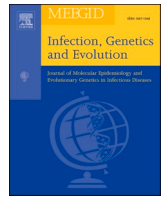




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Research paper

Revisiting the *Pneumocystis* host specificity paradigm and transmission ecology in wild Southeast Asian rodents

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## ABSTRACT

*Pneumocystis* fungi are opportunistic parasites of mammalian lungs whose evolution, ecology and host specificity in natural host populations remain poorly understood and controversial. Using an extensive collection of 731 lung samples from 27 rodent species sampled in five Southeast Asian countries, and nested PCR amplification of mitochondrial and nuclear genes, we investigated the host specificity and genetic structure of *Pneumocystis* lineages infecting wild rodents. We also identified the rodent species playing a central role in the transmission of these parasites using network analysis and centrality measurement and we characterized the environmental conditions allowing *Pneumocystis* infection in Southeast Asia using generalized linear mixed models.

Building upon an unprecedented *Pneumocystis* sampling from numerous rodent species belonging to closely related genera, our findings provide compelling evidence that the host specificity of *Pneumocystis* lineages infecting rodents is not restricted to a single host species or genus as often presented in the literature but it encompasses much higher taxonomic levels and more distantly related rodent host species. The phylogenetic species status at both mitochondrial and nuclear genetic markers of at least three new *Pneumocystis* lineages, highly divergent from *Pneumocystis* species currently described, is also suggested by our data. Our models show that the probability of *Pneumocystis* infection in rodent hosts is positively correlated to environmental variables reflecting habitat fragmentation and landscape patchiness. Synanthropic and habitat-generalist rodents belonging to the *Rattus*, *Sundamys* and *Bandicota* genera played a role of bridge host species for *Pneumocystis* spreading in these heterogeneous habitats, where they can reach high population densities. These are critical findings improving our understanding of the ecology of these enigmatic parasites and the role played by cospeciation and host switches in their evolution. Our results also confirmed the role of land-use change and habitat fragmentation in parasite amplification and spillover in rodents.

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## 1. Introduction

*Pneumocystis* organisms are airborne-transmitted fungal parasites that infect the lungs of numerous mammal species and require a living host at each stage of their life cycle (Aliouat-Denis et al., 2008; Cissé and Hauser, 2018). *Pneumocystis* infection may cause life-threatening pneumonia in immunocompromised individuals but asymptomatic and benign infections are also commonly observed in immunocompetent and healthy hosts (Chabé et al., 2004). Several molecular studies investigating the diversity and host specificity of these fungal parasites in a wide range of mammalian species showed that the *Pneumocystis* genus is highly diversified and includes numerous divergent taxonomic entities characterized by strong host specificity and apparently restricted to a single host species (Akbar et al., 2012; Danesi et al., 2016; Demanche et al., 2001; Hugot et al., 2003; Petružela et al., 2019). However recent studies called this long-standing *Pneumocystis* strict host specificity paradigm into question as the biology, evolution, epidemiology and transmission of these airborne-transmitted organisms in natural host populations remain poorly understood (Babb-Biernacki et al., 2020; Cissé and Hauser, 2018; Latinne et al., 2018; Ma et al., 2018).

*Pneumocystis* infection is common and widespread in both laboratory and wild Old World rodents (Cushion et al., 2004; Danesi et al., 2016; Demanche et al., 2015; Latinne et al., 2018; Palmer et al., 2000; Petružela et al., 2019). To date, only three *Pneumocystis* species infecting rodents have been formally described. *Pneumocystis carinii* (Frenkel, 1999) is the type species of the genus and was first identified in the lungs of laboratory rats (*Rattus norvegicus*). *Pneumocystis wakefieldiae* (Cushion et al., 2004) is the second species described in laboratory rats, while *P. murina* (Keely et al., 2004) is the sole species described in laboratory mice (*Mus musculus*). However, recent studies of *Pneumocystis* parasites infecting rodents from Southeast Asia and Africa revealed a much larger *Pneumocystis* diversity in wild rodents, including many unknown lineages corresponding to potential new species (Latinne et al., 2018; Petružela et al., 2019). Several of these lineages were clearly delineated in phylogenies based on mitochondrial markers, and the level of mitochondrial genetic distance between these lineages and formally recognized species was indicative of phylogenetic species recognition (Latinne et al., 2018). The use of nuclear markers, notoriously difficult to amplify in samples from wild hosts characterized by low *Pneumocystis* loads (Babb-Biernacki et al., 2020; Demanche et al., 2019), would further confirm their specific taxonomic status.

This large diversity of *Pneumocystis* observed in rodents arose from a complex host-parasite evolutionary history, involving a long-lasting process of co-evolution but also several host switches (Latinne et al., 2018; Petružela et al., 2019). Several genera of murid rodents, including the genus *Rattus*, are known to be infected by several divergent *Pneumocystis* lineages, sometimes in co-infection (Icenhour et al., 2006; Latinne et al., 2018; Petružela et al., 2019). Our previous study also demonstrated that most *Pneumocystis* lineages identified in Southeast Asian murid rodents infected several closely related host species, suggesting that the *Pneumocystis* host specificity in rodents is mostly limited to the generic level (stenoxenism) rather than the species level (monoxenism) (Latinne et al., 2018). These results contradict the apparent strict host specificity observed in other mammals (Akbar et al., 2012; Demanche et al., 2001; Hugot et al., 2003) but some authors recently suggested that the inferred monoxenism of *Pneumocystis* in some of these studies was due to the inclusion of only distantly related species and the lack of extensive host intrageneric sampling (Babb-Biernacki et al., 2020).

Using Southeast Asian murid and spalacid rodents as models and an extensive dataset, the objectives of this study were to (1) identify the *Pneumocystis* lineages infecting a large diversity of wild rodents in Southeast Asia and characterize their taxonomic status, host specificity, geographic distribution and genetic structure using both mitochondrial and nuclear markers, (2) identify the rodent species playing a central role in *Pneumocystis* sharing and transmission in natural rodent

communities in Southeast Asia and (3) characterize the environmental conditions and habitat attributes allowing *Pneumocystis* infection in these rodent populations. In the past decades, Southeast Asia has been exposed to accelerating rates of habitat destruction and modification, such as deforestation, urbanization, and agricultural intensification, with consequent impacts on the structure and richness of host-pathogen communities in the region (Wilcove et al., 2013). Characterized by a high taxonomic diversity of rodents, Southeast Asia is considered to be the center of origin and diversification of Murinae rodents, one of the five subfamilies among murid rodents (Schenk et al., 2013), which display various life histories and collectively inhabit a wide range of ecological niches where they pose significant threats to crop production (Singleton et al., 2010) and for human health as rodents host a high proportion of zoonotic pathogens (Han et al., 2015; Meerburg et al., 2009).

## 2. Materials and methods

### 2.1. Study sites and rodent sampling

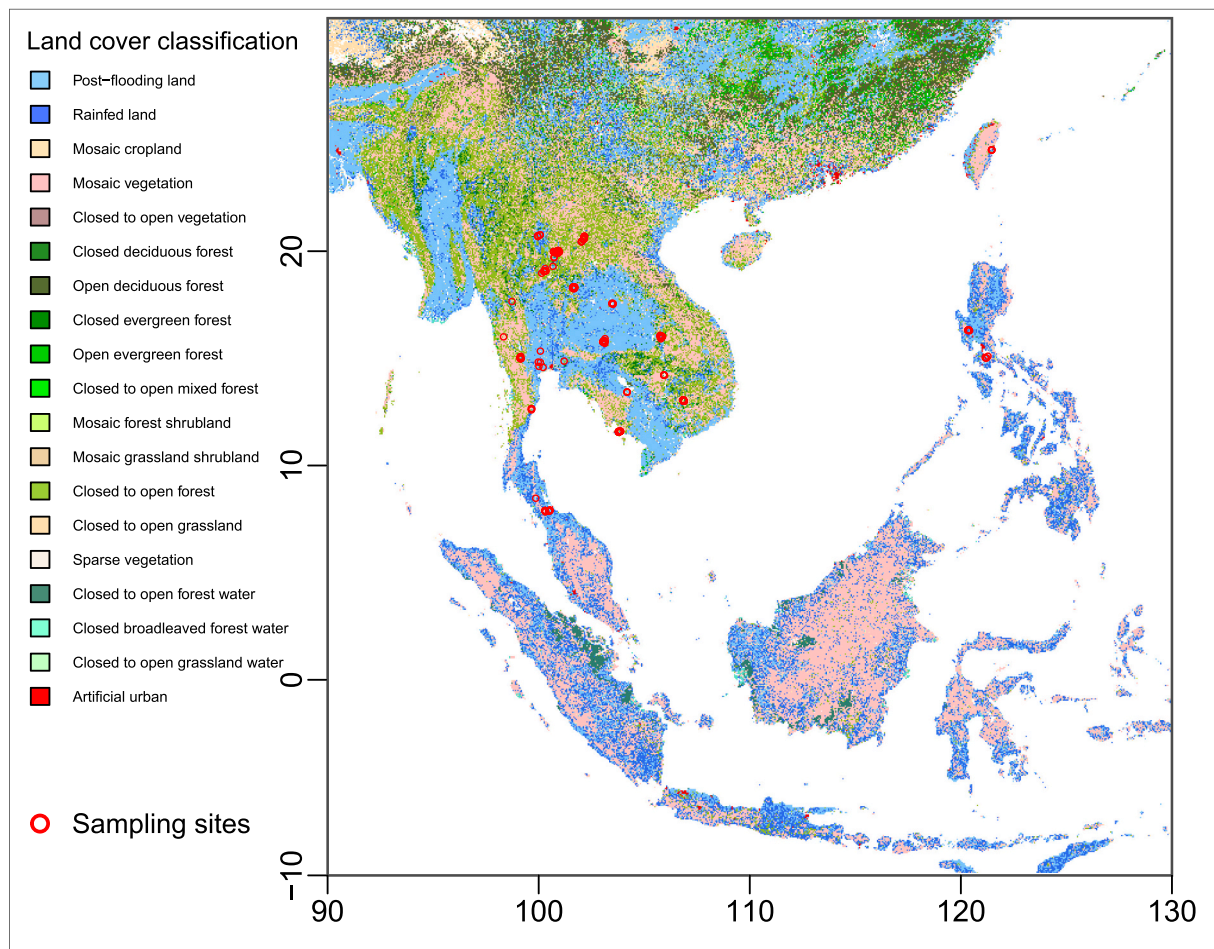
Rodent lung samples were collected at multiple sites located in 19 provinces in five countries of Southeast Asia (Thailand, Cambodia, Lao PDR, Philippines and Taiwan) (Fig. 1) as part of the CERoPath and BiodivHealthSEA projects (Herbretreau et al., 2011; Morand et al., 2015). The lung samples were collected immediately after euthanasia and stored in RNAlater (QIAGEN, France) at  $-20^{\circ}\text{C}$ .

