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Research paper

Evolutionary history of *Pneumocystis* fungi in their African rodent hosts

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ABSTRACT

Pneumocystis is a genus of parasitic fungi infecting lung tissues in a wide range of mammal species, displaying a strong host specificity and patterns of co-speciation with their hosts. However, a recent study on Asiatic murids challenged these patterns reporting several *Pneumocystis* lineages/species shared by different host species or even genera in the Rattini and Murini tribes. Here we screened lung samples of 27 species of African rodents from five families for the presence of *Pneumocystis* DNA. Using reconstructed multi-locus phylogenies of both hosts and parasites, we tested the hypothesis of their co-evolution. We found that *Pneumocystis* is widespread in African rodents, detected in all but seven screened host species, with species-level prevalence ranging from 5.9 to 100%. Several host species carry pairs of highly divergent *Pneumocystis* lineages/species. The retrieved co-phylogenetic signal was highly significant ($p = .0017$). We found multiple co-speciations, sorting events and two host-shift events, which occurred between Murinae and Deomyinae hosts. Comparison of genetic distances suggests higher substitution rates for *Pneumocystis* relative to the rodent hosts on neutral loci and slower rates on selected ones. We discuss life-history traits and population dynamics factors which could explain the observed results.

1. Introduction

The genus *Pneumocystis* is a group of unicellular yeast-like fungi (phylum Ascomycota, class Pneumocystidomycetes). They are obligate parasites inhabiting lung alveoli of a wide range of mammalian species, including humans (Aliouat-Denis et al., 2008). Although benign in most cases, the organism is the cause of life-threatening *Pneumocystis* pneumonia in immunosuppressed human and laboratory mice/rats. Historically, the whole genus was thought to be represented by a single species, *Pneumocystis carinii*, which could spread to humans from rats, since similar cellular forms of *Pneumocystis* were found in lung tissues of both infected humans and rats (Calderón-Sandubete et al., 2002). It was later shown by cross-infection experiments and DNA sequence eanalyzes that *Pneumocystis* is comprised of several species, each specific to their mammalian hosts with, apparently, low ability to infect distantly related mammal species (Aliouat-Denis et al., 2008; Calderón-Sandubete et al., 2002; Cissé and Hauser, 2018). To date, five nominate species of *Pneumocystis* from four host species have been formally described (see review in Aliouat-Denis et al., 2008) - *P. carinii* and *P. wakefieldae* in rats (originally from *Rattus norvegicus*, but recently found also in other rat taxa; Latinne et al., 2017), *P. murina* in house mice (*Mus musculus*), *P. jirovecii* in humans, and *P. oryctolagi* in rabbits (*Oryctolagus cuniculus*). However, many additional *Pneumocystis* lineages have been detected in a wide range of mammalian species, including other rodents and primates, bats, insectivores, carnivores, pigs, lagomorphs and one report found evidence of *Pneumocystis* in a marsupial namely the red kangaroo (Aliouat-Denis et al., 2008; Danesi et al., 2016; Hugot et al., 2003; Laakkonen, 1998; Poelma, 1975; and references therein). For a comprehensive review of *Pneumocystis* biology, life-histories, genomics and evolution, see also Aliouat-Denis et al., 2008; Ma et al., 2016; Cissé and Hauser, 2018.

Cophylogenetic studies of *Pneumocystis* across mammals (Guillot et al., 2005) or in specific groups such as primates (Hugot et al., 2003) or bats (Derouiche et al., 2009) have usually found high numbers of co-speciation events, suggesting that *Pneumocystis* co-speciates with its hosts. However, some studies reported cases of *Pneumocystis* lineages shared by closely related hosts such as in primates (Hugot et al., 2003) or woodmice (Danesi et al., 2016). Recently, a study in Southeast Asian murid rodents reported the occurrence of several *Pneumocystis* lineages/species, each infecting several species or even genera in the Rattini and

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Murini murid tribes (Latinne et al., 2017). This pattern is particularly striking in the Rattini tribe, where for example *P. carinii* was reported in five *Rattus* species (*R. tanezumi*, *R. exulans*, *R. argentiventer*, *R. sakeratensis*), and *P. wakefieldiae* in six species (*R. tanezumi*, *R. exulans, R. nitidus*, *R. andamanensis*, *R. sakeratensis*). Although the authors detected a global signal of co-speciation, they deemed multiple host switches were necessary to explain the observed phylogenetic pattern (Latinne et al., 2017). These results suggest that the specificity of *Pneumocystis* infecting Southeast Asian murid rodents is much lower than that of *Pneumocystis* infecting primates or bats (Derouiche et al., 2009; Guillot et al., 2005). However, lack of host specificity of *Pneumocystis* in the Rattini tribe could be a special case. This rodent clade likely originated in Southeast Asia around 2–3 Mya and it shows a diversification rate three times higher than in other rodents of subfamily Murinae (Rowe et al., 2011). Explosive diversification in Southeast Asia, the centre of Rattini diversity, could thus explain the occurrence of several *Pneumocystis* lineages/species shared by multiple host species. This example, therefore, suggests that the evolutionary history of *Pneumocystis* can be more complex, and that co-speciation alone is insufficient to explain it (Latinne et al., 2017; Cissé and Hauser, 2018).

Rodents are the most diverse order of mammals. In Africa, they represent about 17% of the worldwide rodent diversity and with 463 species, account for almost half of the mammal species of the continent (Happold, 2013). Rodent species from sub-saharan eastern Africa have complex phylogeographic histories, closely linked to the geomorphology of the east African rift system and the climatic dynamics of the region during the Plio-Pleistocene (Monadjem et al., 2015). There is now ample evidence that these species consist of several phylogroups, which were repeatedly separated in allopatry during periods of unfavourable climatic conditions, and consequently came into contact multiple times during climatic optima. A similar pattern of historical population dynamics and phylogeographic structure is present in multiple rodents sampled in this study - e.g. *Mus minutoides*, *Mastomys natalensis*, *Acomys spinosissimus* complex (Bryja et al., 2014; Colangelo et al., 2013; Petružela et al., 2018). Studying parasite populations in these species can, therefore, help to better understand the co-evolution between hosts and parasites in general, and *Pneumocystis* and murids in particular, because of the diversity and complex evolutionary histories that repeat across multiple rodent species.

Detection and phylogenetic studies of *Pneumocystis* are often based on the amplification of short genomic fragments (ca. 250–350 bp) of two markers, the large and small rRNA subunit mitochondrial genes (LSU rRNA and SSU rRNA) by nested PCR (e.g. Derouiche et al., 2009; Guillot et al., 2005; Hugot et al., 2003; Latinne et al., 2017). However, these markers are problematic in phylogenetic studies because of their short sizes, and alignment problems due to numerous gaps. The use of ambiguously aligned regions in *Pneumocystis* phylogenetic analyzes varies from study to study, with some excluding those regions (e.g. Guillot et al., 2005), while others encoding gaps as a fifth state and including them (e.g. Latinne et al., 2017). In mammals, and especially in rodents, the most commonly used marker in phylogenetic reconstructions is the mitochondrial cytochrome *b* (*CYTB*) gene, which is sufficiently variable even at intraspecific level and thanks to its coding nature it is easier to align than rRNA genes. Thus, targeting the orthologous gene of cytochrome *b* in *Pneumocystis*, the apocytochrome *b (COB)*, could be helpful in two ways: to get a less ambiguous marker for phylogenetic reconstruction, and to allow comparison of molecular substitution rates between the host and the fungus (within the limits of the similarity in repair mechanisms of very distant organisms).

