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Genetic diversity and evolution of *Pneumocystis* fungi infecting wild Southeast Asian murid rodents

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Research Article

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Abstract

Pneumocystis organisms are airborne-transmitted fungal parasites that infect the lungs of numerous mammalian species with strong host specificity. In this study, we investigated the genetic diversity and host specificity of *Pneumocystis* organisms infecting Southeast Asian murid rodents through PCR amplification of two mitochondrial genes and tested the co-phylogeny hypothesis among these fungi and their rodent hosts. *Pneumocystis* DNA was detected in 215 of 445 wild rodents belonging to 18 Southeast Asian murid species. Three of the *Pneumocystis* lineages retrieved in our phylogenetic trees correspond to known *Pneumocystis* species, but some of the remaining lineages may correspond to new undescribed species. Most of these *Pneumocystis* species infect several rodent species or genera and some sequence types are shared among several host species and genera. These results indicated a weaker host specificity of *Pneumocystis* species infecting rodents than previously thought. Our co-phylogenetic analyses revealed a complex evolutionary history among *Pneumocystis* and their rodent hosts. Even if a significant global signal of co-speciation has been detected, co-speciation alone is not sufficient to explain the observed co-phylogenetic pattern and several host switches are inferred. These findings conflict with the traditional view of a prolonged process of co-evolution and co-speciation of *Pneumocystis* and their hosts.

Introduction

Pneumocystis organisms are opportunistic and airborne-transmitted fungal parasites that infect the lungs of humans and other mammalian species (Aliouat-Denis *et al.* 2008; Chabé *et al.* 2011). They may induce severe *Pneumocystis* pneumonia in immunocompromised individuals but are also able to colonize asymptomatically the lungs of immunocompetent and healthy hosts, which constitute potential infection sources (Chabé *et al.* 2004). Due to the inability to reproducibly culture these microorganisms *in vitro*, *Pneumocystis* life-cycle and basic biology are still poorly understood (Martinez *et al.* 2013). Most of the available data on these parasites have been obtained using immunosuppressed laboratory animals, but studies focused on wild mammals have also led to important advancements regarding *Pneumocystis* diversity, evolution and life cycle (Aliouat-Denis *et al.* 2008; Chabé *et al.* 2011).

Once considered as a unique taxonomic entity named '*Pneumocystis carinii*', molecular genetic studies have revealed that the *Pneumocystis* genus is highly diversified and includes numerous divergent taxonomic entities characterized by strong host specificity [see Aliouat-Denis *et al.* (2008) for review], as confirmed by the failure of cross-infection experiments (Aliouat *et al.* 1993, 1994; Gigliotti *et al.* 1993). Marked host species-related genetic divergence among *Pneumocystis* species has been observed and specific gene sequences could be attributed to parasites from different host species (Wakefield *et al.* 1998; Demanche *et al.* 2001; Guillot *et al.* 2001; Ma *et al.* 2001; Hugot *et al.* 2003; Akbar *et al.* 2012). The strong host specificity of these fungal parasites suggests that they probably resulted from a long host–parasite co-evolution process that led to co-speciation (Demanche *et al.* 2001; Guillot *et al.* 2001; Derouiche *et al.* 2009). The comparison of the respective phylogenies of *Pneumocystis* and their primate hosts revealed a high number of homologous nodes that may result from co-divergence events between the parasites and their hosts (Guillot *et al.* 2001; Hugot *et al.* 2003).

Despite this large diversity, only five *Pneumocystis* species have been formally described and accepted so far. *Pneumocystis carinii* (Frenkel, 1999) is the type species of the genus and has been 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*

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(Keely *et al.* 2004) is the sole species described in laboratory mice (*Mus musculus*). *Pneumocystis jirovecii* (Frenkel, 1999) infects the lungs of humans. Finally, *P. oryctolagi* (Dei-Cas *et al.* 2006) has been described in Old World rabbits (*Oryctolagus cuniculus*). These *Pneumocystis* species are characterized by marked genetic divergence at several mitochondrial and nuclear loci and by differences in their ultrastructural morphology, growth rate and infectivity (Dei-Cas *et al.* 2006; Aliouat-Denis *et al.* 2008).

