocystis* was compared with different controversial phylogenies (according to the mitochondrial and/or nuclear markers, and morphological characters that were used) for the hosts. This comparison demonstrated that depending upon which topology was accepted for the hosts, at least 61% and up to 77% of the homologous nodes of the respective cladograms of the hosts and parasites could be interpreted to result from co-divergence events [33].

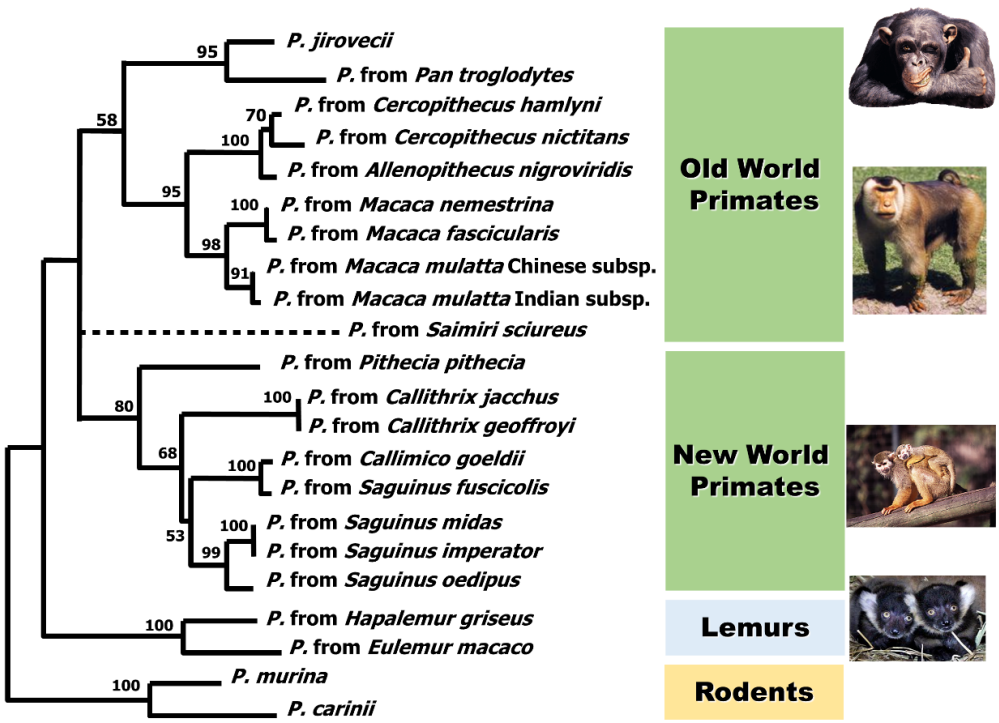

Figure 1 Phylogenetic tree of *Pneumocystis* mtLSU rRNA sequences obtained from 19 non-human primate species (from Demanche *et al.*, 2001).