To investigate the major mode of evolution of *Pneumocystis* in rodents, we sampled lung tissues of 28 African rodent species from five families (Muridae, Bathyergidae, Spalacidae, Gliridae, Nesomyidae) collected in Tanzania, and tested them for *Pneumocystis* presence via polymerase chain reaction (PCR) targeting the *COB* gene. Positive samples were further sequenced at multiple genetic markers and multilocus phylogenetic trees of *Pneumocystis* and their hosts were compared to test the hypothesis of their co-divergence. We also used the *CYTB* gene to estimate the difference in substitution rates between *Pneumocystis COB* and their host *CYTB* genes.

2. Material and methods

2.1. Sampling

We sampled 517 rodent individuals in various habitats across Tanzania, the majority in June 2015 (see Table S1A in Supplementary Material S1). At most localities we conducted a single night survey with intensive trapping effort using three types of traps: Museum Specials snap traps, 14 × 7 cm, Victor Rat snap traps, 17.5 × 8.5 cm, and medium-sized Sherman Traps, 23 × 9.5 × 8 cm. Each trap line consisted of 30 Museum Specials, 15 Victors and 15 Shermans. Usually we set up four lines of 60 traps each at each locality. In order to maximize trapping success and diversity of the sampled mammals, we set the lines to cover a representative proportion of each habitat rather than using a fixed distance or grid. Each trap was baited with peanut butter mixed with sardine fish. Traps were set around 0500 P.M. and checked early morning at 0600 A.M. Individuals caught in the snap traps were directly identified and dissected; those trapped in Sherman traps were killed by cervical dislocation before identification and dissection. Identification in the field was conducted at the genus level based on external morphology and biometric data (length of head, body, tail, ear, and hind feet). For each autopsied animal, a piece of lung was preserved in RNAlater for further genetic analysis. Voucher specimens were preserved in methylated spirits for further taxonomic work and are deposited at the University of South Bohemia, Czech Republic. Field protocols and export of samples were approved with the permission of the Directorate of Research and Postgraduate Studies, Sokoine University of Agriculture, Morogoro, Tanzania, reference no. RPGS/R/8.Vol VI (issued for year 2015). An import permit was provided by the State Veterinary Administration of the Czech Republic, reference no. SVS/2015/061573-G.

2.2. Laboratory procedures

Screening of genetic diversity of *Pneumocystis* was performed by PCR using the KAPA2G Robust HotStart PCR Kit (Kapabiosystems, USA) from DNA extracted from lung samples by JETquick Tissue DNA spin kit (Genomed). The mitochondrial gene encoding for the *COB* of *Pneumocystis* was amplified using the primers COB-F (5'-GGTAGAGCA ATCGT TTTACACG-3′), and COB-R (5'-ATCTTTAAYTCGTATCGGACTTTCC-3′). Primers were newly designed in conserved regions of the t-RNA-Val and rnpB genes flanking COB and based on the alignment of *P. murina* and *P. carinii* mitochondrion genomes (GenBank accession number: JX499144, GU133622, and JX499145). For one reaction 2 µl of DNA was used together with 0.3 µM of each primer, 0.2 µM of dNTPs, 0.5 µM of MgCl2, 2.4 µl of buffer, 6.3 µl of water, and 0.1 unit of polymerase in final volume of 12 µl. The PCR was run for 42 cycles with annealing temperature of 55 °C (1 min) and elongation temperature of 72 °C (1 min). Sanger sequencing of PCR products was commercially performed at GATC Biotech (Germany) and sequences were edited and visually inspected using Geneious 9.0.5 (Kearse et al., 2012). Samples were considered positive for *Pneumocystis* when we obtained a partial or complete sequence of COB.

A subset of the samples distributed across the rodent phylogeny and detected positive after the COB molecular screening ($N = 47$) was selected for further analyzes. We selected two most distant samples from each distinct clade (based on visual inspection of the tree). Selected samples were analyzed at the mitochondrial large subunit ribosomal RNA (*mtLSU-rRNA)* and nuclear dihydrofolate reductase (*DHFR)* genes using the same PCR kit as described above. For *mtLSU-rRNA*, we employed a modified version of the nested PCR by Wakefield (1996). Primers used for the first round were PAZ102-H (5'-GTGTACGTTGCA

AAGTAGTC-3′) and PAZ102-E (5’-GATGGCTGTTTCCAAGCCCA-3′) and for the second round pAZ102-L1 (5’-GTGAAATACAAATCGGACT AGG-3′) and pAZ102-X (5’-TCACTTAATATTAATTGGGGAGC-3′) (Wakefield, 1996). For the first round of the nested PCR, we used 2 μl of DNA, together with 0.3 μM of each primer, 0.2 μM of dNTPs, 0.2 μM $MgCl_2$, 2 μl of buffer, 4.9 μl of water, and 0.1 unit of polymerase in a final volume of 10 μl. The second round was performed in the same final volume but using 0.5 μl of PCR product from the first round as template. Both rounds had 40 cycles of amplification and the same temperature profile (annealing phase 52 °C (1 min 30 s), elongation phase 72 °C (2 min).

For the nuclear *DHFR* gene, we used primers from Ma and Kovacs (2001): sense (5’-ATGAATCAGCAAAAGTCTTTAACATTGATTGTT-3′) and antisense (5’-TTATAAATCTCTTGTCCACATTTCGAATTC-3′). The reaction volume was 1 μl of DNA, 0.3 μM of each primer, 0.2 μM of dNTps, 0.2 μM $MgCl_2$, 2 μl of buffer, 4.1 μl of water and 0.1 unit of polymerase (10 μl in total). We used 42 cycles of amplification with an annealing phase at 51.5 °C (1 min 30 s), and an elongation phase at 72 °C (2 min).

The *CYTB* gene was amplified in all individual rodents positive for *Pneumocystis* following Bryja et al. (2014). Sanger sequencing of all PCR products was performed as described above but using only the forward primers.

2.3. Prevalence of Pneumocystis in African rodents

The prevalence was measured as a direct ratio between positive and sampled host individuals for each species. Confidence intervals for prevalence were estimated using Sterne's Exact method in Quantitative Parasitology 3.0 (Rózsa et al., 2000).