Rodent species were identified in the field using morphological criteria (see [www.ceropath.org/references/rodent\\_field\\_guide](http://www.ceropath.org/references/rodent_field_guide)) and molecular barcoding was used to confirm field identification using the protocols described in Latinne et al. (2013) and Pages et al. (2010). The two mitochondrial lineages belonging to *Rattus tanezumii* were included in this study and referred to as *Rattus tanezumii* R2 and R3 in accordance with Pages et al. (2010). *Rattus tanezumii* R2 and R3 correspond to the *Rattus rattus* Lineage II sensu Aplin et al., 2011 (Aplin et al., 2011) and *Rattus rattus* Lineage IV sensu Aplin et al., 2011 (Aplin et al., 2011), respectively. Data on the animals used as reference specimens for the barcoding assignment are available on the “Barcoding Tool/RodentSEA” section of the CERoPath project web site ([www.ceropath.org](http://www.ceropath.org)).

Rodent species included in this study are neither on the CITES list, nor the IUCN Red List. Animals were treated in accordance with the guidelines of the American Society of Mammalogists, and within the European Union legislation guidelines (Directive 86/609/EEC). Permits from national, regional and local health authorities were obtained before each trapping sessions. Approval notices for rodent trapping and sample collection were provided by the Ministry of Health Council of Medical Sciences, National Ethics Committee for Health Research (NHCHR) in Lao PDR (number 51/NECHR), by the Ethical Committee of Mahidol University, Bangkok, in Thailand (number 0517.1116/661), and by the Animal Care and Use Advisory Committee of National Taiwan Normal University, Taipei, in Taiwan (number 104016). Sample collection from the Philippines was conducted with permission from the Department of Environment and Natural Resources under Wildlife Gratuitous Permit number R4A-WGP-08-2013-LAG-002.

### 2.2. *Pneumocystis* screening

Samples from 731 rodent individuals belonging to 27 species (Table 1) were screened for the presence of *Pneumocystis* spp. in their lungs through the amplification of two *Pneumocystis* mitochondrial genes, the large subunit rRNA (mtLSU rRNA) and the small subunit rRNA (mtSSU rRNA). The 731 samples screened for this study include 445 rodents from a previous study (Latinne et al., 2018) and 286 new individuals, most of them coming from localities and host species not sampled in Latinne et al. (2018). DNA was extracted from lung tissue using the QIAamp DNA mini kit (QIAGEN, France) according to the manufacturer's protocol. The mtLSU rRNA and the mtSSU rRNA



**Fig. 1.** Southeast Asia land cover map and rodent sampling sites for this study. Land cover map was obtained from the ESA GlobCover 2009 project ([http://due.esrin.esa.int/page\\_globcover.php](http://due.esrin.esa.int/page_globcover.php)).

*Pneumocystis* mitochondrial genes were then amplified by nested PCR using a high-fidelity DNA polymerase (QIAGEN HotStarTaq) and PCR protocols described in Latinne et al. (2018). Samples were considered positive for *Pneumocystis* infection when a PCR product of the expected size (250–350 bp) was amplified either at mtLSU rRNA or mtSSU rRNA or at both loci. Several negative controls were included in each series of DNA extraction and PCR to detect possible cross-contamination.

A subset of strong positive samples from each host species were selected for PCR amplification of a *Pneumocystis* nuclear gene, the dihydropteroate synthase (DHPS) gene. A nested PCR using the external primers A<sub>HUM</sub> (5'-GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3') and B<sub>HUM</sub> (5'-CAT AAA CAT CAT GAA CCC G-3') (Lane et al., 1997) and internal primers C<sub>PRIM</sub> (5'-CCC CCA CTT ATA TCA-3') and D<sub>PRIM</sub> (5'-GGG GGT GTT CAT TCA-3') (Demanche et al., 2001) was performed for each sample using a touchdown PCR cycling program as described in Demanche et al. (2001). Sequencing reactions were performed from both ends on an ABI 3730 XL automated DNA sequencer.

### 2.3. Phylogenetic analysis

Sequences were aligned using the ClustalW algorithm and alignments were subsequently refined manually. The mtSSU rRNA hyper-variable regions, corresponding to the insertions located at positions 22–354 of the mtSSU rRNA sequence of *Pneumocystis carinii* (KX257138) and 213–446 of the mtSSU rRNA sequence of *Pneumocystis* sp. L11 (KX257150) were removed from the alignment as it was not possible to align these regions with other sequences unambiguously, following a strategy similar to the one used in Latinne et al. (2018). Gaps were coded

as a fifth state. GenBank Accession numbers of all *Pneumocystis* sequences generated in this study (MW665237–MW665323, MW694941) and previously published sequences included in our analysis are available in the Supplementary Table S1 Table and Supplementary Table S2.

Phylogenetic trees were obtained using the Maximum Likelihood (ML) inference implemented in PhyML 3.0 (Guindon et al., 2010) and the Bayesian inference implemented in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) using the mtLSU rRNA (389 bp with indels), mtSSU rRNA (256 bp without hypervariable regions) and DHPS (717 bp) datasets separately and the mtLSU rRNA and mtSSU rRNA concatenated dataset (645 bp with indels). The transition/transversion ratio, the proportion of invariable sites and the gamma distribution parameter of the PhyML analysis were estimated and the substitution model automatically selected (Lefort et al., 2017). The starting tree was determined by BioNJ analysis of the datasets. Robustness of the tree was assessed by 1000 bootstrap replicates. Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling of the Bayesian analysis was performed with 4 chains run for  $6.10^6$  generations with one tree sampled every 1000 generations, using default parameters as starting values. A 50% Majority-rule consensus tree was generated after discarding 10% of trees as burn-in values. All distinct sequence types identified in our dataset using DAMBE (Xia, 2018) were included in these phylogenetic trees. Several *Pneumocystis* reference sequences were included in the phylogenetic analysis (Supplementary Table S3). Sequences from *P. jiroveci* and *Pneumocystis* from *Macaca* sp. were used as outgroups in the DHPS phylogenetic analysis (Supplementary Table S3). We also reconstructed a median-joining network including all *Pneumocystis wakefieldiae* mtLSU rRNA sequences using Network 10.0 (Bandelt et al., 1999) to assess the

**Table 1**  
Numbers of tested and *Pneumocystis* positive individuals for each rodent species.

Family	Number of individuals tested	Number of <i>Pneumocystis</i> positive individuals
Tribe		
Species		
<b>Spalacidae</b>		
Rhizomyini		
<i>Cannomys badius</i>	5	5 (100%)
<i>Rhizomys pruinosus</i> *	1	1 (100%)
<b>Muridae</b>		
Hydromyini		
<i>Apomys banahao</i> *		
Murini		
<i>Mus caroli</i>	12	8 (66.7%)
<i>Mus pahari</i> *	5	2 (40%)
<i>Mus cervicolor</i>	26	10 (38.5%)
<i>Mus cookii</i>	23	5 (21.7%)
Rattini		
<i>Rattus norvegicus</i>	58	48 (82.8%)
<i>Bandicota indica</i>	27	21 (77.8%)
<i>Rattus nitidus</i>	16	12 (75%)
<i>Sundamys muelleri</i> *	9	6 (66.7%)
<i>Berylmys bowersi</i>	8	5 (62.5%)
<i>Leopoldamys neilli</i> *	5	3 (60%)
<i>Berylmys berdmorei</i>	25	14 (56%)
<i>Niviventer fulvescens</i>	16	8 (50%)
<i>Rattus sakeratensis</i>	25	12 (48%)
<i>Bandicota savilei</i>	25	11 (44%)
<i>Rattus exulans</i>	138	49 (35.5%)
<i>Leopoldamys herberti</i>	12	4 (33.3%)
<i>Rattus tanezumi</i> R3	120	39 (32.5%)
<i>Rattus tanezumi</i> R2	79	34 (31.6%)
<i>Rattus argentiventer</i>	17	5 (29.4%)
<i>Leopoldamys sabanus</i> *	4	1 (25%)
<i>Rattus andamanensis</i>	9	2 (22.2%)
<i>Rattus tiomanicus</i> *	19	4 (21.1%)
<i>Maxomys surifer</i>	35	5 (14.3%)
<i>Rattus everetti</i> *	2	0

\* Species not included in Latinne et al. (2018).

intraspecific relationships within *P. wakefieldiae*.