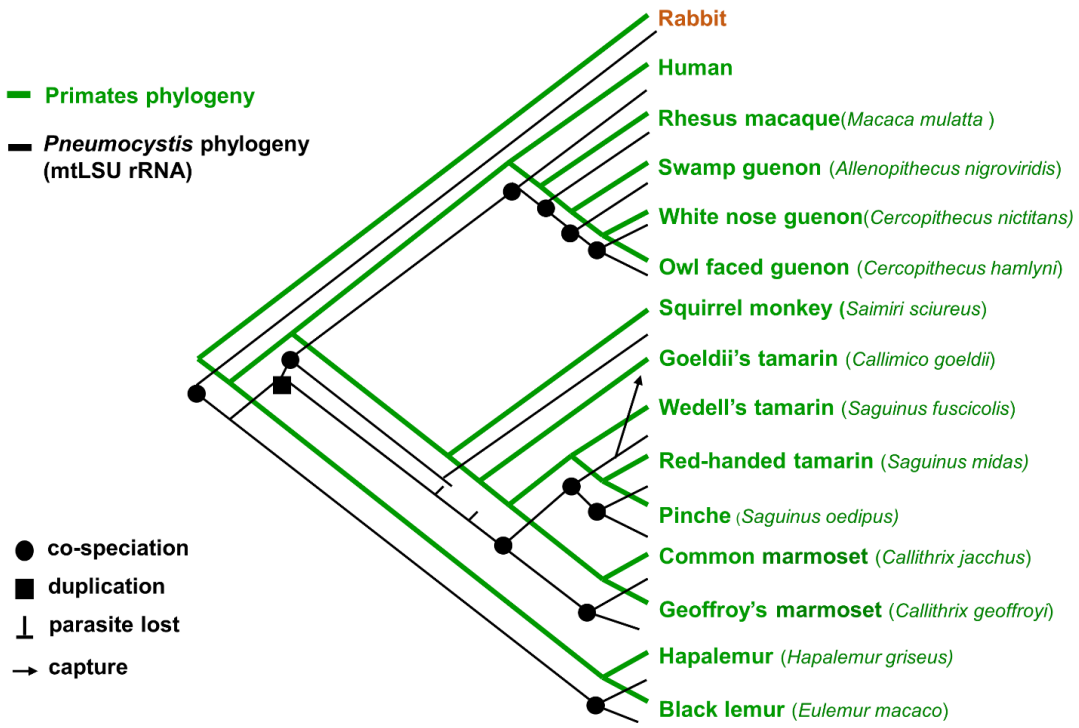

Figure 2. Comparison of *Pneumocystis* and primate's phylogenies with TreeMap 1.0b (from Demanche, 2003).

The phenomenon of co-speciation was also reported in bats. This group of mammals is particularly interesting because of its high biodiversity, in terms of species and ecological characteristics. Bats are widely distributed in various ecosystems and constitute one of the largest groups of mammals, second in the number of species after rodents and first in the number of individuals [37]. The genetic diversity of *Pneumocystis* was studied in lung samples from 19 bat species that were collected from diverse biotopes in New and Old Worlds [2]. *Pneumocystis* was detected by nested PCR at both mtLSU and mitochondrial small subunit (mtSSU) rRNA loci. This study yielded valuable information on *Pneumocystis* biology and transmission. Indeed, ecological and behavioral factors (elevation, crowding, and migration) seemed to influence the *Pneumocystis* carriage. Eleven bat species belonging to five families were found to harbor *Pneumocystis* DNA. For each bat species carrying *Pneumocystis* DNA, at least one novel sequence was amplified at one and/or both loci, suggesting that each species of bats could be harboring a specific species of *Pneumocystis*. Similarly, the data showed that genetic divergence in bat-derived *Pneumocystis* organisms paralleled the phylogenetic divergence existing among corresponding hosts, *Pneumocystis* phylogeny mirrored its host phylogeny, also suggesting co-evolution (Figure 3). Moreover, the link between genetic variability of *Pneumocystis* isolated from populations of the same migrating bat species *Tadarida brasiliensis* and their geographical area could be exploited in terms of phylogeography [2, 38]. In this case, *Pneumocystis* species genotypes were used as proxies at the phylogeographical scale.