2.4. Phylogenetic analyzes of Pneumocystis and African rodents

Alignments for particular markers were produced in AliView v. 1.18 (Larsson, 2014). We included sequences from *P. murina* and *P. carinii* for all markers (GenBank AN: JX499144 and AF321304 for *COB*, JX499144 for *mtLSU-rRNA (P. murina)*, AF17556 and AF322061 for *DHFR*) and multiple mtLSU-rRNA sequences of *P. carinii* (AN: KX257075, KX257070, and KX257082) and *P. wakefieldiae* (AN: FJ475121, KX257125, KX257121 and KX257123) to increase the robustness of the analysis as no sequences were available for *DHFR* and *COB* for *P. wakefieldiae* and the *mtLSU-rRNA* sequences were short. In the analysis of *mtLSU-rRNA* we removed all positions with gaps as there is no general accepted model of evolution for the gap state. jModelTest2 (Darriba et al., 2012) was employed to ascertain the best-scoring model of substitution for each partition. For each marker, we tested five substitution schemes, including models with unequal base frequencies and rate variation among sites with four categories (= 25 models tested for each marker). Table 1 shows a summary of selected substitution models. Phylogenetic analyzes of *Pneumocystis* were performed via Bayesian Inference for each marker separately, and the whole concatenated dataset using MrBayes v. 3.2.4 (Ronquist et al., 2012), with two independent runs starting with random trees, with three heated and one cold chain per run. Five million generations per run were performed (sampling every 1000th). The first 25% of results were discarded as burn-in. Sequences of *P. jirovecii* were used as outgroups in all analyzes (GenBank AN: KU521999 for *COB*, DQ269976 for *DHFR*, and KU693284 for *mtLSU-rRNA*).

Table 1
Summary of the selected substitution models used in phylogenetic analyzes.

Organism	Locus	Type	Best model
Pneumocystis	COB	Mitochondrial	HKY + G
	mtLSU rRNA	Mitochondrial	GTR + G
	DHFR	Nuclear	HKY + G
Rodent	CYTB	Mitochondrial	GTR + G
	RAG1	Nuclear	HKY + G
	GHR1	Nuclear	HKY + G
	IRBP	Nuclear	HKY
	BRCA1	Nuclear	GTR + G

We also performed phylogenetic tree reconstruction for the host based on *CYTB* sequences of rodent specimens sequenced in this study (used in the co-path analysis – see below), and on *CYTB* supplemented by sequences of four nuclear markers: recombination activation gene 1 (RAG1), interphotoreceptor retinoid-binding protein (IRBP), growth hormone receptor 1 (GHR1) and breast cancer gene 1 (BRCA1); used in the analyzes of host-parasite co-evolution – see below) downloaded for particular rodent species from GenBank (see Table S1B in Supplementary materials S1 for more details). The Bayesian phylogenetic tree of rodents was computed in MrBayes v. 3.2.4 using the same parameters as for the phylogenetic reconstruction of *Pneumocystis* (see Table 1 for summary of substitution models).

2.5. Estimation of the relative rate of molecular evolution

The matrix of mean *p*-distances among 4-fold degenerate sites from orthologous mitochondrial *COB* and *CYTB* markers (from *Pneumocystis* and their hosts, respectively) was calculated in MEGA v. 6 (Tamura et al., 2011). The distances were then compared using a scatterplot using R-3.2.3 (R core development team, 2016). We used the 4-fold degenerate sites because these themselves are not expected to be under selection. Although the scatterplot vizualisation is not a statistical test, it provides a useful insight into the rate of molecular evolution of both hosts and parasites. In addition, we performed a complementary analysis, using the same procedure, but with the previously discarded sites (other than 4-fold degenerate) to vizualise the difference between sites under only indirect (and/or mild) versus direct selection pressure. We performed a standard linear regression (with intercept constrained to 0) to investigate the general trend and employed the Wald test for the significance of slope difference between regression line and null hypothesis, represented here by equality (slope = 1).

Next, we performed a co-path analysis (Page, 1996), which is a method similar to the comparison of the molecular divergence rates, but uses only phylogenetic distances between co-evolving tips of the multilocus phylogenetic tree (i.e. tips which share a node representing a host-shift event are excluded). The analysis was performed using R-3.2.3. The employed procedure was the same as with the previous two vizualisations. In our dataset, we, therefore, omitted the taxa from the so-called “Deo-lineage” (see below). Outgroups were removed from both molecular divergence and co-path analyzes.

2.6. Analysis of host-parasite co-evolution

To analyze the level of co-divergence between rodents and their *Pneumocystis* we used the combination of a distance-based method, which assess the degree of host-parasite phylogenetic congruency and tests them against the random distributions, and an event-based method (for in-depth discussion of various cophylogenetic methods and their theoretical frameworks, see e.g. De Vienne et al., 2013). For this part of the analyzes, we reduced the inferred phylogenetic tree to one *Pneumocystis* tip per host species. For those species of rodent hosts which harboured two different *Pneumocystis* lineages, we used two samples, one from each respective *Pneumocystis* lineage (see S1A for the list of individuals). Outgroups were removed.

Patristic distance matrices were calculated from the multi marker phylogenies inferred by Bayesian Inference and used in a distance-based co-phylogeny analysis in AxParafit (Stamatakis et al., 2007), a standard algorithm used in distance-based analyzes. This analysis was executed in CopyCat v. 2.02 (Meier-Kolthoff et al., 2007) with 100,000 permutations.

The event-based co-phylogenetic analysis was performed in Core-Pa v0.3 (Merkle et al., 2010) using the topology inferred via phylogenetic analyzes. This approach allows for visualization of the co-phylogeny and reconstructs the co-phylogenetic scenario by assigning a cost to each co-evolutionary event and then reconstructs the tree minimizing the costs. The least-cost solution is considered the best one, and is statistically tested against a randomized set of host-parasite tree mappings. The result is considered significant if the cost of the best-scoring tree is significantly lower than the cost of the random tree (Merkle et al., 2010; De Vienne et al., 2013). The rationale behind choosing Core-Pa software is that it does not require a priori cost assignment, which is a problem plaguing similar software. Correct assignment of costs is nearly impossible without detailed knowledge of host-parasite relationships and it was shown that the reconstruction varies wildly based on the cost values used (du Toit et al., 2013). Default options with automatic cost assignment were used and the algorithm was run for 100,000 random cycles.

3. Results

3.1. Molecular screening and prevalence of Pneumocystis in African rodents

Molecular screening for the presence of *Pneumocystis* DNA in lung samples revealed that the genus *Pneumocystis* is a widespread pathogen among African rodents as it was detected in 20 out of 27 screened species from families Muridae, Gliridae and Bathyergidae (Table 2). A total 168 pneumo-positive samples were obtained out of 517 tested individuals. No positive results were obtained in families Nesomyidae and Spalacidae. The absence of *Pneumocystis*-positive samples in the seven species might be caused by low number of screened individuals in particular species (from 1 to 5, only in *Acomys wilsoni* $N = 14$; Table 2). The prevalence in different species was highly variable, ranging from 0 to 100%, but some of these values must be taken with caution because of low sample size in some species (see confidence intervals for the prevalence in Table 2). In species with $N > 10$, the prevalence ranged from 5.9 to 35.7%, with two exceptions: the subterranean silvery mole-rat *Heliophobius argenteocinereus* ($N = 10$) showed a very high prevalence (90%, CI [55.4–99.5%]), while all samples of the Wilson's spiny mouse *Acomys wilsoni* living in arid savannahs were *Pneumocystis*-negative (0%, CI [0–23.8%]). The most numerous rodent species was the Natal multimammate mouse, *Mastomys natalensis* ($N = 332$) with a prevalence of *Pneumocystis* infection of 34.0% (CI [29.0–39.3%]) (Table 2). From the 47 samples selected for our subsequent genetic analyzes, we were only able to amplify and sequence 10 DNA samples for *DHFR* and 35 for *mtLSU-rRNA* (see S1A for details and accession numbers on the successful samples). There were no heterozygous sequencing chromatograms in any marker, therefore, no evidence of multiple *Pneumocystis* infections of individual hosts in our dataset.