The aim of this study was to investigate the genetic diversity and host specificity of *Pneumocystis* organisms infecting wild Southeast Asian murid rodents belonging to the subfamilies Rhizomyinae and Murinae and to test the co-phylogeny hypothesis among these fungal parasites and their rodent hosts. Murid rodents are the most diverse mammalian family, including more than 700 species (Musser and Carleton, 2005). Southeast Asia is considered to be the centre of origin and diversification of Murinae rodents from where they dispersed to other Old World regions (Schenk *et al.* 2013). The exceptionally high diversification of these rodents in Southeast Asia is the result of several significant radiations during the last 15 million years (Chaimanee and Jaeger, 2001; Rowe *et al.* 2011; Schenk *et al.* 2013). Murid rodents display various life histories and collectively inhabit a wide range of ecological niches where they occupy diversified habitats, from cities to agricultural fields to primary forests. They are also the reservoirs and vectors of many pathogens of zoonotic importance (Meerburg *et al.* 2009; Blasdell *et al.* 2015). Due to their high taxonomic and ecological diversity and the high prevalence of *Pneumocystis* among wild rodents (Mazars *et al.* 1997; Palmer *et al.* 2000; Chabé *et al.* 2010; Demanche *et al.* 2015; Danesi *et al.* 2016), Southeast Asian murid rodents represent highly relevant models to understand the evolutionary interactions of *Pneumocystis* species and their mammalian hosts.

Material and methods

Sampling

A total of 445 Southeast Asian wild murid rodents (Rodentia, Myomorpha, Muroidea, Muridae) from 18 species belonging to the subfamilies Rhizomyinae (genus *Cannomys*) and Murinae (genera *Mus* belonging to the Murini tribe and *Maxomys*, *Leopoldamys*, *Niviventer*, *Berylmys*, *Bandicota* and *Rattus* belonging to the Rattini tribe) were tested for the presence of *Pneumocystis* in their lungs. These specimens were collected in 12 localities corresponding to several habitat types (human settlements, forests and cultivated areas) in Thailand, Lao P.D.R. and Cambodia (Fig. 1). Sample collection has spanned more than 10 years (1998–2009). The lung samples were collected immediately after euthanasia and stored in RNAlater (QIAGEN, France) at −20 °C. Rodent species included in the study are neither on the CITES list, nor the Red List (IUCN). 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). Each sampling campaign was validated by the national, regional and local health authorities. Approval notices for trapping and investigation of rodents were provided by the Ministry of Health Council of Medical Sciences, National Ethics Committee for Health Research (NHCHR) Lao PDR, number 51/NECHR, and by the Ethical Committee of Mahidol University, Bangkok, Thailand, number 0517.1116/661.

Field identifications of captured rodents were made based on geographical and morphological criteria according to Lekagul and McNeely (1988), Corbet and Hill (1992), Aplin *et al.* (2003) and Francis (2008). These field identifications were then confirmed using molecular barcoding implemented in a web tool (http://data.ceropath.org/) (Pages *et al.* 2010; Galan *et al.* 2012; Latinne *et al.* 2013). In Southeast Asia, *Rattus tanezumi* is characterized by two divergent and paraphyletic mitochondrial lineages but these lineages are undistinguishable according to nuclear markers and morphological data (Pages *et al.* 2010, 2013). These two mitochondrial lineages belonging to *R. tanezumi* were included in this study and referred to as *R. tanezumi R2* and *R3* in accordance with Pages *et al.* (2010).

Fig. 1. Map of rodent sampling localities.

DNA extraction, polymerase chain reaction (PCR) and sequencing

DNA was extracted from lung tissue using the QIAamp DNA mini kit (QIAGEN, France) according to the manufacturer's protocol. Two mitochondrial genes of *Pneumocystis*, the large subunit rRNA (mtLSU rRNA) and the small subunit rRNA (mtSSU rRNA), were amplified by nested PCR using a high-fidelity DNA polymerase (QIAGEN HotStarTaq). The first round of PCR was performed using the external primers pAZ102-H and pAZ102-E for mtLSU rRNA (Wakefield *et al.* 1990) and the external primers pAZ112-10F and pAZ112-10R for mtSSU rRNA (Tsolaki *et al.* 1998). The second round of PCR was performed when the first-round PCR was negative using the internal primers pAZ102-X and pAZ102-W for mtLSU rRNA (Wakefield, 1996; Chabé *et al.* 2010) and the internal primers pAZ112-13 and pAZ112-14 for mtSSU rRNA (Tsolaki *et al.* 1998). PCR mixtures and conditions for both mtLSU rRNA and mtSSU rRNA were as described in Chabé *et al.* (2004) except that a touchdown PCR cycling program with decreasing annealing temperatures from 65 °C to 55 °C (−1 °C/cycle) for the ten first cycles, was used for the mtLSU rRNA first-round PCR. DNA of *P. murina* was used as a positive control for each PCR round. Moreover, several negative controls were included in each series of DNA extraction and PCR to detect possible cross-contamination. Amplification products were visualized using electrophoresis. Samples were considered as positive for *Pneumocystis* infection when a PCR product of the expected

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size (250–350 bp) was amplified either at mtLSU rRNA or mtSSU rRNA or at both loci. When non-specific bands were obtained, the QIAEX II Gel Extraction Kit (QIAGEN) was used to extract and purify amplification products of the expected size from agarose gel. Sequencing reactions were performed from both ends by GenoScreen (Pasteur Institute of Lille, France) on an ABI 3730 XL automated DNA sequencer.