#### 2.4. Network

A bipartite network connecting rodent host species ( $n = 26$ ) with all detected *Pneumocystis* lineages was created using the package tnet (Opsahl, 2007) in R (R Core Team, 2020). Host rodent species and *Pneumocystis* lineage associations were built on lineage assignments delineated in our phylogenetic trees (see Results). We then transformed this bipartite network into a unipartite network depicting the pattern of shared *Pneumocystis* parasites by rodent host species using the tnet package in R. The role of each host species within the network was examined using its centrality measurement. A central host node is highly connected to other host nodes and thus is supposed to have a greater transmission potential of *Pneumocystis*. We calculated the eigenvalue centrality (EC) of each host species using the 'evcent' function from the igraph package (Csardi and Nepusz, 2006) in R.

#### 2.5. Statistical analysis and environmental data

Nineteen land use land cover variables (Fig. 1 and Supplementary Table S4) and several environmental and bioclimatic variables (Digital Elevation Model, human population density, normalized difference vegetation index (NDVI), precipitation, and temperature) were extracted from various sources (Supplementary Table S5) for a 500-m buffer around each individual rodent from which samples were collected using the R packages raster (Hijmans et al., 2015), rgdal (Bivand et al., 2019),

dismo (Hijmans et al., 2017) and rgeos (Bivand and Rundel, 2017). We then extracted the size of each land use class in each 500-m buffer and computed two additional composite variables, open forest cover (Closed to open forest + Closed to open mixed forest) and mosaic land cover (Mosaic vegetation + Mosaic cropland). We also estimated the fragmentation of the land cover within each buffer by estimating sumFRAC, a metrics calculated using the function PatchStat from the SDMTools package (VanDerWal et al., 2014) in R, which reflects shape complexity across a range of spatial scales and is computed as the logarithm of patch perimeter (m) divided by the logarithm of patch area. All variables were transformed to satisfy normality and homoscedasticity by dividing their size by the total area of the buffer in order to get a percentage of occupation for each variable. The final list of variables observed in the buffer areas around each trapped rodent and used in our models includes: urban cover, forest cover (closed deciduous forest), open forest cover, open vegetation (closed to open vegetation), open grassland (closed to open grassland), mosaic vegetation cover, mosaic land cover, rain fed land cover, waterbodies cover, sumFRAC, temperature, slope, elevation, precipitation, NDVI and human population density.

To assess the links between the probability of *Pneumocystis* infection in rodents and their environment, we modeled the presence/absence probability of the parasite as a function of these environmental variables using a GLMM with logit function and random effects (for sites) using the package lme4 (Bates et al., 2015) implemented in R. Statistical analyses were performed for all *Pneumocystis* spp. and then separately for *P. wakefieldiae* in mainland sites (Thailand, Cambodia, Lao PDR) and *P. wakefieldiae* in island sites (Philippines) to assess potential differences in transmission routes of the same *Pneumocystis* species in different environments. We focused on *P. wakefieldiae* as it was the species for which we had the highest number of positive rodent samples and sampling locations (see Results).

We evaluated support for competing models by investigating the relationship between the occurrence of *Pneumocystis* parasite and all explanatory variables. We used likelihood-based methods to quantify the alternative models and estimate their parameters. AIC adjusted for sample size (AICc) was used to assess the relative information content of the models. Performance of models was also assessed by estimating the percentage of deviance explained using maximum likelihood R2 and AUC with the pROC (Robin et al., 2011) and MuMin (Barton and Barton, 2015) packages. Selection of the best competing models was made using package glmulti version 1.0.7.2 (Calcagno and De Mazancourt, 2010) implemented in R, which allows the exploration of all models using automated model selection and model-averaging procedure using a genetic algorithm. Visualization of the predicted response, *Pneumocystis* infection, was made using the packages sjmisc, sjlabelled and sjPlot (Lüdtke, 2018; Lüdtke and Lüdtke, 2017).

### 3. Results

#### 3.1. *Pneumocystis* infection prevalence

*Pneumocystis* DNA was detected in 319 samples (43.6%) belonging to 26 Southeast Asian species; only *Rattus everetti* yielded no positive sample (Table 1). The six Spalacidae samples belonging to the species *Cannomys badius* (5/5) and *Rhizomys pruinosus* (1/1) were all positive (100%) while the prevalence of *Pneumocystis* infection in Muridae species varied from 14.3% (5/35) in *Maxomys surifer* to 82.8% (48/58) in *Rattus norvegicus* (Table 1).

#### 3.2. *Pneumocystis* diversity and phylogenetic trees

We obtained 253 mtLSU rRNA sequences, corresponding to 102 distinct sequence types, and 166 mtSSU rRNA sequences, corresponding to 61 distinct sequence types, after screening 731 rodent samples (Supplementary Table S1). The mitochondrial concatenated dataset (mtLSU rRNA + mtSSU rRNA), excluding some hypervariable and non-

alignable regions of mtSSU rRNA, included 140 distinct sequence types (Supplementary Table S1). Additionally, we were able to obtain 57 *Pneumocystis* DHPS sequences, corresponding to 37 distinct sequence types, from 16 rodent host species (Supplementary Table S2).

A total of 16 *Pneumocystis* phylogenetic lineages, 13 of them infecting Southeast Asian rodents, were identified by taking the topology of the mtLSU rRNA (Supplementary Fig. S1 and S2), mtSSU rRNA (Supplementary Fig. S3 and S4), mitochondrial concatenated (Fig. 2 and Supplementary Fig. S5) and DHPS (Fig. 3 and Supplementary Fig. S6) phylogenetic trees into account. These lineages were named using the same terminology as in Latinne et al. (2018). Co-infection was detected in 15 *R. norvegicus*, one *R. argentiventer*, one *N. fulvescens* and one *B. indica* samples whose mtLSU rRNA and mtSSU rRNA sequence types did not belong to the same phylogenetic lineage (Supplementary Table S1). The mitochondrial sequence types from these samples were not concatenated in the combined dataset but were included separately to avoid any phylogenetic misinterpretation in our concatenated trees.

The topologies of the ML (Fig. 2) and Bayesian (Supplementary Fig. S5) mitochondrial concatenated trees were mostly similar. The first lineage to diverge after the outgroup, Lineage 15, included a single highly divergent mtSSU rRNA sequence (HSSU7) isolated in a co-infected *N. fulvescens* sample (CLS14) whose mtLSU rRNA sequence type belonged to Lineage 7 (*Pneumocystis* from *Niviventer* sp., *Leopoldamys* spp. and *Maxomys* sp.). This highly divergent lineage was also observed in the mtSSU rRNA trees (Supplementary Fig. S3 and S4). A well-supported clade including Lineage 1 (*Pneumocystis* from wild Danish *R. norvegicus*, referred to as *Pc* f. sp. *rattus-terti* in Palmer et al. (2000)), Lineage 2 (*Pneumocystis* from wild Danish *R. norvegicus*, referred to as *Pc* f. sp. *rattus-quarti* in Palmer et al. (2000) and from Southeast Asian *Rattus exulans*) and Lineage 3 (*Pneumocystis* from *Cannomys* sp. and *Rhizomys* sp.) was next to diverge in the mitochondrial concatenated trees (Fig. 2 and Supplementary Fig. S5). These three lineages were also first to diverge in both mtLSU rRNA phylogenetic trees (Supplementary Fig. S1 and S2) but not in the mtSSU rRNA trees (Supplementary Fig. S3 and S4). Another clade including the remaining 12 lineages among which phylogenetic relationships were poorly resolved was retrieved in the ML and Bayesian mtLSU rRNA and mitochondrial concatenated trees (Fig. 2 and Supplementary Fig. S1, S2 and S5). Lineages 5 and 14 were closely related to a *Pneumocystis* sequence from cricetid rodent (*Microtus*) (Lineage 4) in all mitochondrial trees. Lineage 14 included sequences isolated in two *S. muelleri* samples and a co-infected *B. indica* sample whose mtLSU rRNA sequence type belonged to Lineage 11 (*Pneumocystis* from *Bandicota* spp.) (Supplementary Table S1). The phylogenetic placement of Lineage 13 (*Pneumocystis* from *Apomys* sp.) in the mitochondrial concatenated trees remained unclear as only mtSSU rRNA sequences were available for this lineage. Lineage 13 was nested within Lineage 5 (*Pneumocystis* from *Rattus* spp. and *Bandicota* sp.) in the Bayesian mitochondrial concatenated tree (Supplementary Fig. S5) but corresponded to an independent and divergent lineage in the ML concatenated tree (Fig. 2). The Bayesian and ML mtSSU rRNA trees (Supplementary Fig. S3 and S4) confirmed the phylogenetic independence of Lineage 13 and therefore we considered it as a separate lineage. *P. murina* (Lineage 8) was paraphyletic in the ML mtLSU rRNA tree but was monophyletic in other trees.