3.2. Genetic diversity and phylogenetic reconstruction of Pneumocystis

The final alignments were 1143 bp for *COB*, 178 bp for *mtLSU-rRNA* and 599 bp for *DHFR*. Bayesian inference analysis of the concatenated multilocus dataset of *Pneumocystis* revealed a phylogenetic pattern which resembles the rodent phylogeny (Fig. 1). The multi-locus tree was primarily driven by *COB* topology, but the tree from the *DHFR* analysis yielded congruent topology. On the other hand, the tree resulting from *mtLSU-rRNA* had numerous polytomies (low support of most nodes), likely a result of the limited amount of information in such short length sequences. Nevertheless, the tree showed a high support for basal divergence between *Pneumocystis* from Murid and Bathyergid/Glirid hosts, and also for the majority of sister tree tips (see Supplementary material S2 for the gene trees). The first splits to three major clades correspond to the three rodent families (Bathyergidae, Gliridae, Muridae). The diversification within Muridae roughly corresponds to three subfamilies (Murinae, Deomyinae, and Gerbillinae), and clades within subfamily Murinae are almost concordant with its tribal classification (Rattini, Murini, Arvicanthini, Praomyini).

Interestingly, the analysis revealed the existence of two very divergent lineages of *Pneumocystis* in murine rodents. Besides the widespread lineage found in most murine genera ("Mur-lineage" in Fig. 1), another distinct lineage was found in two genera of Praomyini (*Mastomys* and *Praomys*), and one genus of Arvicanthini (*Aethomys*). The latter is phylogenetically nested within the clade grouping *Pneumocystis* from *Acomys* and *Lophuromys*, i.e. subfamily Deomyinae ("Deo-lineage" in Fig. 1). As an illustration, the divergence between the two *Mastomys Pneumocystis* lineages is about 18% on *COB*. The mean divergence on

Table 2
Prevalence of *Pneumocystis* in screened African rodent taxa.

Taxonomy	Species	No. tested	No. positive	Prevalence (%) [CI – 95%]
Bathyergidae	*Heliophobius argenteocinereus*	10	9	90.0 [55.4–99.5]
Gliridae	*Graphiurus murinus*	13	4	30.8 [11.3–58.7]
Gliridae	*Graphiurus* sp. indet. (Killimanjaro)	1	1	100.0 [5.0–100]
Muridae: Deomyinae	*Acomys ngurui*	9	1	11.1 [0.6–44.3]
Muridae: Deomyinae	*Acomys muzei*	4	3	75.0 [24.9–98.7]
Muridae: Deomyinae	*Acomys ignitus*	1	0	0 [0–95.0]
Muridae: Deomyinae	*Acomys wilsoni*	14	0	0 [0–23.8]
Muridae: Deomyinae	*Lophuromys kilonzoi*	2	1	50.0 [2.5–97.5]
Muridae: Deomyinae	*Lophuromys makundii*	7	1	14.3 [0.7–55.4]
Muridae: Gerbilliinae	*Gerbilliscus vicinus*	26	3	11.5 [3.2–30.4]
Muridae: Murinae: Arvicanthini	*Aethomys hindei*	3	0	0 [0–63.2]
Muridae: Murinae: Arvicanthini	*Aethomys chrysophilus*	11	3	27.3 [7.9–59.6]
Muridae: Murinae: Arvicanthini	*Aethomys kaiseri*	9	2	22.2 [4.1–55.8]
Muridae: Murinae: Arvicanthini	*Grammomys surdaster*	10	3	30.0 [8.7–61.9]
Muridae: Murinae: Arvicanthini	*Lemniscomys striatus*	1	1	100.0 [5.0–100]
Muridae: Murinae: Arvicanthini	*Lemniscomys rosalia*	7	3	42.9 [12.9–77.5]
Muridae: Murinae: Arvicanthini	*Rhabdomys dilectus*	4	2	50.0 [9.8–90.2]
Muridae: Murinae: Murini	*Mus minutoides*	14	5	35.7 [15.3–62.9]
Muridae: Murinae: Murini	*Mus triton*	17	6	35.3 [16.6–59.4]
Muridae: Murinae: Otomyini	*Otomys angoniensis*	1	0	0 [0–95.0]
Muridae: Murinae: Praomyini	*Hylomyscus arcimontensis*	1	1	100.0 [5.0–100]
Muridae: Murinae: Praomyini	*Mastomys natalensis*	332	113	34.0 [29.0–39.3]
Muridae: Murinae: Praomyini	*Myomyscus brockmani*	2	2	100.0 [22.4–100]
Muridae: Murinae: Praomyini	*Praomys delectorum*	8	4	50.0 [19.3–80.7]
Nesomyidae	*Saccostomus umbriventer*	5	0	0 [0–50.0]
Nesomyidae	*Steatomys* sp. indet.	2	0	0 [0–77.6]
Spalacidae	*Tachyoryctes splendens*	3	0	0 [0–63.2]