Pneumocystis species-specific PCR

To estimate the proportion of *Rattus* specimens co-infected by both *P. carinii* and *P. wakefieldiae*, a randomly selected subset of 91 infected *Rattus* specimens belonging to *R. nitidus*, *R. norvegicus*, *R. exulans*, *R. andamanensis*, *R. sakeratensis*, *R. tanezumi R2* and *R. tanezumi R3* were tested with species-specific primers. First-round mtLSU rRNA PCR products obtained with the universal primers pAZ102-H and pAZ102-E were used as templates for the second round of PCR using primers RC1/RC2 and RR1/RR2. These primer pairs developed by Palmer *et al.* (1999) were used to amplify a portion of the mtLSU rRNA gene of *P. carinii* and *P. wakefieldiae*, respectively. Amplification products were then visualized using electrophoresis.

Sequence alignments and phylogenetic reconstructions

Sequence alignments were performed in BioEdit 7·0·9·0 (Hall, 1999) using ClustalW algorithm and subsequently refined by eye. The mtLSU rRNA (395 bp including indels) and mtSSU rRNA (872 bp including indels) genes were then concatenated in a combined dataset. Two concatenated alignments were created, one including the hypervariable and ambiguous regions of mtSSU rRNA and one excluding these regions. Gaps were coded as a fifth state.

Molecular phylogenies were estimated by Bayesian inference (BI) and Maximum Likelihood (ML) approaches on the mtLSU rRNA and mtSSU rRNA datasets separately and on the two concatenated datasets (with and without mtSSU rRNA hypervariable regions) including all distinct sequence types. We also added to our dataset reference sequences from *P. murina*, *P. carinii* and *P. wakefieldiae* as well as *Pneumocystis* sequences available on GenBank and isolated from other Muroid rodents belonging to Muridae and Cricetidae (Table 1). Sequences of *Pneumocystis* isolated from *Ctenodactylus gundi* (Rodentia, Hystricomorpha, Ctenodactylidae) were used as outgroups in our phylogenetic trees (Table 1). The most suitable model of DNA substitution (GTR + gamma for each dataset) was determined for each dataset using jMODELTEST 0·1 (Posada, 2008). Bayesian analyses were performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed with five chains run for five million generations with one tree sampled every 1000 generations, using default parameters as starting values. A 50% majority-rule consensus tree was then generated in PAUP 4.0b10 (Swofford, 1998) with burn-in values of 300 000 generations. ML analyses were performed using PhyML 3.0 (Guindon *et al.* 2010). The transition/transversion ratio, the proportion of invariable sites and the gamma distribution parameter were estimated. The starting tree was determined by BioNJ analysis of the datasets. Robustness of the tree was assessed by 1000 bootstrap replicates.

The net genetic distance among the main *Pneumocystis* lineages recovered in the phylogenetic trees was computed for the mtLSU rRNA dataset in Mega 4.1 (Tamura *et al.* 2007) under Jukes-Cantor model with complete deletion of gaps or with pairwise-deletion of gaps.

Co-phylogenetic analyses between Pneumocystis and Southeast Asian Rattini

In order to test the congruence between phylogenies of *Pneumocystis* and their rodent hosts, we first used two global-fit

Table 1. *Pneumocystis* reference and outgroup sequences used in phylogenetic analyses and their GenBank accession number

Host species	*Pneumocystis* species	mtLSU rRNA GenBank accession number	mtSSU rRNA GenBank accession number
Muridae (Rodentia, Myomorpha, Muroidea):			
Mus musculus	*Pneumocystis murina*	JX499144	JX499144
Rattus norvegicus (laboratory rats)	*Pneumocystis carinii*	JX499145 U20169 U20170 U20171 U20172	JX499145 / / / /
Rattus norvegicus (laboratory rats)	*Pneumocystis wakefieldiae*	U20173	See in (Hunter and Wakefield 1996)
Rattus norvegicus (wild Danish rats)	*Pneumocystis* sp. (*Pc* f. sp. *rattus-quarti*)	AF308809	/
Rattus norvegicus (wild Danish rats)	*Pneumocystis* sp. (*Pc* f. sp. *rattus-tertii*)	AF308808	/
Rattus norvegicus (wild Danish rats)	*Pneumocystis* sp. (*Pc* f. sp. *rattus-secundi*)	AF308807	/
Apodemus sylvaticus	*Pneumocystis* sp.	KF384955 KF384971 KF384990	KF384913 KF384929 KF384934
Cricetidae (Rodentia, Myomorpha, Muroidea):			
Microtus agrestis	*Pneumocystis* sp.	AY279099	/
Outgroup: Ctenodactylidae (Rodentia, Hystricomorpha):			
Ctenodactylus gundi	*Pneumocystis* sp.	KX257170	KX257171