Lineage 9 (*Pneumocystis carinii*), Lineage 10 (*Pneumocystis* from *Berylmys* spp.), Lineage 11 (*Pneumocystis* from *Bandicota* spp.) and Lineage 12 (*Pneumocystis wakefieldiae*) were closely related and belonged to a well-supported clade. An additional lineage (Lineage 16) was created to encompass a single mtSSU rRNA sequence (HSSU8) isolated in a co-infected *R. argentiventer* sample (CLS19) whose mtLSU rRNA sequence type belonged to *P. carinii*. This mtSSU rRNA sequence type was related to Lineages 10, 11 and 12 but our mtSSU rRNA phylogenetic trees (Supplementary Fig. S3 and S4) clearly showed that it did not belong to any of these lineages. Co-infections by both *P. wakefieldiae* (Lineage 12) and *P. carinii* (Lineage 9) were detected in 15 *R. norvegicus* samples characterized by a mtLSU rRNA sequence type closely related to

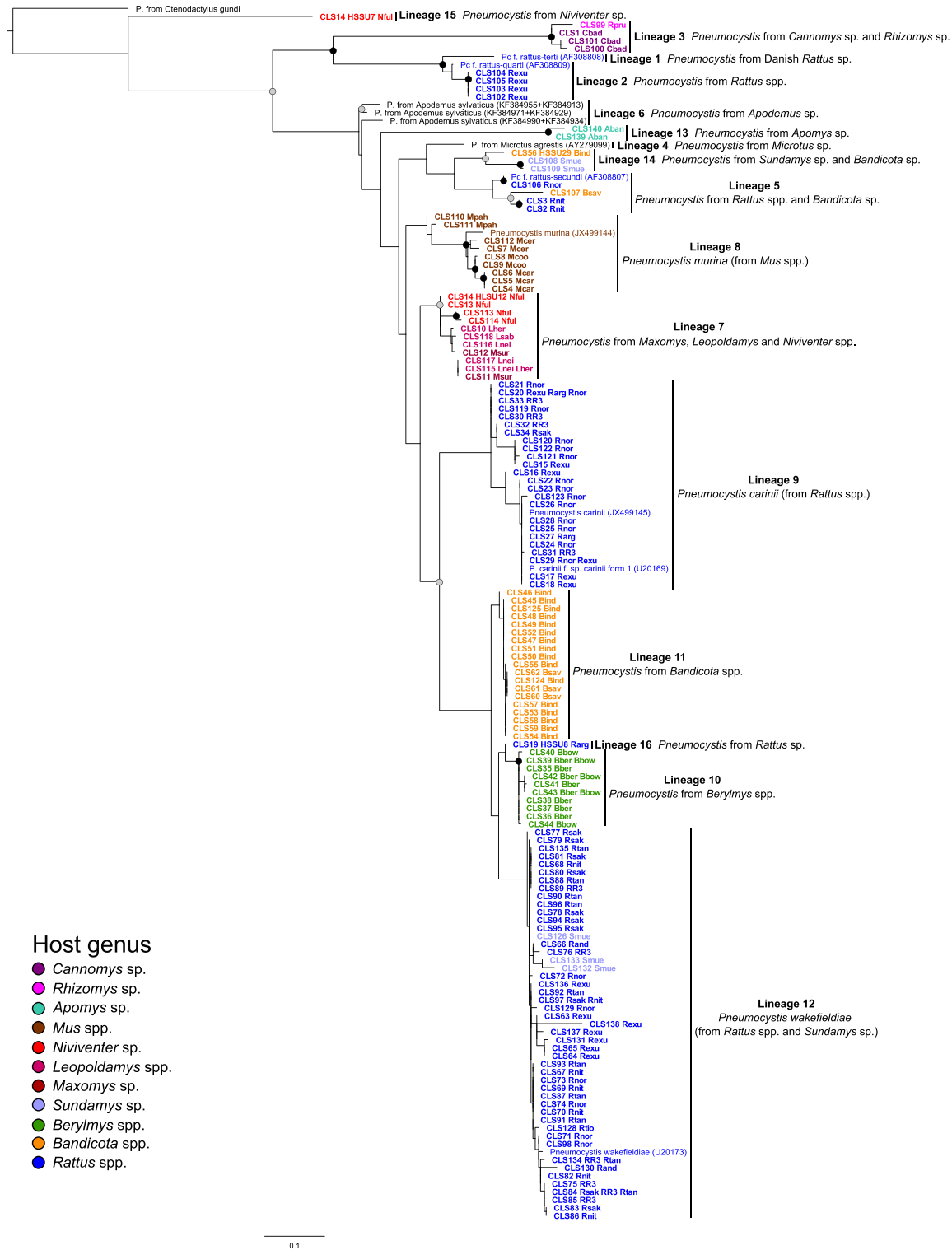
*P. carinii* while their mtSSU rRNA sequence type was closely related to *P. wakefieldiae* (Supplementary Table S1).

Most lineages identified in the mitochondrial phylogenetic trees were also retrieved and well-supported in the DHPS tree (Fig. 3). The first *Pneumocystis* sequences to diverge within the tree ingroup were those from *Mus* spp. (*Pneumocystis murina*). However, the DHPS sequence type identified in our *Mus pahari* sample (HDHPS1) whose mtLSU rRNA and mtSSU rRNA sequences belonged to Lineage 8 (*P. murina*) did not form a monophyletic clade with the DHPS reference sequence from *P. murina*. DHPS Lineage 7 (including *Pneumocystis* from *Leopoldamys* and *Niviventer*), Lineage 5 (including *Pneumocystis* from *Rattus*), Lineage 14 (including *Pneumocystis* from *Sundamys*), Lineage 9 (*Pneumocystis carinii*, including sequences from *Rattus*) and Lineage 10 (including *Pneumocystis* from *Berylmys*) were next to diverge (Fig. 3). The two DHPS sequence types identified in *Bandicota indica* and corresponding to the mitochondrial Lineage 11 were paraphyletic and closely related to Lineage 12 (*P. wakefieldiae*) which included DHPS sequences detected in *Rattus* spp. and *Sundamys muelleri*. Even after several attempts, we were not able to obtain DHPS sequences from samples belonging to the mitochondrial Lineages 2, 3, 13, 15, and 16.

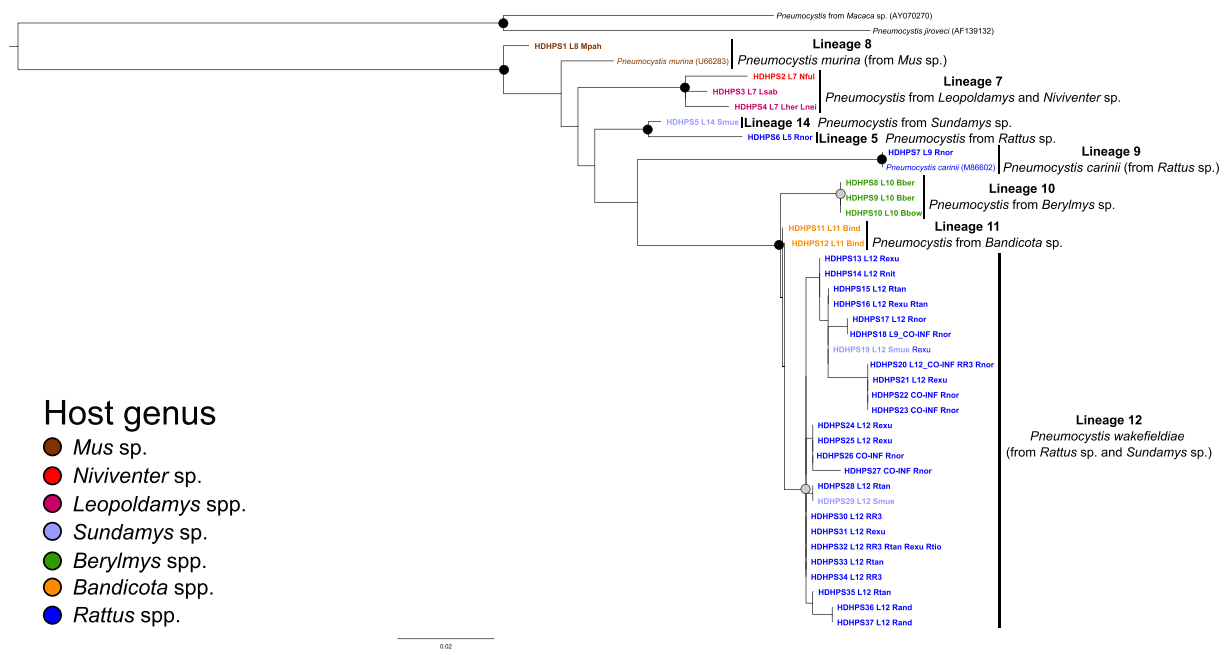
The only discrepancy between the mitochondrial and nuclear *Pneumocystis* lineage identification of samples for which both types of markers were obtained was observed for a *Rattus norvegicus* individual whose both mtLSU rRNA and mtSSU rRNA mitochondrial sequence types clustered within Lineage 9 (*P. carinii*) while its DHPS sequence type belong to Lineage 12 (*P. wakefieldiae*). Mitochondrial and nuclear markers yielded similar identification for all other samples. The DHPS sequence type of all *R. norvegicus* samples co-infected by both *P. wakefieldiae* and *P. carinii* according to mitochondrial markers corresponded to *P. wakefieldiae*.