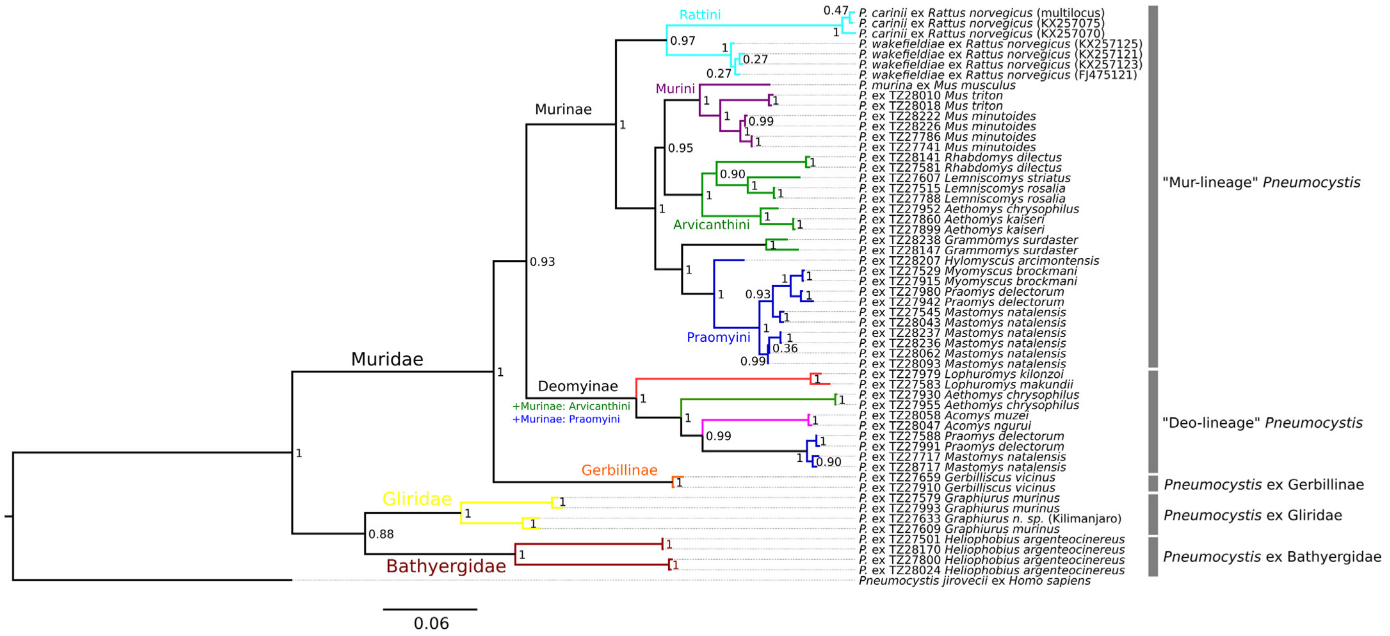

Fig. 1. Bayesian phylogeny of *Pneumocystis* based on concatenated multi-locus dataset. Node labels denote posterior probability support of each node. Different colours indicate the taxonomic position of hosts (i.e. families/tribes).

COB between Mur- and Deo- lineages is 16.8%, with within-group divergences being 8.8% for Mur-lineage and 10.0% for Deo-lineage. Occurrence of two distinct *Pneumocystis* lineages was also found in two other rodent families. While the sample TZ27633 is probably a new genetically divergent species of dormouse (*Graphiurus*), two of the three remaining *Pneumocystis*-positive individuals are genetically very similar (they likely belong to *G. murinus)*, but they harbour two very distinct *Pneumocystis* lineages (7.7% divergence on *COB*), one of which is present in the new species as well (Fig. 1). All examined *Heliophobius* mole-rats genetically belong to the same phylogeographic lineage (sensu Bryja et al., 2018), but their *Pneumocystis* also form two very distinct clades (13.8% divergence). The two species of *Pneumocystis* from *Rattus norvegicus* formed sister lineages.

The reconstructed host phylogeny (Supplementary material S2D) used for this part of analyzes matched the topology of previously published phylogenetic works on murid rodents (Fabre et al., 2012; Schenk et al., 2013).

3.3. Estimation of the relative rate of molecular evolution and the co-path analysis

The majority of the 4-fold degenerate sites distance comparisons (Fig. 2a) are situated above the equality line (black line in Fig. 2a), hinting at higher rate of divergence of the *Pneumocystis COB* compared to the host *CYTB*. The regression slope ($a = 1.53$) was significantly different from equality ($F = 867.7$, $p < .001$). In stark contrast, the analysis using the complementary dataset with sites potentially under direct selection (Fig. 2b) yielded the opposite result, with much less molecular divergence observed for *Pneumocystis COB* ($a = 0.52$), again significantly different from equality ($F = 8140.1$, $p < .001$).

The co-path analysis returned the branch length comparison values grouped into three discrete clusters (see Fig. 2c) corresponding to the taxonomic levels being compared, but otherwise having a linear trend showing a higher rate of divergence for the parasite than for host ($a = 1.31$). The results were again statistically significant ($F = 1261.7$, $p < .001$).

3.4. Co-divergence between rodents and Pneumocystis

The AxParafit analysis yielded a highly significant result on the 5% probability level for the congruence hypothesis (ParaFit global = 0.19801, $p = .00001$), with 18 individual links being significant on the 5% level (Table 3). The best-scoring co-phylogenetic reconstruction from the CorePa analysis is displayed in Fig. 3, with quality score < 0.001, and total cost of 5.44. The retrieved co-phylogenetic signal was highly significant with $p = .0017$. It consisted of 12 co-speciations, 21 lineage sorting events (in which the species still evolve together, albeit the divergence times may differ - thus leading to unequal branch lengths), eight duplications and two host shifts (corresponding to *A. chrysophilus* and *Praomys* + *Mastomys* ancestors bearing "Deo-lineage" of *Pneumocystis*). *Pneumocystis wakefieldae* and the second lineage in *H. argenteocinereus* were identified as duplications from *Pneumocystis carinii* and the first lineage of *Heliophobius*-specific *Pneumocystis*, respectively.

4. Discussion

4.1. Pneumocystis is widespread among rodents

We report the occurrence of *Pneumocystis* in 20 East African rodent species, supporting the hypothesis that *Pneumocystis* is ubiquitous throughout the mammalian class (Aliouat-Denis et al., 2008; Latinne et al., 2017). Although seven screened species of rodents yielded no positive results, this fact alone should not be interpreted as evidence of *Pneumocystis* absence due to the small number of screened individuals and/or possible false negatives due to mutations in primer sequences. The most numerous rodent species was *M. natalensis*. The observed prevalence in this species (34%) can be thus be considered a reliable estimate and it is similar to the average prevalence (36%) previously reported in recent study on various mammal species (Danesi et al., 2016). Interestingly, there was a high prevalence of *Pneumocystis* in the subterranean silvery mole-rat *H. argenteocinereus* (90% [55.4–99.5], $N = 10$). Although this can be also an artefact again due to small sample size, it is possible that subterranean environment present favourable conditions for *Pneumocystis* spread (note here that *H. argenteocinereus* is a solitary rodent). More samples and comparison with