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methods: ParaFit (Legendre *et al.* 2002) and PACo (Procrustean Approach to Cophylogeny) (Balbuena *et al.* 2013) implemented in R 3.2.2 (R Core Team, 2013) using the packages ape and vegan. These methods assess the degree of congruence between host and parasite trees using matrices of patristic distances and test its significance against a random distribution (999 permutations for Parafit and 100 000 for PACo). ParaFit also identifies the host–parasite associations significantly contributing to the co-phylogenetic structure.

We then used the heuristic approach with a genetic algorithm implemented in Jane 4.0 (Conow *et al.* 2010). This event-based method assigns different costs to five evolutionary events (i.e. co-speciation, duplication, host switch, loss and failure to diverge) used to map the parasite phylogeny to the host one and finds a mapping that minimizes the total cost. The least cost solution is considered as the best solution and is then statistically tested by comparison with the costs obtained after randomization of the parasite tree and tip mappings. The two phylogenies are considered as significantly congruent if the cost of the best solution is lower than the costs expected by chance. We tested four different sets of costs for each type of evolutionary event: (1) co-speciation = 0, duplication = 1, host switch = 2, loss = 1, failure to diverge = 1 (default cost scheme of Jane); (2) co-speciation = 0, duplication = 1, host switch = 1, loss = 1, failure to diverge = 1; (3) co-speciation = 0, duplication = 1, host switch = 2, loss = 2, failure to diverge = 1; and (4) co-speciation = −1, duplication = 0, host switch = 0, loss = 0, failure to diverge = 0, this cost scheme maximizes the number of inferred co-speciation events. The analyses were performed with 500 generations and population size of 300. The cost of the best solution was compared with the costs found in 1000 randomizations of both tip mapping and parasite tree topologies.

These co-phylogenetic analyses were limited to the Rattini tribe, the most diverse Murinae tribe in Southeast Asia. This tribe includes, among others, the genera *Maxomys*, *Leopoldamys*, *Niviventer*, *Berylmys*, *Bandicota* and *Rattus* and a robust phylogeny is already available and well accepted (Pages *et al.* 2010; Fabre *et al.* 2013). The *Pneumocystis* tree was compared to the phylogeny of the Rattini tribe obtained by Fabre *et al.* (2013) on the basis of one mitochondrial (cytb) and two nuclear (*IRBP* and *GHR*) genes. The *Pneumocystis* input tree was therefore pruned to include only the six main lineages identified in Southeast Asian Rattini (lineages 5, 7, 9, 10, 11, 12).

Results

Pneumocystis *genetic diversity in murid rodents in Southeast Asia*

Pneumocystis DNA was detected in 215 out of 445 (48.3%) wild murid rodents in Southeast Asia. Of these 215 positive individuals, 24% were positive after PCR mtLSU1, 29% after PCR mtSSU1, 41% after PCR mtLSU2 and 23% after PCR mtSSU2. We observed an important variation in the frequency of *Pneumocystis* infection among Muridae species, it varied from 90·9% in *R. norvegicus* to only 14·3% in *Leopoldamys herberti* (Table 2).

After sequencing, we obtained 130 valid mtLSU rRNA sequences and 77 valid mtSSU rRNA sequences. In total, valid sequences were obtained at both mtLSU rRNA and mtSSU rRNA for 77 specimens. A total of 69 distinct sequence types were identified among our dataset for mtLSU rRNA and 43 for mtSSU rRNA (Tables 3 and 4). The concatenated dataset (mtLSU rRNA + mtSSU rRNA) included 97 distinct sequence types (92 when the hypervariable regions of mtSSU rRNA were excluded) (Supplementary Tables S1 and S2). Most sequence types of both loci are specific to one Muridae species with the exception of HLSU17, HLSU20, HLSU62, HLSU64, HLSU68, HSSU32, HSSU33 and HSSU43 that are shared by several *Rattus* species, HLSU27 and HLSU29 that are shared by two *Berylmys* species and HSSU6 that is shared by both *Maxomys surifer* and *L. herberti* (Tables 3 and 4). These shared sequence types have been isolated at different time periods in various localities (Supplementary Tables S3 and S4).