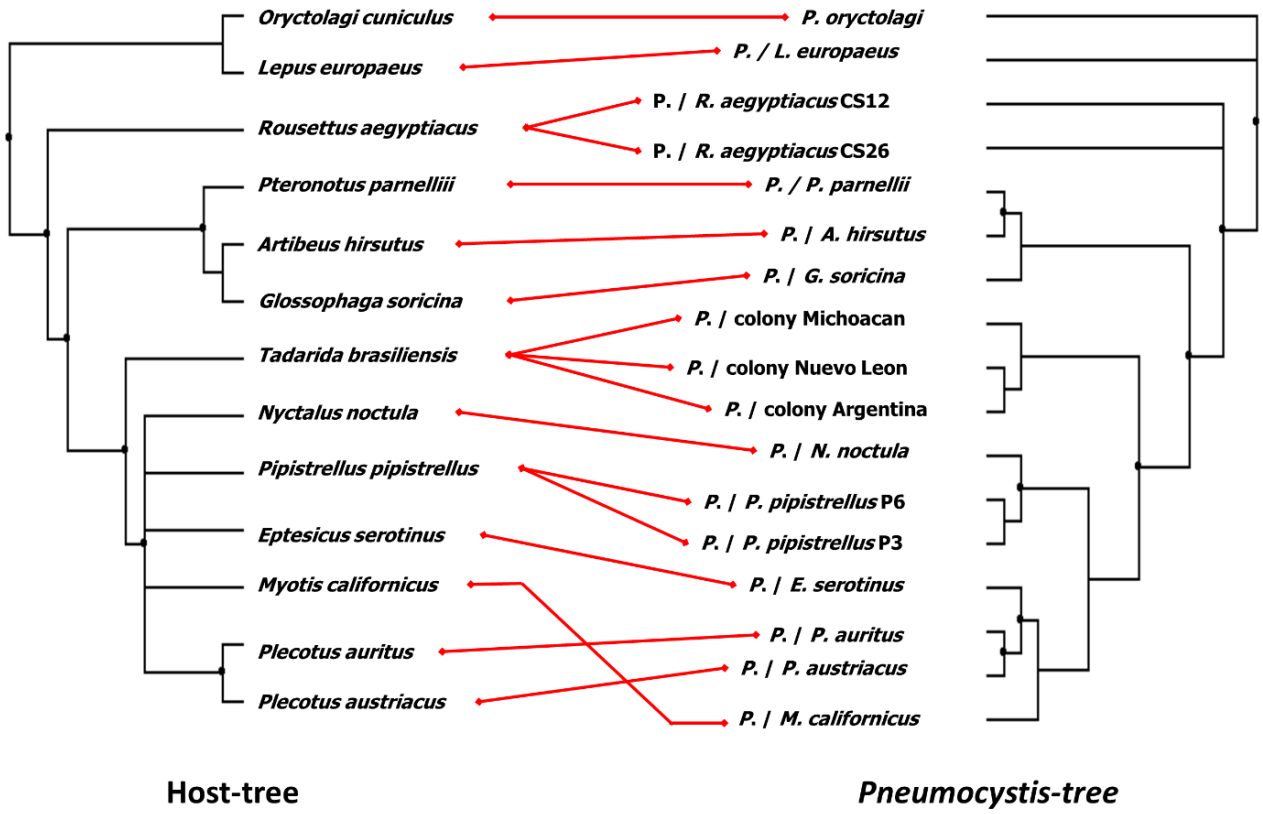

Figure 3 Parallel phylogenies of *Pneumocystis* mtSSU rRNA sequences and their bat hosts (from Derouiche *et al.*, 2009).

Similar findings were obtained at the *Pneumocystis* infra-specific level for *Apodemus sylvaticus*, the common wood mouse [5]. The presence of *Pneumocystis* DNA was assessed by nested PCR at mtLSU and mtSSU rRNA loci. The results demonstrated a very high variability among wood mouse-derived *Pneumocystis* organisms with a total number of 30 distinct combined mtLSU and mtSSU sequence types. However, the genetic divergence among these sequence types was very low (less than 3.9%), and the presence of several *Pneumocystis* species within *A. sylvaticus* was considered unlikely. The analysis of the genetic structure of wood mouse-derived *Pneumocystis* revealed two distinct groups: the first group comprised *Pneumocystis* from wood mice collected from continental Spain, France, and the Balearic Islands, and the second group included *Pneumocystis* from wood mice collected from continental Italy, Corsica, and Sicily. These two genetic groups were in accordance with the two lineages currently described within the host species *A. sylvaticus*. *Pneumocystis* DNA polymorphism seems to be related to the geographical distribution of wood mice, and the analysis of *Pneumocystis* genetic diversity is thus in agreement with the evolutionary history of *A. sylvaticus* in Europe. *Pneumocystis* organisms are known to be possibly carried by their specific hosts during their migration and evolution [5]. Many micro-organisms can be used as a proxy at a phylogenetic or phylogeographical scale to gain insights into the host phylogeny or migrations [39-43]. However, to our knowledge, *Pneumocystis* is the only organism that can simultaneously serve as a good phylogeographical and evolutionary marker for its hosts.

3. Some Exceptions that Prove the Rule

Till date, only five *Pneumocystis* species have been formally described and accepted based on the requirements of the International Code of Botanical Nomenclature (ICBN). *Pneumocystis jirovecii* [11] is present in humans; *P. oryctolagi* [18] has been described in rabbits (*Oryctolagus cuniculus*); *P. murina* [19] is the sole species described in laboratory mice (*Mus musculus*), whereas two species have been described in the laboratory rats (*Rattus norvegicus*), namely *P. carinii* [20] and *P. wakefieldiae* [17].