Five of the 13 *Pneumocystis* mitochondrial lineages infecting Southeast Asian rodents are not restricted to a single rodent genus host (Fig. 2). Lineage 3 encompasses *Pneumocystis* from *Cannomys* and *Rhizomys*. Lineage 14 includes *Pneumocystis* sequences isolated in *Sundamys* and *Bandicota* species and is closely related to Lineage 5 which includes *Pneumocystis* sequences isolated in *Rattus* and *Bandicota* species and a sequence identified as *Pc* f. sp. *rattus-secundi* in wild *Rattus norvegicus* from Denmark (Palmer et al., 2000). Lineage 7 includes *Pneumocystis* sequences from *Leopoldamys*, *Maxomys* and *Niviventer* species while sequence types closely related to *P. wakefieldiae* and forming Lineage 12 were identified in several *Rattus* species but also in *Sundamys muelleri*. Most of the remaining *Pneumocystis* lineages identified in several Southeast Asian rodent individuals (Lineages 2, 8, 9, 10 and 11) included sequence types isolated in several congeneric rodent species and were not restricted to a single rodent host species. It is also interesting to note that most *Pneumocystis* lineages commonly identified in Southeast Asia were distributed in large and mostly overlapping areas and were not restricted to a single region or country (Fig. 4). Lineage 13 (*Pneumocystis* from *Apomys banahao*) is restricted to a single location in the Philippines as its host species (*Apomys banahao*) is endemic to Mt. Banahaw (Heaney et al., 2011) and was not trapped in our other study sites. Lineages 15 and 16 are also restricted to a single location as they were each identified in a single individual.

The median-joining network representing the intraspecific phylogenetic relationships among *P. wakefieldiae* mtLSU rRNA sequence types clearly showed that the most prevalent *P. wakefieldiae* sequence types were shared among several *Rattus* species and locations (Fig. 5). The mtLSU rRNA sequence types identified in *Sundamys muelleri* were closely related to *P. wakefieldiae* sequence types identified in *Rattus* spp. and distinct by only three and seven mutations (Fig. 5A). No clear structure related to host taxonomy was observed, but it is interesting to note that sequence types identified in *R. exulans* were mostly distinct from those identified in other *Rattus* species. No geographic structure was observed either, with sequences from all locations widespread in the network (Fig. 5B).



**Fig. 2.** Maximum Likelihood (ML) tree (GTR + G) depicting phylogenetic relationships among *Pneumocystis* from Southeast Asian rodents inferred from concatenated mtLSU rRNA and mtSSU rRNA sequences excluding hypervariable regions. Nodes with strong statistical support (bootstrap support >75%) are indicated with a black dot while nodes with moderate statistical support (bootstrap support >60%) are indicated with a grey dot. Node supports from within some lineages were removed for clarity of presentation. Sequences from Southeast Asian rodents are in bold, other reference sequences are from GenBank (accession numbers in brackets). *Pneumocystis* sequences from rodent genera distributed in Southeast Asia are colored according to their host genus and host species of each sequence type is indicated in the sequence name. Aban: *Apomys banahao*; Bind: *Bandicota indica*; Bsav: *Bandicota savilei*; Bber: *Berylmys berdmorei*; Bbow: *Berylmys boweri*; Cbad: *Cannomys badius*; Lher: *Leopoldamys herberti*; Lnei: *Leopoldamys neilli*; Lsab: *Leopoldamys sabanus*; Msur: *Maxomys surifer*; Mcar: *Mus caroli*; Mcar: *Mus cervicolor*; Mcoo: *Mus cookii*; Mpah: *Mus pahari*; Nful: *Niviventer fulvescens*; Rarg: *Rattus argenteventer*; Rand: *Rattus andamanensis*; Rexu: *Rattus exulans*; Rnit: *Rattus nitidus*; Rnor: *Rattus norvegicus*; Rpru: *Rhizomys pruinosus*; RR3: *Rattus tanezumii* R3; Rsak: *Rattus sakaretensis*; Rtan: *Rattus tanezumii* R2; Rtio: *Rattus tiomanicus*; Smue: *Sundamys muelleri*.



**Fig. 3.** Maximum Likelihood (ML) tree (HKY85 + G) depicting phylogenetic relationships among *Pneumocystis* from Southeast Asian rodents inferred from dihydropteroate synthase (DHPS) gene sequences. Nodes with strong statistical support (bootstrap support >75%) are indicated with a black dot while nodes with moderate statistical support (bootstrap support >60%) are indicated with a grey dot. Node supports from within lineages were removed for clarity of presentation. Sequences from Southeast Asian rodents are in bold, other reference sequences are from GenBank (accession numbers in brackets). *Pneumocystis* lineages from rodent genera distributed in Southeast Asia are colored according to their host genus and host species of each sequence type is indicated in the sequence name. Aban: *Apomys banahao*; Bind: *Bandicota indica*; Bsav: *Bandicota savilei*; Bber: *Berylmys berdmorei*; Bbow: *Berylmys bowersi*; Cbad: *Cannomys badius*; Lher: *Leopoldamys herberti*; Lnei: *Leopoldamys neilli*; Lsab: *Leopoldamys sabanus*; Msur: *Maxomys surifer*; Mcar: *Mus caroli*; Mcer: *Mus cervicolor*; Mcoo: *Mus cookii*; Mpah: *Mus pahari*; Nful: *Niviventer fulvescens*; Rarg: *Rattus argentiventer*; Rand: *Rattus andamanensis*; Rrexu: *Rattus exulans*; Rnit: *Rattus nitidus*; Rnor: *Rattus norvegicus*; Rpru: *Rhizomys pruinosus*; RR3: *Rattus tanezumii* R3; Rsak: *Rattus sakaretensis*; Rtan: *Rattus tanezumii* R2; Rtio: *Rattus tiomanicus*; Smue: *Sundamys muelleri*.

### 3.3. Network and *Pneumocystis* sharing among rodent species

The unipartite network describing the interactions among rodent species based on the host species and *Pneumocystis* lineage associations identified in our phylogenetic trees (Supplementary Table S6) is presented in Fig. 6. Six clusters were clearly identified within the network and only one of them (Cluster 2) included a single rodent host species (*Apomys banahao*). Two of the remaining clusters (Clusters 1 and 6) included several congeneric species (*Berylmys* spp.; *Mus* spp.) while the other three (Clusters 3, 4, and 5) included species from several host genera (*Leopoldamys*/*Niviventer*/*Maxomys*; *Rhizomys*/*Cannomys*; *Bandicota*/*Sundamys*/*Rattus*). These clusters were not interacting together, indicating that *Pneumocystis* transmission was compartmentalized and occurred only between rodent species and genera belonging to the same cluster (Fig. 6).

Most species were characterized by low centrality values (<0.01), except those of Cluster 5 who shared several *Pneumocystis* lineages (Table 2). Among these species, *Rattus norvegicus*, *Bandicota indica*, *Bandicota savilei*, *Rattus nitidus* and *Sundamys muelleri* had the highest centrality values (centrality >0.7; Table 2) and contributed the most to the sharing of various *Pneumocystis* lineages among rodent species. *Rattus nitidus*, *Sundamys muelleri* and *Bandicota savilei* were also characterized by a high centrality (Fig. 6 and Table 2).

### 3.4. Fine-scale habitat characteristics and *Pneumocystis* infection

Of the variables considered in the global GLMM including all *Pneumocystis* lineages, mosaic land cover and elevation were each important in explaining the *Pneumocystis* infection probability of rodents according to the best model (Table 3 and Supplementary Fig. S7). A significant positive association ( $p = 0.037$ ) between sites characterized by a patchwork of mosaic of habitats and infection of rodents with any

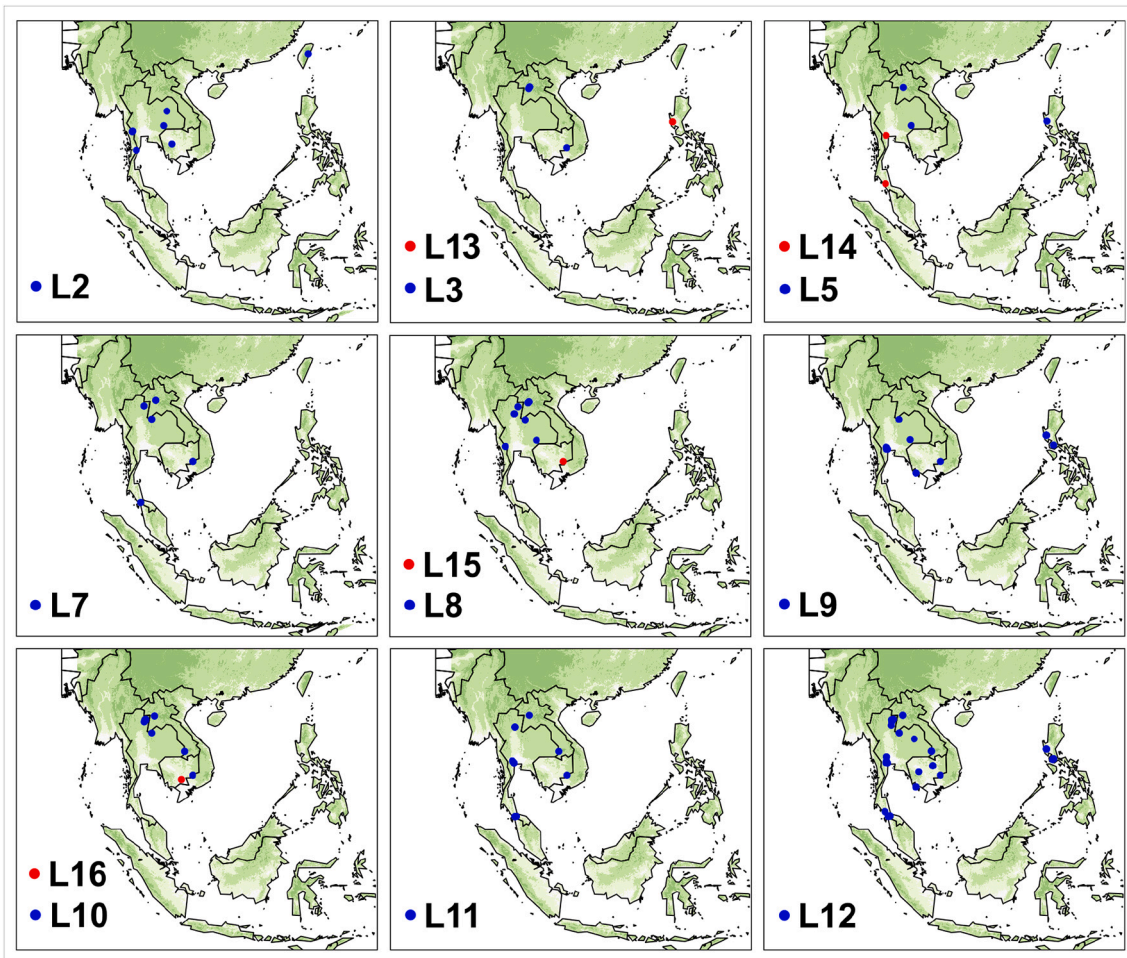
*Pneumocystis* lineages was observed (Supplementary Fig. S7). Although the performance of the model is statistically significant with an AUC = 0.64 (0.59–69), the percentage of deviance explained by the best model and estimated by maximum likelihood  $R^2$  is low ( $R^2c = 0.13$ ) (Table 3 and Supplementary Fig. S8).