Table 2. Numbers of tested and *Pneumocystis* positive murid rodent samples

Species	Number of samples tested	Number of *Pneumocystis* positive samples (%)
Cannomys badius	1	1
Rattus norvegicus	33	30 (90·9%)
Bandicota indica	26	21 (80·8%)
Rattus nitidus	15	11 (73·3%)
Mus caroli	11	8 (72·7%)
Berylmys bowersi	7	5 (71·4%)
Rattus exulans	45	29 (64·4%)
Berylmys berdmorei	24	14 (58·3%)
Niviventer fulvescens	9	5 (55·5%)
Rattus sakeratensis	24	12 (50%)
Bandicota savilei	21	10 (47·6%)
Rattus tanezumi R2	50	21 (42%)
Rattus argentiventer	15	6 (40%)
Mus cervicolor	22	8 (36·4%)
Rattus tanezumi R3	84	22 (26·2%)
Rattus andamanensis	4	1 (25%)
Mus cooki	22	5 (22·7%)
Maxomys surifer	25	5 (20%)
Leopoldamys herberti	7	1 (14·3%)

Pneumocystis *co-infection within the genus* Rattus

Positive PCR amplification using the *Pneumocystis* species-specific primers was obtained in only 56 out of the 91 infected *Rattus* specimens that were tested. *Pneumocystis wakefieldiae* was found alone in 43 specimens (76·8%), *P. carinii* in seven specimens (12·5%) while six individuals (10·7%) were positive for both *P. wakefieldiae* and *P. carinii*.

Phylogenetic reconstructions

The two mitochondrial loci used in this study were first used separately to reconstruct phylogenetic trees. They yielded poorly-resolved phylogenies but they mostly recovered similar lineages with the exception of taxa not represented in the mtSSU rRNA matrix (data not shown). The mtLSU rRNA and mtSSU rRNA genes were then concatenated in a single matrix.

The ML tree topology of the concatenated dataset, including the hypervariable regions of mtSSU rRNA retrieved 12 main *Pneumocystis* lineages (Fig. 2). A weakly-supported group (node B, BS = 59, BP = 0·86) including two *Pneumocystis* sequences derived from Danish wild *R. norvegicus* and referred to as *Pc* f. sp. *rattus-tertii* (lineage 1) and *Pc* f. sp. *rattus-quarti* (lineage 2) in Palmer *et al.* (2000) and *Pneumocystis* from *Cannomys badius* (lineage 3) was the first to diverge within the ingroup (Fig. 2). Then a

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Table 3. Host range, phylogenetic lineage (numbers refer to Fig. 2) and GenBank accession number of mtLSU rRNA sequence types isolated in Southeast Asian murid rodents

	Cannomys badius	*Mus caroli*	*Mus cervicolor*	*Mus cooki*	*Maxomys surifer*	*Niviventer fulvescens*	*Leopoldamys herberti*	*Berylmys berdmorei*	*Berylmys bowersi*	*Bandicota indica*	*Bandicota savilei*	*Rattus nitidus*	*Rattus norvegicus*	*Rattus exulans*	*Rattus andamanensis*	*Rattus argentiventer*	*Rattus sakeratensis*	*Rattus tanezumi R3*	*Rattus tanezumi R2*	Phylogenetic lineage (species)	GenBank Accession number
n LSU	1	7	2	5	3	2	1	11	4	18	6	10	17	11	1	3	11	9	8		
HLSU1	X																			3	KX257058
HLSU2												X								5	KX257059
HLSU3												X								5	KX257060
HLSU4		X																		8 (*P. murina*)	KX257061
HLSU5			X																	8 (*P. murina*)	KX257062
HLSU6				X																8 (*P. murina*)	KX257063
HLSU7				X																8 (*P. murina*)	KX257064
HLSU8							X													7	KX257065
HLSU9					X															7	KX257066
HLSU10					X															7	KX257067
HLSU11						X														7	KX257068
HLSU12						X														7	KX257069
HLSU13														X						9 (*P. carinii*)	KX257070
HLSU14														X						9 (*P. carinii*)	KX257071
HLSU15														X						9 (*P. carinii*)	KX257072
HLSU16														X						9 (*P. carinii*)	KX257073
HLSU17														X		X				9 (*P. carinii*)	KX257074
HLSU18													X							9 (*P. carinii*)	KX257075
HLSU19													X							9 (*P. carinii*)	KX257076
HLSU20													X	X		X				9 (*P. carinii*)	KX257077
HLSU21																		X		9 (*P. carinii*)	KX257078
HLSU22																		X		9 (*P. carinii*)	KX257079
HLSU23																		X		9 (*P. carinii*)	KX257080
HLSU24																		X		9 (*P. carinii*)	KX257081
HLSU25																	X			9 (*P. carinii*)	KX257082
HLSU26								X												10	KX257083
HLSU27								X	X											10	KX257084