In order to determine whether the strains of *Pneumocystis* infecting wild rats differ from those in laboratory rats and thus avoid biases derived from conventional breeding, a study looked for *Pneumocystis* spp. that could be encountered in wild rats (*R. norvegicus*) in Thailand. *Pneumocystis* DNA was detected in 57.7% of these animals, although the presence of *Pneumocystis* organisms was never associated with typical *Pneumocystis* pneumonia [3]. The two *Pneumocystis* species *P. carinii* and *P. wakefieldiae* were found in wild rats from Thailand. *P. carinii* and *P. wakefieldiae* were found alone in 19% and 23% of rats, respectively, and 7.7% of the rats were colonized by both species. In this work, a new variant sequence of *P. wakefieldiae* was also identified in wild rats in the same geographical area [3].

Interestingly, Southeast Asia is considered to be the center of origin and diversification of Murinae rodents, from where they have been known to spread to other parts of the Old World [44]. Murid rodents belong to the most diverse mammalian family (Muridae) and comprise over 700 species [45]. In addition, they occupy a wide range of ecological niches in diverse habitats, ranging from cities to agricultural fields and primary forests. Thus, due to their high taxonomical and ecological diversity and the high prevalence of *Pneumocystis* described in wild rodents [3, 5, 28, 29, 46, 47], murid rodents in Southeast Asia are considered to be extremely relevant models for understanding the evolutionary interactions of *Pneumocystis* species and their mammalian

hosts. This was precisely the subject of a recently published study [34], in which the genetic diversity and host specificity of *Pneumocystis* organisms infecting wild Southeast Asian murid rodents was investigated through PCR sequencing of two mitochondrial genes (mtLSU rRNA and mtSSU rRNA) followed by checking the co-phylogeny hypothesis among *Pneumocystis* spp./ strains and their rodent hosts. *Pneumocystis* organisms were detected in 48.3% (215/445) of these wild rodents belonging to 18 Southeast Asian murid species and 8 genera [34]. In total, 69 distinct *Pneumocystis* sequence types were identified for mtLSU rRNA and 43 for mtSSU rRNA. Interestingly, some *Pneumocystis* sequence types could be shared between several host species and genera. For example, although most sequence types of two loci were specific to a murid species, it was surprising to observe that 7 mtLSU rRNA sequence types were common to several *Rattus* species or were found in two *Berylmys* species. Similarly, of the 43 types of mtSSU rRNA sequences identified, 4 were common to several *Rattus* species or shared between *Maxomys surifer* and *Leopoldamys herberti*. It should be noted that these types of shared sequences have been isolated at different time points and in different locations, thus confirming the relevance of these results. Also, the three *Pneumocystis* species already described in rats and mice can infect several rodent species. *P. murina* can be recovered from the lungs of various species of the *Mus* genus, and both *P. carinii* and *P. wakefieldiae* can be found alone or together in the lungs of various *Rattus* species. Co-phylogenetic analyses revealed a complex evolutionary history among *Pneumocystis* species/lineages and their rodent hosts. Even if a significant global co-speciation signal has been detected, it is alone insufficient to explain the observed co-phylogenetic pattern, suggesting that several host switches have probably occurred. These results, therefore, suggest a lower host specificity of *Pneumocystis* species than what was previously thought in rodents.

The lower host specificity of *Pneumocystis* species/lineages colonizing wild rodents than that of primates and bats could be attributed to similarities in physiological, cellular architecture, and/or immune systems among these closely related rodent species that diverged quite recently. Indeed, the origin of the *Rattus* genus, that contains 66 species [45], is relatively recent, estimated at approximately 2 to 3 million years, and its diversification rate is more than three times higher than that for other Murinae rodents [48], suggesting that these rodents are still in the process of speciation.

In the coevolution theory, parasites infecting micromammals (small-bodied with short lifespans, high reproduction rates, and high population densities) have lower host specificity than those adapted to long-lived hosts with more stable population densities [49]. It is interesting to note that humans are infected by only one *Pneumocystis* species, whereas wild rats, according to the results mentioned above, may be co-infected by two or more *Pneumocystis* species [3, 34]. These results may indicate a relaxation of strict host specificity in small mammals colonized by these fungi.