According to the GLMMs including *P. wakefieldiae* only, sumfrac, a metrics reflecting the fragmentation of the land cover ( $p = 0.006$ ), open forest cover ( $p = 0.055$ ) and NDVI ( $p = 0.048$ ) were significantly associated to *P. wakefieldiae* infection in mainland sites (Thailand, Cambodia and Lao PDR) while slope was the variable significantly correlated to *P. wakefieldiae* infection in insular sites of the Philippines ( $p = 0.017$ ) (Table 3 and Supplementary Fig. S9). The percentage of deviance explained by these models was estimated by maximum likelihood  $R^2c$  to 0.38 and 0.35, respectively, indicating a good prediction of these best models, with good statistical performance given by AUC, respectively 0.80 (0.76–0.83) and 0.79 (0.75–0.83) (Table 3 and Supplementary Fig. S8).

## 4. Discussion

### 4.1. *Pneumocystis* diversity and host specificity in murine rodents

This study further investigates the *Pneumocystis* evolution, diversity and host specificity in wild rodents from Southeast Asia using both mitochondrial and nuclear markers. Our phylogenies reveal a huge *Pneumocystis* diversity in murid rodents and do not reflect any global pattern of co-speciation as *Pneumocystis* lineages detected in spalacid (Lineage 3) and cricetid rodents (Lineage 4) are all closely related to lineages identified in murid rodents (Lineages 1/2 and Lineages 14/5, respectively) while several murid rodent genera (*Rattus*, *Sundamys*, *Bandicota*, and *Niviventer*) are each infected by two to four highly divergent *Pneumocystis* lineages characterized by distinct evolutionary



**Fig. 4.** Geographic distribution of the 13 *Pneumocystis* lineages identified in Southeast Asian rodents. Map background correspond to elevation. L2, Lineage 2 (*Pneumocystis* sp. from *Rattus*); L3, Lineage 3 (*Pneumocystis* sp. from *Cannomys* and *Rhizomys*); L5, Lineage 5 (*Pneumocystis* sp. from *Rattus*, *Sundamys* and *Bandicota*); L7, Lineage 7 (*Pneumocystis* sp. from *Maxomys*, *Leopoldamys* and *Niviventer*); L8, Lineage 8 (*Pneumocystis murina*); L9, Lineage 9 (*Pneumocystis carinii* from *Rattus*); L10, Lineage 10 (*Pneumocystis* sp. from *Berylmys*); L11, Lineage 11 (*Pneumocystis* sp. from *Bandicota*); L12, Lineage 12 (*Pneumocystis wakefieldiae* from *Rattus* and *Sundamys*); Lineage 13 (*Pneumocystis* sp. from *Apomys* sp.); L14, Lineage 14 (*Pneumocystis* sp. from *Sundamys* sp. and *Bandicota* sp.); L15, Lineage 15 (*Pneumocystis* sp. from *Niviventer* sp.); L16, Lineage 16 (*Pneumocystis* sp. from *Rattus* sp.)

pathways, revealing a complex evolutionary history of *Pneumocystis* parasites in rodents.

Our nuclear phylogenetic tree confirmed the phylogenetic and evolutionary independence of most *Pneumocystis* lineages identified in our mitochondrial phylogenetic trees at both mitochondrial and nuclear markers. The phylogenetic concordance of mitochondrial and nuclear genes confirms the genetic distinctiveness of Lineage 5 (*Pneumocystis* from *Rattus* and *Bandicota*), Lineage 14 (*Pneumocystis* from *Sundamys* and *Bandicota*) and Lineage 7 (*Pneumocystis* from *Maxomys*, *Leopoldamys* and *Niviventer*) and further suggest their phylogenetic species status according to the Genealogical Concordance Phylogenetic Species Recognition under the Phylogenetic Species Concept (PSC), which uses the phylogenetic concordance of multiple unlinked genes as a criterion to indicate evolutionary independence of lineages and is currently the most widely used species criterion for fungi (Taylor et al., 2000; Giraud et al., 2008). This was also previously indicated by the higher levels of mtDNA genetic distance among these lineages than between other currently recognized *Pneumocystis* species (Latinne et al., 2018). Lineage 15 is clearly the most divergent of the *Pneumocystis* lineages identified in this study and was detected in a single *Niviventer* sample, co-infected by Lineage 7. However, this lineage is represented by a single mtSSU rRNA sequence and mtLSU rRNA and DHPS sequences belonging to this lineage are not available. Therefore, additional sequences are needed before

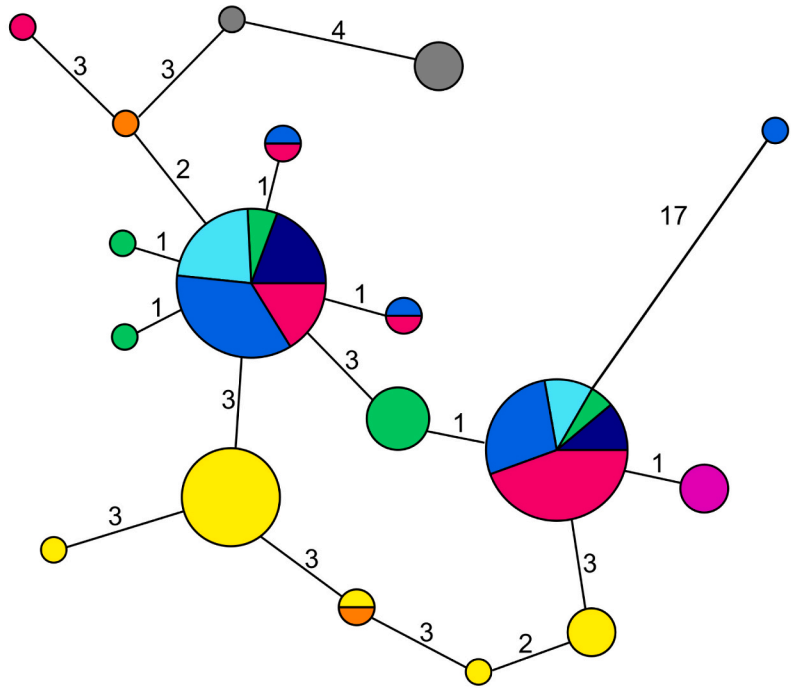
we can confirm the specific status of this lineage. Lineage 10 (*Pneumocystis* from *Berylmys*) and Lineage 11 (*Pneumocystis* from *Bandicota*) are characterized by specific mitochondrial and nuclear sequence types but are less clearly delineated in our nuclear phylogenetic trees where they are closely related to *P. wakefieldiae* (Lineage 12). This pattern could be explained by genetic drift after lineage divergence resulting in these distinctive sequence types but insufficient mutation events to create a pattern of reciprocal monophyly at the slow evolving DHPS gene (Weber et al., 2019). These results do not allow us to confirm the phylogenetic species status of these two lineages. Lineage 16, identified in a *R. argentiventer* sample co-infected by *P. carinii* (Lineage 9), is closely related to these three lineages but its phylogenetic position remains unclear as only one mtSSU rRNA sequence is available. Additional sequences are needed before we can fully characterize this lineage.