(*Continued*)

Table 3. (*Continued.*)

	Cannomys badius	*Mus caroli*	*Mus cervicolor*	*Mus cooki*	*Maxomys surifer*	*Niviventer fulvescens*	*Leopoldamys herberti*	*Berylmys berdmorei*	*Berylmys bowersi*	*Bandicota indica*	*Bandicota savilei*	*Rattus nitidus*	*Rattus norvegicus*	*Rattus exulans*	*Rattus andamanensis*	*Rattus argentiventer*	*Rattus sakeratensis*	*Rattus tanezumi R3*	*Rattus tanezumi R2*	Phylogenetic lineage (species)	GenBank Accession number
HLSU28									X											10	KX257085
HLSU29								X	X											10	KX257086
HLSU30									X											10	KX257087
HLSU31										X										11	KX257088
HLSU32										X										11	KX257089
HLSU33										X										11	KX257090
HLSU34										X										11	KX257091
HLSU35										X										11	KX257092
HLSU36										X										11	KX257093
HLSU37										X										11	KX257094
HLSU38										X										11	KX257095
HLSU39										X										11	KX257096
HLSU40										X										11	KX257097
HLSU41										X										11	KX257098
HLSU42											X									11	KX257099
HLSU43											X									11	KX257100
HLSU44														X						12 (*P. wakefieldiae*)	KX257101
HLSU45														X						12 (*P. wakefieldiae*)	KX257102
HLSU46															X					12 (*P. wakefieldiae*)	KX257103
HLSU47												X								12 (*P. wakefieldiae*)	KX257104
HLSU48												X								12 (*P. wakefieldiae*)	KX257105
HLSU49												X								12 (*P. wakefieldiae*)	KX257106
HLSU50												X								12 (*P. wakefieldiae*)	KX257107
HLSU51													X							12 (*P. wakefieldiae*)	KX257108
HLSU52													X							12 (*P. wakefieldiae*)	KX257109
HLSU53													X							12 (*P. wakefieldiae*)	KX257110
HLSU54													X							12 (*P. wakefieldiae*)	KX257111
HLSU55																		X		12 (*P. wakefieldiae*)	KX257112

(*Continued*)

Table 3. (*Continued.*)

	Cannomys badius	*Mus caroli*	*Mus cervicolor*	*Mus cooki*	*Maxomys surifer*	*Niviventer fulvescens*	*Leopoldamys herberti*	*Berylmys berdmorei*	*Berylmys bowersi*	*Bandicota indica*	*Bandicota savilei*	*Rattus nitidus*	*Rattus norvegicus*	*Rattus exulans*	*Rattus andamanensis*	*Rattus argentiventer*	*Rattus sakeratensis*	*Rattus tanezumi R3*	*Rattus tanezumi R2*	Phylogenetic lineage (species)	GenBank Accession number
HLSU56																		X		12 (*P. wakefieldiae*)	KX257113
HLSU57																	X			12 (*P. wakefieldiae*)	KX257114
HLSU58																	X			12 (*P. wakefieldiae*)	KX257115
HLSU59																	X			12 (*P. wakefieldiae*)	KX257116
HLSU60																	X			12 (*P. wakefieldiae*)	KX257117
HLSU61																	X			12 (*P. wakefieldiae*)	KX257118
HLSU62												X					X	X		12 (*P. wakefieldiae*)	KX257119
HLSU63																			X	12 (*P. wakefieldiae*)	KX257120
HLSU64																		X	X	12 (*P. wakefieldiae*)	KX257121
HLSU65																			X	12 (*P. wakefieldiae*)	KX257122
HLSU66																			X	12 (*P. wakefieldiae*)	KX257123
HLSU67																			X	12 (*P. wakefieldiae*)	KX257124
HLSU68												X					X		X	12 (*P. wakefieldiae*)	KX257125
HLSU69													X							12 (*P. wakefieldiae*)	KX257126

Table 4. Host range, phylogenetic lineage (numbers refer to Fig. 2) and GenBank accession number of mtSSU rRNA sequence types isolated in Southeast Asian murid rodents