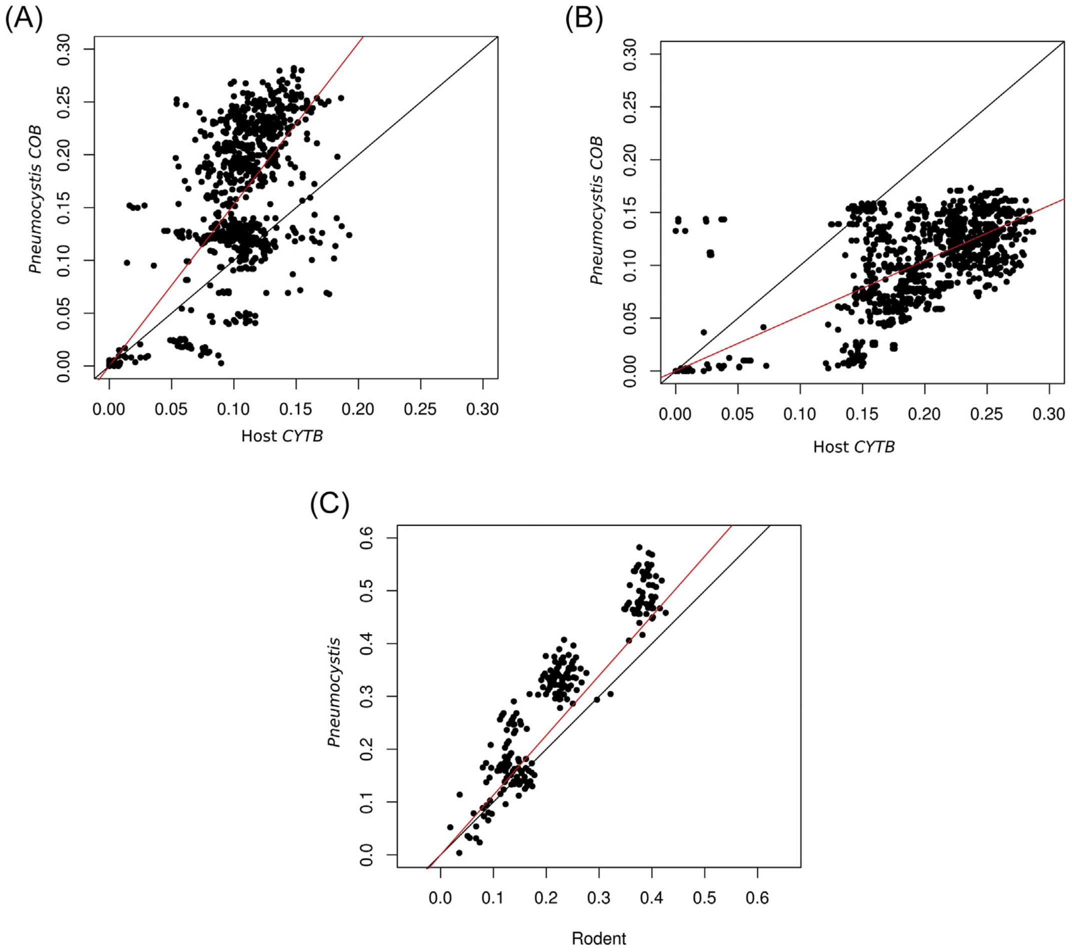

Fig. 2. (a) Plot of pairwise genetic distances at *CYTB* of rodent hosts vs. pairwise genetic distances at *COB* of *Pneumocystis* (both including 4-fold degenerate sites only). The black line denotes equality, the red line is a regression line from the linear model. The regression slope is equal to 1.53. (b) Plot of pairwise genetic distances at *CYTB* of rodent hosts vs. pairwise genetic distances at *COB* of *Pneumocystis,* both excluding 4-fold degenerate sites. The black line denotes equality, the red line is a regression line from the linear model. The regression slope is equal to 0.52. (c) Plot of pairwise phylogenetic distances (branch lengths) comparisons from coevolving taxa (Co-paths). The black line denotes equality, the red line is a regression line from the linear model. The regression slope is equal to 1.13. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other subterranean mammals are needed to test this hypothesis. It should be also noted that our positives-detection method may be less sensitive than the widely used nested PCR of rRNA genes, which may detect even very low abundance of *Pneumocystis* because of the "nested" nature of the PCR. Very likely most individuals in wild populations exhibit low amounts of *Pneumocystis* in their lungs (Aliouat-Denis et al., 2008; Danesi et al., 2016), which may have not been detected by our screening approach.

4.2. Genetic diversity of Pneumocystis

Apart from sequences from the three described species introduced for the comparison with new sequences (*P. wakefieldae*, *P. carinii* and *P. murina*), every single other lineage might potentially correspond to a new undescribed *Pneumocystis* species, i.e. in a single study we potentially found up to 25 new *Pneumocystis* species.

For the purposes of *Pneumocystis* species recognition, a Phylogenetic Species Concept was developed for *Pneumocystis* sp. (Stringer et al., 2001). It states that two distinct *Pneumocystis* lineages can be considered separate species if their divergence on a certain marker exceeds that of a *P. carinii* and *P. wakefieldae*. The authors suggest using *mtLSU-rRNA* as a standard marker for these purposes.

Unfortunately, the phylogenetic species concept based on sequences encoding *mtLSU-rRNA* (sensu Stringer et al., 2001) could not be applied for the species delimitation of *Pneumocystis* in our study, since we sequenced only a short part of this marker (178 bp) containing very limited information. In fact, the resulting tree had very low node supports and resolution. Furthermore, we did not obtain any *COB* or *DHFR* sequences of *P. wakefieldae* which would allow us to extrapolate the species concept to other genetic markers. The constraints introduced by the nature of our dataset preclude us from further elaborations on the Phylogenetic Species Concept as per sensu Stringer et al. (2001).

4.3. Molecular divergence comparisons

We have found that *Pneumocystis COB* exhibits higher differentiation rates than rodent *CYTB* on the 4-fold degenerate sites (Fig. 2a), which should be free from any selective pressure. This is in agreement with the generally accepted assumption that parasites evolve faster than their hosts. However, one should remain cautious making such an interpretation, because the observed pattern could just reflect a higher accumulation of neutral polymorphism without any evolutionary implications.

In contrast to this finding, the remaining (non-4-fold degenerate) sites exhibited the opposite pattern (Fig. 2b). When using all the sites except the 4-fold degenerate ones, we find that *Pneumocystis* has much lower differentiation rates on *COB* than the host. The most likely explanation would be that there is a strong negative purifying selection

Table 3
Results of AxParaFit co-phylogenetic analysis (+/− denotes significance of the link). F1 and F2 denote F-statistic value.

Sig.	Parasite	Host	*P*-value 1	P-value 2	F1	F2
−	*Pneumocystis carinii*	*Rattus norvegicus*	0.12663	0.12663	0.00285	0.01544
−	*Pneumocystis wakefieldiae*	*Rattus norvegicus*	0.06797	0.06797	0.00315	0.01902
+	*Pneumocystis murina*	*Mus musculus*	0.03493	0.03493	0.00542	0.02939
+	*P.* ex *Mus triton*	*Mus triton*	0.02341	0.02341	0.00624	0.03380
+	*P.* ex *Mus minutoides*	*Mus minutoides*	0.01667	0.01667	0.00638	0.03458
+	*P.* ex *Rhabdomys dilectus*	*Rhabdomys dilectus*	0.03531	0.03531	0.00670	0.03629
+	*P.* ex *Lemniscomys striatus*	*Lemniscomys striatus*	0.03903	0.03903	0.00794	0.04302
+	*P.* ex *Lemniscomys rosalia*	*Lemniscomys rosalia*	0.02736	0.02736	0.00580	0.03140
+	*P.* ex *Aethomys chrysophilus*	*Aethomys chrysophilus*	0.03506	0.03506	0.00520	0.02816
+	*P.* ex *Aethomys kaiseri*	*Aethomys kaiseri*	0.03351	0.03351	0.00603	0.03266
+	*P.* ex *Grammomys surdaster*	*Grammomys surdaster*	0.04090	0.04090	0.00567	0.03072
+	*P.* ex *Hylomyscus arcimontensis*	*Hylomyscus arcimontensis*	0.03155	0.03155	0.00461	0.02495
−	*P.* ex *Mastomys natalensis*	*Mastomys natalensis*	0.06443	0.06443	0.00417	0.02257
−	*P.* ex *Praomys delectorum*	*Praomys delectorum*	0.05942	0.05942	0.00514	0.02787
−	*P.* ex *Myomyscus brockmani*	*Myomyscus brockmani*	0.06296	0.06296	0.00472	0.02557
+	*P.* ex *Lophuromys kilonzoi*	*Lophuromys kilonzoi*	0.02737	0.02737	0.01609	0.08717
+	*P.* ex *Lophuromys makundii*	*Lophuromys makundii*	0.02678	0.02678	0.01459	0.07904
+	*P.* ex *Acomys muzei*	*Acomys muzei*	0.03349	0.03349	0.01224	0.06629
+	*P.* ex *Acomys ngurui*	*Acomys ngurui*	0.03760	0.03760	0.01138	0.06164
−	*P.* ex *Gerbilliscus vicinus*	*Gerbilliscus vicinus*	0.12662	0.12662	0.00369	0.02000
+	*P.* ex *Graphiurus* n.sp.	Graphiurus n.sp.	0.00049	0.00049	0.06648	0.36014
+	*P.* ex *Graphiurus murinus*	*Graphiurus murinus*	0.00030	0.00030	0.06944	0.37619
+	*P.* ex *Heliophobius argenteocinereus* A	*Heliophobius argenteocinereus*	0.00118	0.00118	0.06133	0.33226
+	*P.* ex *Heliophobius argenteocinereus* B	*Heliophobius argenteocinereus*	0.00447	0.00447	0.05387	0.32485
−	*P.* ex *Aethomys chrysophilus* ("Deo-lineage")	*Aethomys chrysophilus*	0.94638	0.94638	0.00299	0.00923
−	*P.* ex *Praomys delectorum* ("Deo-lineage")	*Praomys delectorum*	0.87612	0.87612	0.00205	0.00635
−	*P.* ex *Mastomys natalensis* ("Deo-lineage")	*Mastomys natalensis*	0.86827	0.86827	0.00193	0.00598