A large dataset of sequences from additional nuclear genes would definitely clarify the taxonomic status of each of the lineages identified in this study. However, sequencing of single copy *Pneumocystis* nuclear genes from wild animals is particularly difficult because of the low fungal load in wild samples and the current lack of reproducible in-vitro culture system of these parasites (Chabé et al., 2010; Cissé and Hauser, 2018; Hauser, 2019). The use of high-throughput sequencing could overcome these difficulties but presents its own technical challenges when applied to *Pneumocystis* studies (Demanche et al., 2019). High-



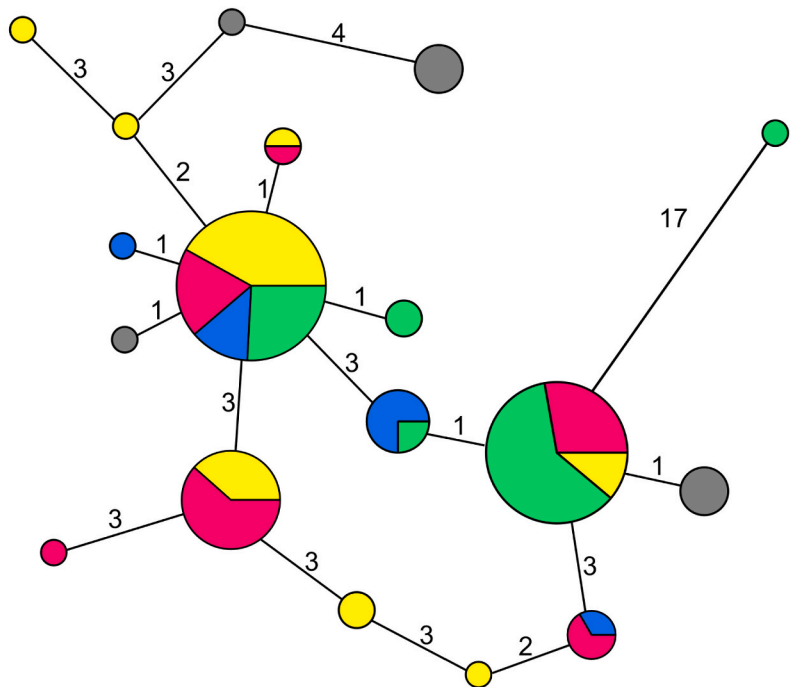
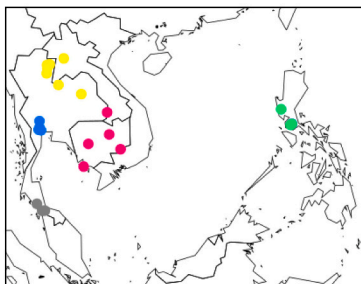
### A. *P. wakefieldiae* - Hosts

- *Rattus norvegicus*
- *Rattus nitidus*
- *Rattus exulans*
- *Rattus andamanensis*
- *Rattus tiomanicus*
- *Rattus sakeratensis*
- *Rattus tanezumi* R2
- *Rattus tanezumi* R3
- *Sundamys muelleri*

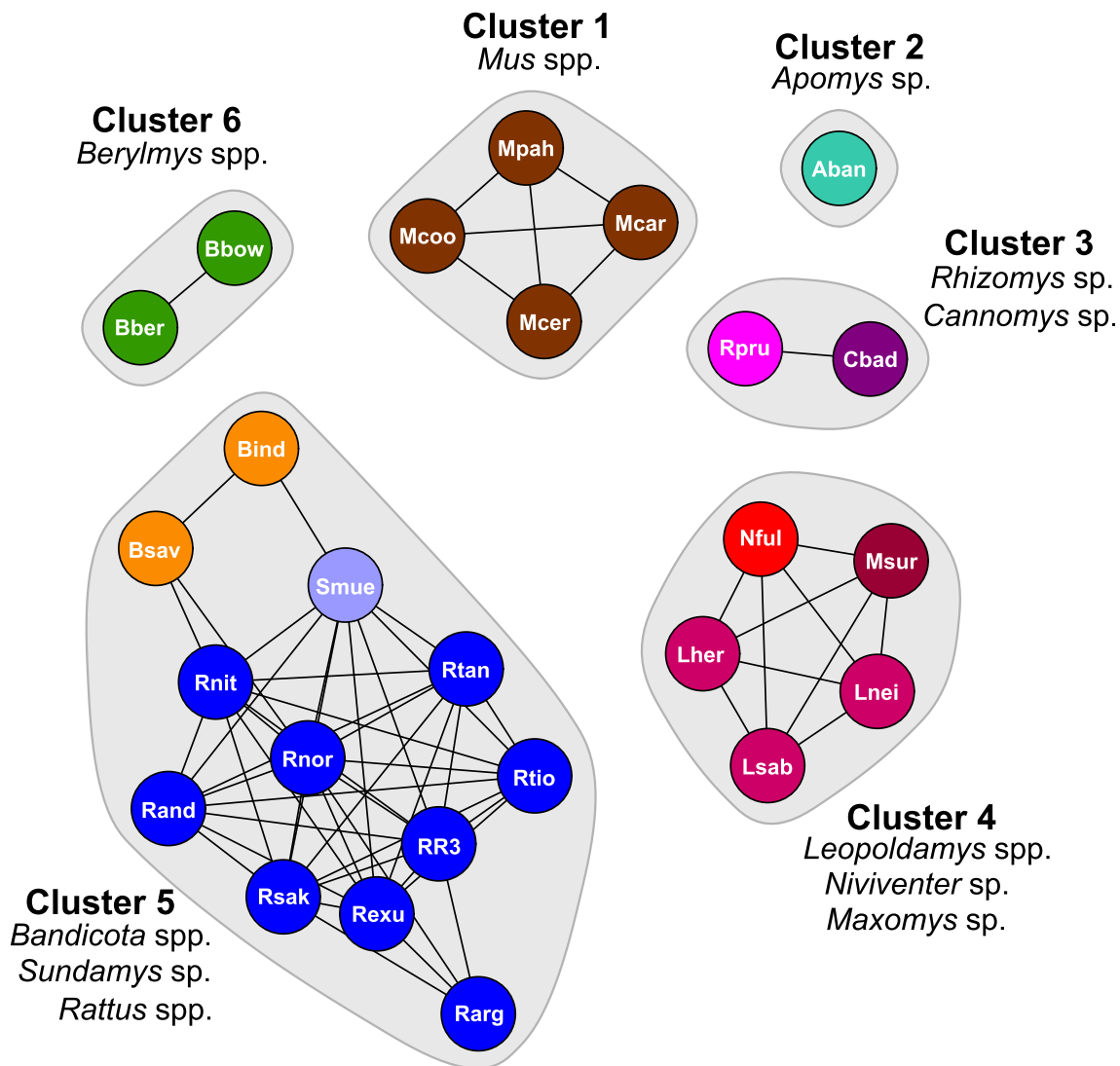


### B. *P. wakefieldiae* - Localities

- North
- East
- West
- South
- Philippines



**Fig. 5.** Median-joining network based on mtLSU rRNA sequences from *Pneumocystis wakefieldiae*. Circles correspond to distinct sequence types and circle sizes are proportional to the number of animals sharing this haplotype. The number of mutations between sequence types is indicated above branches. Circles are colored according to the rodent host species (A) or sampling localities (B). The insert map shows the rodent sampling localities and their colors correspond to the Philippines and each sampling region within mainland Southeast Asia; North, East, West, South.



**Fig. 6.** Unipartite network and clusters identified based on shared *Pneumocystis* lineages among rodent hosts are highlighted by different colors. The links among nodes (node = species) of unipartite networks depict shared *Pneumocystis* lineages. Aban: *Apomys banahao*; Bind: *Bandicota indica*; Bsav: *Bandicota savilei*; Bber: *Berylmys berdmorei*; Bbow: *Berylmys bowersi*; Cbad: *Cannomys badius*; Lher: *Leopoldamys herberti*; Lnei: *Leopoldamys neilli*; Lsab: *Leopoldamys sabanus*; Msur: *Maxomys surifer*; Mcar: *Mus caroli*; Mcer: *Mus cervicolor*; Mcoo: *Mus cookii*; Mpah: *Mus pahari*; Nful: *Niviventer fulvescens*; Rarg: *Rattus argentiventer*; Rand: *Rattus andamanensis*; Rexu: *Rattus exulans*; Rnit: *Rattus nitidus*; Rnor: *Rattus norvegicus*; Rpru: *Rhizomys pruinosus*; RR3: *Rattus tanezumi* R3; Rsak: *Rattus sakarensis*; Rtan: *Rattus tanezumi* R2; Rtio: *Rattus tiomanicus*; Smue: *Sundamys muelleri*.

throughput sequencing would also improve the detection of co-infection of a single host by multiple *Pneumocystis* lineages as Sanger sequencing may not be able to detect some minority genetic variants. Several instances of co-infections were identified in this study and two of the 13 lineages identified in Southeast Asian rodents (Lineages 15 and 16) were detected only in co-infection with other lineages and seem to be characterized by low prevalence in rodent populations. It is likely that some other rare sequence types were not detected by Sanger sequencing in some samples.

Based on an extended sampling of congeneric rodent host species, including 27 species belonging to 11 genera, this study reveals an even weaker host specificity of *Pneumocystis* in rodents than suggested by our previous study which included less host species (Latinne et al., 2018). In addition to Lineage 7, which has been detected in three rodent genera, *Leopoldamys*, *Maxomys* and *Niviventer*, by both kinds of genetic markers, we identified four additional *Pneumocystis* lineages infecting multiple rodent genera. This study provides the first demonstration and compelling evidence of *P. wakefieldiae* (Lineage 12) natural infection in a rodent genus other than *Rattus*. Indeed, our phylogenetic trees and

median-joining network clearly showed that both the mitochondrial and nuclear *Pneumocystis* sequence types identified in several *Sundamys muelleri* samples from Thailand are very closely related to *P. wakefieldiae* sequence types identified in multiple *Rattus* species and belong to the same evolutionary unit. Closely related mitochondrial and nuclear sequence types belonging to Lineages 5 and 14 were also detected in *Rattus*, *Sundamys*, and *Bandicota*. These results are very robust as our mitochondrial findings were confirmed by the nuclear marker used in this study, the DHPS gene. We can exclude the hypothesis that these results could be due to laboratory contaminations as the mtLSU rRNA, mtSSU rRNA and DHPS sequence types isolated in *Sundamys* and *Bandicota* and belonging to Lineages 5, 12 and 14 are unique and distinct from those isolated in *Rattus* and have not been previously isolated in any other samples screened for this study. Finally, the mitochondrial Lineage 3 has also been detected in two spalacid rodent genera, *Cannomys* and *Rhizomys*, but these findings have not been confirmed by nuclear genes as we were not able to obtain DHPS sequences from these two genera.