	Cannomys badius	*Mus caroli*	*Mus cervicolor*	*Mus cooki*	*Maxomys surifer*	*Niviventer fulvescens*	*Leopoldamys herberti*	*Berylmys berdmorei*	*Berylmys bowersi*	*Bandicota indica*	*Bandicota savilei*	*Rattus nitidus*	*Rattus norvegicus*	*Rattus exulans*	*Rattus andamanensis*	*Rattus argentiventer*	*Rattus sakeratensis*	*Rattus tanezumi R3*	*Rattus tanezumi R2*	Phylogenetic lineage (species)	GenBank Accession number
n SSU	1	6	2	5	2	1	1	7	2	6	2	4	14	3	0	2	8	5	6		
HSSU1	X																			3	KX257127
HSSU2		X																		8 (*P. murina*)	KX257128
HSSU3		X																		8 (*P. murina*)	KX257129
HSSU4			X																	8 (*P. murina*)	KX257130
HSSU5				X																8 (*P. murina*)	KX257131
HSSU6					X		X													7	KX257132
HSSU7						X														7	KX257133
HSSU8																X				9 (*P. carinii*)	KX257134
HSSU9													X							9 (*P. carinii*)	KX257135
HSSU10													X							9 (*P. carinii*)	KX257136
HSSU11													X							9 (*P. carinii*)	KX257137
HSSU12													X							9 (*P. carinii*)	KX257138
HSSU13													X							9 (*P. carinii*)	KX257139
HSSU14																X				9 (*P. carinii*)	KX257140
HSSU15													X							9 (*P. carinii*)	KX257141
HSSU16																		X		9 (*P. carinii*)	KX257142
HSSU17								X												10	KX257143
HSSU18								X												10	KX257144
HSSU19								X												10	KX257145
HSSU20								X												10	KX257146
HSSU21								X												10	KX257147
HSSU22									X											10	KX257148
HSSU23									X											10	KX257149
HSSU24										X										11	KX257150
HSSU25										X										11	KX257151
HSSU26										X										11	KX257152
HSSU27										X										11	KX257153

(*Continued*)

Table 4. (*Continued.*)

	Cannomys badius	*Mus caroli*	*Mus cervicolor*	*Mus cooki*	*Maxomys surifer*	*Niviventer fulvescens*	*Leopoldamys herberti*	*Berylmys berdmorei*	*Berylmys bowersi*	*Bandicota indica*	*Bandicota savilei*	*Rattus nitidus*	*Rattus norvegicus*	*Rattus exulans*	*Rattus andamanensis*	*Rattus argentiventer*	*Rattus sakeratensis*	*Rattus tanezumi R3*	*Rattus tanezumi R2*	Phylogenetic lineage (species)	GenBank Accession number
HSSU28											X									11	KX257154
HSSU29										X										11	KX257155
HSSU30											X									11	KX257156
HSSU31													X							12 (*P. wakefieldiae*)	KX257157
HSSU32												X							X	12 (*P. wakefieldiae*)	KX257158
HSSU33													X						X	12 (*P. wakefieldiae*)	KX257159
HSSU34												X								12 (*P. wakefieldiae*)	KX257160
HSSU35												X								12 (*P. wakefieldiae*)	KX257161
HSSU36																	X			12 (*P. wakefieldiae*)	KX257162
HSSU37														X						12 (*P. wakefieldiae*)	KX257163
HSSU38														X						12 (*P. wakefieldiae*)	KX257164
HSSU39																	X			12 (*P. wakefieldiae*)	KX257165
HSSU40																	X			12 (*P. wakefieldiae*)	KX257166
HSSU41																			X	12 (*P. wakefieldiae*)	KX257167
HSSU42																		X		12 (*P. wakefieldiae*)	KX257168
HSSU43																		X	X	12 (*P. wakefieldiae*)	KX257169