present, which is reducing the genetic diversity at these sites. It should be noted that *COB* is an important part of oxygen metabolism pathways and *Pneumocystis* is strictly aerobic, lung dwelling organism with specific needs for stable oxygen supply (Ma et al., 2016). We suggest this could explain the observed reductions because this oxygen may well be a limiting factor within the *Pneumocystis* environment and possibly even a source of competition among various *Pneumocystis* strains, and thus imposing a limit on genetic diversity of oxygen metabolism related markers. This hypothesis could be in principle tested by comparing the substitution rates on relevant sites of multiple (both parasitic and free-living) organisms with different demands on oxygen supply.

These results should be, however, interpreted with caution, as the neutrality of 4-fold degenerate sites was recently challenged (Chamary et al., 2006; Kunstner et al., 2011). However, even if there indeed is a selective pressure acting on the 4-fold degenerate sites, there is still justification for using these comparisons, since one can reasonably expect the degree of selection on 4-fold degenerate sites to be considerably lower than on the rest of the sites (see e.g. Fig. 1 in Kunstner et al., 2011), therefore allowing us to make such comparisons.

4.4. Host-parasite co-phylogenetic pattern

The results of phylogenetic analyses are suggestive of a co-divergence pattern between African rodents and their *Pneumocystis* parasites, thus in line with Farenholz's rule, stating that parasite phylogeny mirrors that of its hosts (Brooks, 1985). This co-phylogenetic pattern was also supported strongly by the co-phylogenetic analyzes, which favour co-speciation as the major explanation of our data. The comparisons of molecular and phylogenetic distances also favour this general picture. The co-path analysis revealed a linear trend with several clumps of branch length comparison points, corresponding to comparisons between species, tribes and, higher-order taxa respectively (Fig. 3). This is not surprising since our dataset contained a high number of lineage sorting events, which generally produce unequal branch lengths.

Three species of murid rodents, namely *Aethomys chrysophilus*, *Praomys delectorum* and *Mastomys natalensis* were found to harbour two different *Pneumocystis* lineages (although never in the same individuals -), in all three cases, the second lineage did not cluster with the other one found in each species in question, but instead clustered with *Pneumocystis* obtained from rodents belonging to the Deomyinae subfamily - i.e. genera *Lophuromys* and *Acomys*. We call the cluster of related *Pneumocystis* lineages from Deomyinae subfamily + the three "non-standard" lineages from the aforementioned species a "Deo-lineage" as opposed to "Mur-lineage" - i.e. other *Pneumocystis* found in rodents from the Murinae subfamily (see Fig. 1a). This pattern suggests a possibility of parallel evolution of two independent *Pneumocystis* lineages: the ancestors of this *Pneumocystis* lineage switched hosts between Deomyinae and Murinae subfamilies. After that, the "Deo-lineage" co-diverged in murine rodents in parallel with the "Mur-lineage". An alternative possibility is that there were multiple shifts from Deomyinae to other taxa, which do not form a single parallel phylogeny. This could perhaps reflect an evolutionary adaptation, which allows *Pneumocystis* species from Deo-lineage to switch its host more frequently, however, this is a speculation on our part and the observed pattern could be coincidental in nature. Currently, the data favour the second scenario, because Core-Pa analysis identified the shifts as two separate events. Nevertheless, this could be an artefact of the analyzes: the events identified as host-shifts could represent a mislabelled sorting event. This cannot be ruled out, since *Pneumocystis* and mammals share a very long evolutionary history. More exhaustive sampling of various rodent species would be needed for further development and testing of these hypotheses. Also, it should be noted that parasites with even a high degree of specialization can colonize new host species (Araujo et al., 2015; Hoberg and Brooks, 2008).

There are some additional incongruencies between rodent and fungal phylogenies within the subfamily Murinae. For example, there is an evident mismatch at the *Pneumocystis* ex *Grammomys surdaster*, clustered apart from other *Pneumocystis* obtained from Arvicanthini. As co-phylogenetic scenario and co-path analysis suggest, these are best explained as lineage-sorting events, which probably do not reflect some radical host-shift events within the *Pneumocystis* subtree.

Overall, the cospeciation pattern is present and well corroborated by the analyzes. Our results are consistent with the results of the study on *Pneumocystis* in primates (Hugot et al., 2003), but in partial contrast with the recent study on *Pneumocystis* in Asian rats (Latinne et al., 2017), where they found less significant congruence among rodents and