According to the most recent phylogenies of murine rodents where

**Table 2**

Network centrality measurement (Eigenvalue centrality values) of each *Pneumocystis* rodent host species.

Species	Eigenvalue centrality values
<i>Rattus norvegicus</i>	1
<i>Bandicota savilei</i>	0.87
<i>Bandicota indica</i>	0.81
<i>Rattus nitidus</i>	0.78
<i>Sundamys muelleri</i>	0.72
<i>Rattus tanezumi</i> R3	0.65
<i>Rattus exulans</i>	0.65
<i>Rattus sakarensis</i>	0.65
<i>Rattus argentiventer</i>	0.38
<i>Rattus tiomanicus</i>	0.33
<i>Rattus tanezumi</i> R2	0.33
<i>Rattus andamanensis</i>	0.33
<i>Apomys banahao</i>	<0.001
<i>Berylmys berdmorei</i>	<0.001
<i>Berylmys bowersi</i>	<0.001
<i>Cannomys badius</i>	<0.001
<i>Leopoldamys herberti</i>	<0.001
<i>Leopoldamys neilli</i> <i>Leopoldamys neilli</i>	<0.001
<i>Leopoldamys sabanus</i>	<0.001
<i>Mus caroli</i>	<0.001
<i>Mus cervicolor</i>	<0.001
<i>Mus cookii</i>	<0.001
<i>Mus pahari</i>	<0.001
<i>Maxomys surifer</i>	<0.001
<i>Niviventer fulvescens</i>	<0.001
<i>Rhizomys pruinosus</i>	<0.001

the divisions (major evolutionary clades) within each murine tribe were redefined (Rowe et al., 2019), the three genera infected by Lineage 7 belong to two distinct divisions, namely the *Maxomys* division (including, among others, *Maxomys*) and the *Dacnomys* division (including, among others, *Leopoldamys* and *Niviventer*). The genera infected by *P. wakefieldiae* (Lineage 12) and Lineages 5 and 14 also belong to distinct divisions, the *Bunomys* division (including, among others, *Sundamys*) and the *Rattus* division (including, among others, *Rattus* and *Bandicota*) (Rowe et al., 2019). These findings therefore contradict the strict host specificity paradigm in *Pneumocystis* often repeated in the literature and demonstrate that the host specificity of *Pneumocystis* lineages infecting rodents is not restricted to a single host species or even genus but it encompasses much higher taxonomic levels and more distantly related host rodent species than commonly presented in the literature (Demanche et al., 2019; Hauser, 2019).

The divergence between the *Dacnomys* and *Maxomys* divisions is dated back to 7.5 Mya, while the *Bunomys* and *Rattus* divisions diverged around 3.6 Mya (Rowe et al., 2019), suggesting that the *Pneumocystis* lineages identified in this study have persisted in these highly distinct rodents without diverging for several million years while maintaining

**Table 3**

Results of the best GLMM explaining the occurrence of *Pneumocystis* in rodents from 19 provinces in Thailand, Lao PDR, Cambodia, Taiwan and the Philippines as a function of surrounding habitat characteristics around individually trapped rodents obtained from land cover layers (see Fig. 1 and Supplementary Table S4).

	Explanatory habitat and socio-environmental factors	Estimate (SD)	P	Log likelihood, deviance (DF)	R2m, R2c	AUC (CI)
<i>Pneumocystis</i> / all rodents/ all localities	mosaicland	0.20 (0.09)	<b>0.037</b>	−469.2, 938.5 (711)	0.02, 0.13	0.70 (0.66–0.73)
	elevation	0.17 (0.01)	0.093			
<i>P. wakefieldiae</i> / <i>Rattus</i> spp. / mainland SEA localities	sumFrac	0.44 (0.16)	<b>0.006</b>	−167.7, 335.4 (352)	0.02, 0.38	0.80 (0.76–0.83)
	forestopen	0.49 (0.27)	<b>0.055</b>			
<i>P. wakefieldiae</i> / <i>Rattus</i> spp. / insular SEA localities	ndvi	−0.47 (0.24)	<b>0.048</b>	−62.7, 125.5110)	0.03, 0.35	0.79 (0.75–0.83)
	slope	−2.46 (1.03)	<b>0.017</b>			
	ndvi	0.76 (0.45)	0.099			
	Precipitation	0.64 (0.37)	0.084			

Estimate of the logit function with SD = standard deviation; residual deviance with DF = degree of freedom, AUC = accuracy with 95% confidence interval. R2m (marginal) is the variance explained by fixed factors while R2c (conditional) is the variance explained by both fixed and random factors (i.e. the entire model).

the ability to recognize and evade the immune systems of all these species. Therefore, the *Pneumocystis* diversity currently observed in murine rodents did not arise from recent co-speciation events along with their hosts. The latest radiations occurring within the murine rodents in Southeast Asia (Rowe et al., 2019) were not followed by *Pneumocystis* co-speciation and did not lead to the emergence of new *Pneumocystis* lineages. This was supported by the species divergence time estimates from other studies showing that most *Pneumocystis* species diverged much before their hosts and did not co-speciate along with them (Ma et al., 2018).

Our findings stress the importance and necessity of using extensive sampling of closely related mammal host species and genera to fully assess the exact host range and host specificity of *Pneumocystis* species and detect the rarer lineages whose prevalence are lower than the most common lineages. This study also confirms that the proverbial monoxenism and co-evolution of *Pneumocystis* and their hosts often presented in the literature could be a sampling artifact rather than a biological reality as recently suggested by Babb-Biernacki et al. (2020). Future exhaustive studies of *Pneumocystis* diversity in taxa other than rodents will help determine whether the relaxed stenoxenism of *Pneumocystis* in murine rodents observed in this study is an exception or the rule among mammalian hosts.

#### 4.2. Factors influencing *Pneumocystis* infection and transmission

The epidemiology and transmission of *Pneumocystis* in natural host populations remain poorly understood to date and, to our knowledge, this study is the first to investigate the factors explaining the probability of infection of wild rodents by these airborne-transmitted organisms in their natural habitat. The global *Pneumocystis* prevalence level observed in this study (43.6%) is similar to the prevalence found in other wild rodent studies (Danesi et al., 2018; Petruželka et al., 2019). Our models suggest that the probability of *Pneumocystis* infection in rodent hosts is positively correlated to environmental variables reflecting habitat fragmentation and landscape patchiness (i.e. mosaic land, sumfrac and forest open). This correlation is observed both when our models include all *Pneumocystis* lineages and when only *P. wakefieldiae* on mainland sites is included. These environmental factors may affect *Pneumocystis* transmission rate and dynamics via impacts on rodent diversity and density. Several studies have shown that the ecological edge effect in fragmented areas and ecosystem boundaries influences both host species richness and population densities (Borremans et al., 2019; Mendoza et al., 2019). As *Pneumocystis* organisms are directly transmitted via airborne spread of cysts from infected to susceptible hosts without an environmental source (Ma et al., 2018), this increased *Pneumocystis* prevalence in fragmented habitats may be related to increased contact between rodent individuals in these habitats. A study in bats showed that crowding favored *Pneumocystis* airborne transmission between bat

individuals (Akbar et al., 2012). Similar effect of landscape heterogeneity and fragmentation on infection prevalence has been suggested for other parasites and pathogens infecting rodents in various tropical regions of the world (McCauley et al., 2015; Morand et al., 2019; Pumhom et al., 2015; Suzán et al., 2009). Some specific micro-environmental conditions may also favor *Pneumocystis* infection as we found it is positively correlated to elevation, slope and precipitation levels. An effect of elevation and precipitation on *Pneumocystis* infection rate was also observed in bats (Akbar et al., 2012) and macaques (Demanche et al., 2003), respectively.

Our network analysis confirmed that the transmission of each *Pneumocystis* lineage occurred among a small number of rodent species and genera, sometimes phylogenetically distantly related as discussed above, with no interactions among host species belonging to distinct clusters identified in the network. Centrality measurement showed that the rodent species belonging to Cluster 5 and infected by several *Pneumocystis* lineages, such as several *Rattus* species but also *Sundamys muelleri* and *Bandicota savilei*, contributed the most to the sharing of *Pneumocystis* lineages among rodent species included in this study. Differences in habitat preference and social structure may explain these findings (Bordes et al., 2007; Bordes et al., 2017). Most of rodent species with high centrality value are synanthropic and habitat-generalist rodents whose abundance increased in fragmented and human-dominated landscapes of mainland Southeast Asia (Morand et al., 2019). Similar to our findings in *Pneumocystis*, the prevalence of some other pathogens in these species also increased in this type of habitat (Morand et al., 2019). *B. savilei*, *B. indica*, *R. exulans*, *R. tanezumi*, *R. sakeratensis*, and *R. norvegicus* were already previously identified as bridge host species contributing to most parasite transmission in Southeast Asian ecosystems in a previous study focusing on helminths and several zoonotic pathogens (Bordes et al., 2017). The present study demonstrated that the same synanthropic and generalist rodent species played a similar role of hub in spreading various *Pneumocystis* lineages in heterogeneous habitats, where they can reach high population densities. Rodent species with a strong preference for forest habitat (*Maxomys surifer* and *Leopoldamys* spp.) or dry fields (*Berymys bowersi*) (Morand et al., 2019) were infected by a single *Pneumocystis* lineage and were characterized by very low centrality value in our network and did not significantly contribute to the *Pneumocystis* cross-species transmission.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2021.104978>.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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