Another exception regarding the strong host specificity of *Pneumocystis* has been reported in macaques. The circulation of *Pneumocystis* organisms within a social organization of healthy crab-eating macaques (*Macaca fascicularis*) living in a natural setting in France was studied for two years with biological samples collected monthly [50]. *Pneumocystis* DNA was detected every month in several animals within the colony. The study demonstrated a relatively high prevalence of *Pneumocystis* DNA in the upper respiratory tract of healthy macaques (33.6% of PCR-positive nasal swabs). Moreover, the presence of *Pneumocystis* DNA was frequently detected from nasal swab samples by nested PCR at mtLSU and mtSSU rRNA loci throughout the study. The fungi are

always aerially transmitted; the transmission being favored by co-housing with transiently infected healthy animals. Specific *Pneumocystis* DNA sequence types were harbored by *M. fascicularis*. No *Pneumocystis* from other primate species including humans was found despite co-housing with other primate species. However, it is important to note that one sequence type was common to samples from *Macaca mulatta* and *Macaca fascicularis* from the same research center. Three nasal swab samples from three different individuals of the *Macaca fascicularis* colony and one *Macaca mulatta* lung tissue sample were similar [51]. A study about phylogenetic relationships among *Pneumocystis* from Asian macaques using mitochondrial rRNA sequences demonstrated that two genetic macaque-derived groups could be considered as distinct *Pneumocystis* species. Surprisingly, these *Pneumocystis* species were recovered from both *M. mulatta* and *M. fascicularis*. The lack of host specificity in macaque-derived *Pneumocystis* could be explained by the hypothesis that the rhesus and crab-eating macaques belong to the same entity (maybe species?) or that they have not speciated completely and are still in the process of speciation like murid rodents from South Asia. This hypothesis is strongly supported by several investigations related to the classification of the genus *Macaca* and the possibility of natural hybridization between the rhesus and crab-eating macaques [52-55]. The divergence time between the two species is also very recent and has been estimated to be 1.3 million years [55].

The last example challenges the strict stenoxenism of *Pneumocystis* at the host intra-generic level. Indeed, an identical *Pneumocystis* lineage (based on mtLSU rRNA sequence) was found in the lungs of *Apodemus flavicollis* and *A. sylvaticus* [46].

4. Challenging mtLSU and mtSSU rRNA Genetic Markers to Study *Pneumocystis* Genetic Diversity

Only two mitochondrial genes, i.e., mtLSU and mtSSU rRNAs have been historically used in all epidemiological and phylogenetically related studies to explore the genetic diversity of *Pneumocystis* in mammals. To strengthen and confirm the obtained results, additional markers such as nuclear genes (found in a single copy in *Pneumocystis* genomes) could be used; these could also confirm the hypothesis for *Pneumocystis* species delineation and co-speciation. However, it should be kept in mind that wild animals carry very low fungal load in their lungs, i.e., they usually do not develop *Pneumocystis* pneumonia [3] and that the amplification of single-copy genes could be problematic. To overcome the difficulty of obtaining the sufficient quantity of *Pneumocystis* micro-organisms and high purity of genomic DNA for genome sequencing, two approaches, namely purification of parasites and a Whole Genome Amplification (WGA) before next-generation sequencing (NGS), could be implemented. One of the major problems encountered is the removal of host cells and DNA in the purification step, a problem that was also faced by several authors while sequencing *Pneumocystis* genomes [7, 10, 26, 56, 57]. However, Martinez et al. [58] described an original, reproducible, and efficient method for separating trophic and cystic forms of *P. carinii* using a high-speed cell sorter. Large amounts of highly purified (99.6 ± 0.3%) *Pneumocystis* trophic and cystic forms were thus obtained. Therefore, combining this purification approach with a WGA of *Pneumocystis* organisms present in the lungs of wild mammals would certainly be a good approach to sequence these *Pneumocystis* genomes.

Moreover, reports on mitochondrial gene dynamics during *P. jirovecii* infection have shown *Pneumocystis* mitochondrial genomes to be significantly plastic in terms of variation in copy number [59] and genetic diversity, including heteroplasmy [60]. Thus, the interpretation of results

of Sanger sequencing from lung samples of these infected rodents could be questioned. Indeed, Sanger sequencing is not the optimal method for identifying mixed *Pneumocystis* infections owing to its inability to detect minority alleles. Application of other methods such as NGS would make it possible to overcome this shortcoming and to study precisely *Pneumocystis* co-infection patterns in animals.

Author Contributions

MC, CD and JG contributed equally to this work.

Competing Interests

The authors have declared that no competing interests exist.

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