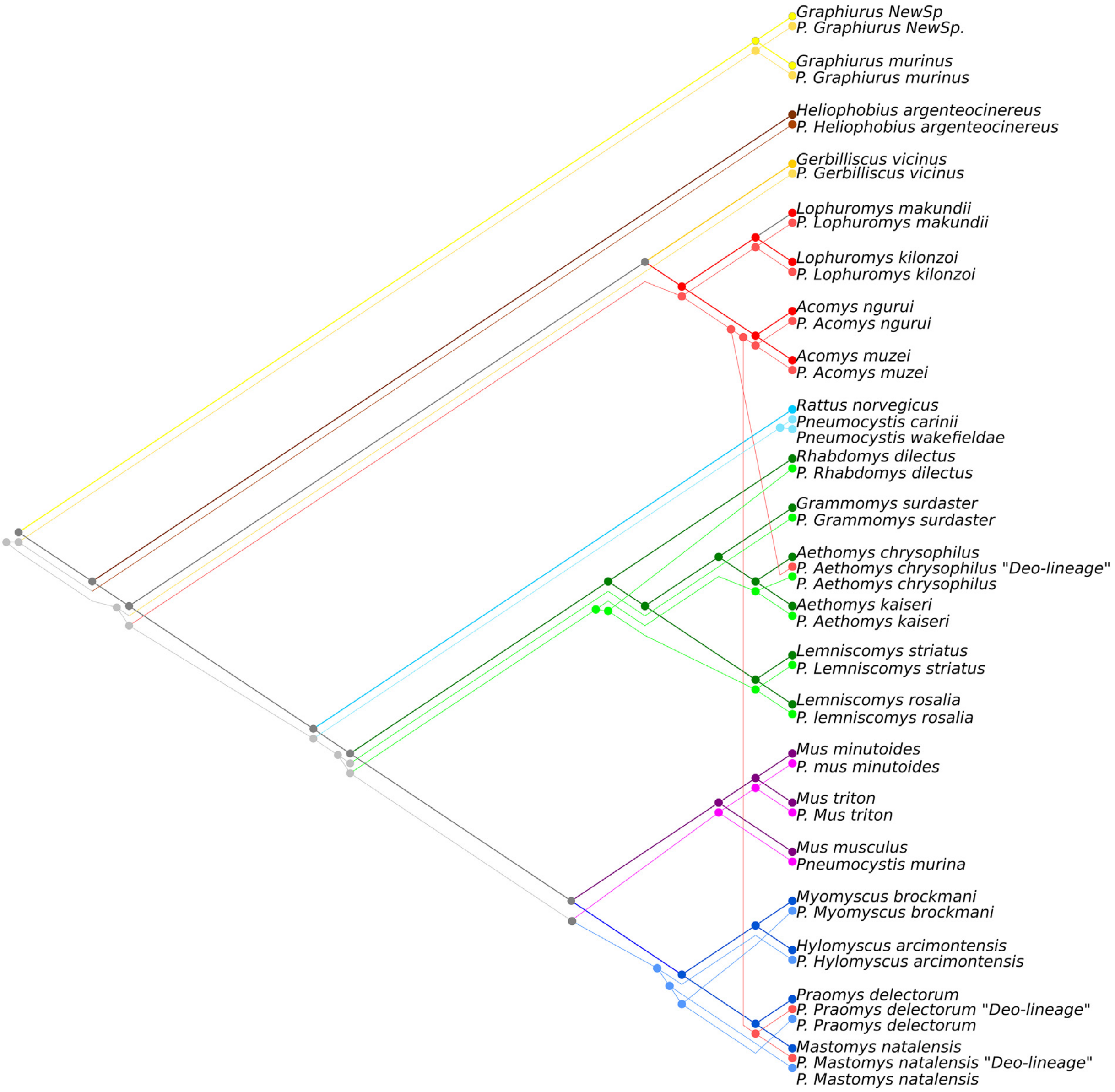

Fig. 3. The best-scoring co-evolutionary scenario obtained from CorePa. Darker tree represents the phylogeny of rodent hosts, while the lighter tree is for *Pneumocystis*. The branch colours represent different tribes/subfamilies.

their pathogens. Instead, they have observed that multiple (closely related) rat species can be simultaneously infected by *P. wakefieldae,* with the latter lacking any significant genetic diversity. The authors proposed physiological, immunological and cellular similarities between recently diverged rodent species, which fail to provide a barrier to *Pneumocystis* cross-infection, as an explanation for the observed pattern. Further, multiple hosts were infected by a single *Pneumocystis* lineage, which was also found in the case of *Macaca mulatta* and *M. fascicularis* (Hugot et al., 2003), suggesting that *Pneumocystis* can infect closely related species.

We suggest that the observed partial incongruences between otherwise coevolving parasite and host phylogenies should not be cause for surprise. The initial studies, which established the co-speciation concept, were performed usually on very small datasets (Aliouat-Denis et al., 2009; Cissé and Hauser, 2018 and references therein) leading to an oversimplified picture of the process. More importantly, in the case of *Pneumocystis*, there is little reason to presume complete one-to-one phylogenetics correspondence since it is an airborne pathogen which can spread horizontally, as well as vertically and has no known mating structures (Aliouat-Denis et al., 2009; Cissé and Hauser, 2018), meaning it has the potential to mix freely within host populations and, therefore, strict co-speciation should be observed only in a limited number of evolutionary scenarios, such as vicariant events, which split the host population into multiple isolated ones, preventing gene flow between the *Pneumocystis* populations. In a case of host speciating due to isolation by distance, i.e. forming a primary hybrid zone, there is a wide room for continued gene flow of *Pneumocystis* between the diverging host populations, leading to various effects, such as delayed parasite speciation or minor topology incongruences. For example, one can envision a scenario where two diverging host populations develop their own parasitic lineages, but the parasite from one host population shifts into the other and competitively excludes its sister lineage, or evolves towards sympatry. The same could apply for a secondary contact scenario. Moreover, a simple lineage sorting, which we commented on previously, can also introduce such incongruences - especially in the branch lengths.

On the other hand, other mechanisms, such as *Pneumocystis* adaptations to live in a given host species can represent a counterbalancing force to those mentioned above, driving towards greater host specificity in general and constraining possibilities for infecting other host lineages over longer periods of time. In that case, one should expect well developed host-specificity in older host-parasite couples and exceptions to this rule in newly diverged host species, since there is a wide room for preferential host-switch between the closely related host species in sympatry. Observation of *P. wakefieldae* infecting related species of rats (a tribe which has undergone a rapid adaptive radiation in recent history) from Latinne et al. (2017), would count as an example of such dynamics.

4.5. *Two different Pneumocystis in the same host species*

We found several cases when a single host species is infected by two very distinct *Pneumocystis* at the same locality. This is best demonstrated by the sympatric occurrence of "Mur-lineage" and "Deo-lineage" of *Pneumocystis* in three species of murine rodents (*M. natalensis*, *P. delectorum* and *A. chrysophilus*), but also by very divergent *Pneumocystis* in genetically non-diverse mole-rats and dormice. All examined *Heliophobius* mole-rats belong to the same clade distributed in northern Tanzania (Bryja et al., 2018), and the two *Pneumocystis* lineages pinpoint to an occurrence of multiple parasitic lineages within a single host population (although no coinfections have been evidenced yet).

On the other hand, *P. wakefieldae* very likely do not belong to the "Deo-lineage" of murine *Pneumocystis*. We failed to sequence this species at *COB,* and used only the *mtLSU-rRNA* sequence from GenBank. While its close relation to *P. carinii* cannot thus be firmly established (because of low information content in this marker), it is not without interest that Latinne et al. (2017), achieved similar results with much more robust dataset, indicating that *P. wakefieldae* is much closer to the *P. carinii* than to *Pneumocystis* from other murine tribes.

The following are the supplementary data related to this article.

Authors' contributions

JGB and JB designed the study, JB, JGB, CS and AK collected the field samples, AB and JP genotyped the material, JP analyzed the data, and JP, JB, SJEB and JGB contributed to the first version of the manuscript. All authors provided comments to the final version of the manuscript.

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Declaration of interest

None.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103934.

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