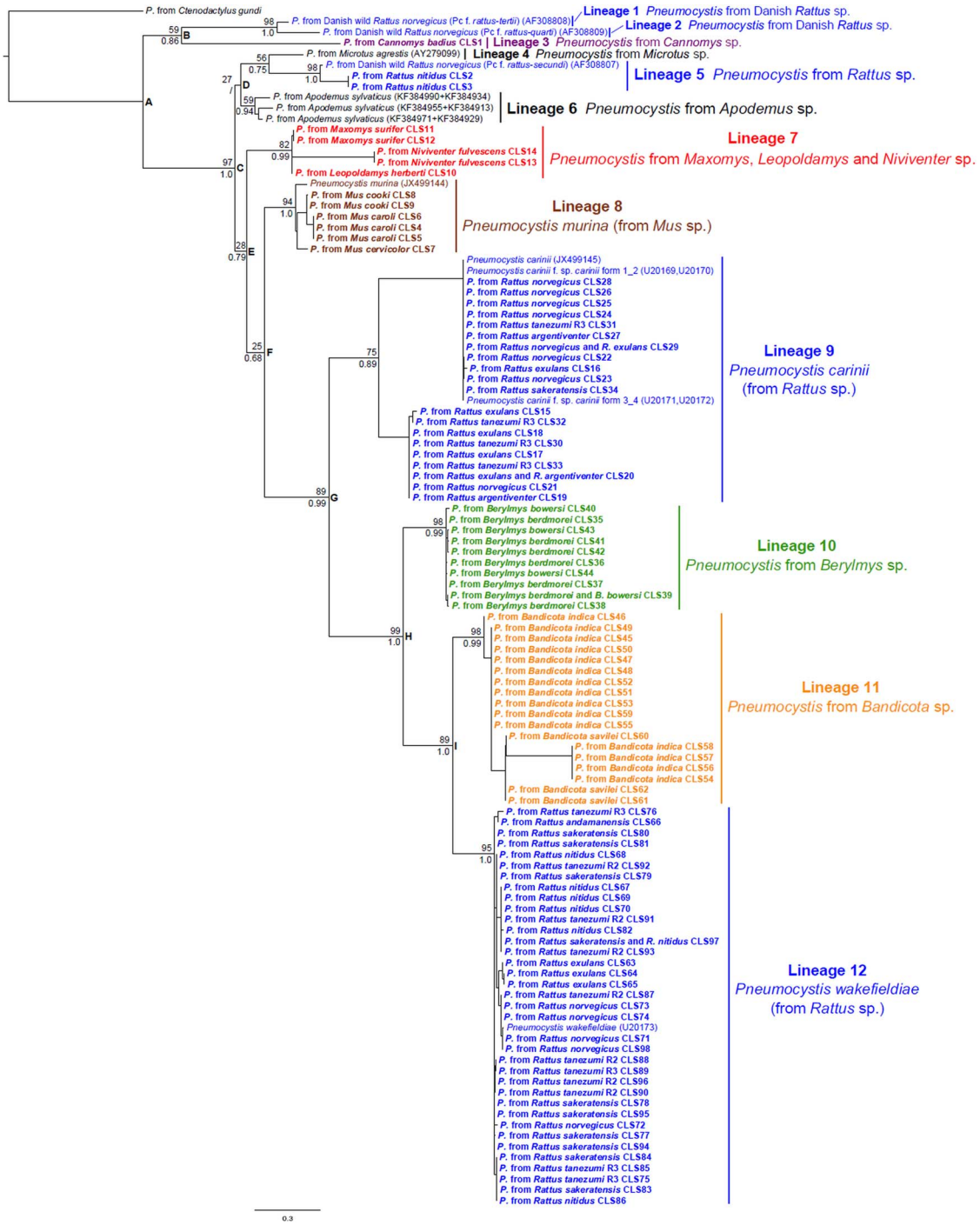

Fig. 2. Maximum Likelihood (ML) tree (GTR + G) depicting phylogenetic relationships among *Pneumocystis* from murid rodents inferred from concatenated mtLSU rRNA and mtSSU rRNA sequences including mtSSU rRNA hypervariable regions. Bootstrap support (%, 1000 replicates) and posterior probabilities of nodes are indicated above and below the branches, respectively. Node supports from within lineages were removed for clarity of presentation. Sequences from Southeast Asian murid rodents are in bold, other sequences are from GenBank (accession numbers in brackets). *Pneumocystis* lineages from murid rodent genera distributed in Southeast Asia are coloured according to their host genus.

well-supported group (node C, BS = 97, BP = 1·0) included *Pneumocystis* sequence types derived from Murinae rodents with the exception of one sequence from *Microtus agrestis* (Rodentia, Cricetideae). Several weakly-supported nodes (nodes D, E and F) leading to five well-supported lineages were also identified, corresponding to *Pneumocystis* from *Microtus agrestis* (lineage 4), *Pneumocystis* from Danish wild *R. norvegicus* (*Pc* f. sp. *rattus-secundi*) and two Southeast Asian *R. nitidus* (lineage 5, BS = 98, BP = 1·0), *Pneumocystis* from *Apodemus sylvaticus* (lineage 6, BS = 59, BP = 0·94), *Pneumocystis* from *Maxomys*, *Leopoldamys* and *Niviventer* (lineage 7, BS = 82, BP = 0·99), and *Pneumocystis* from *Mus* (*P. murina*, lineage 8, BS = 94, BP = 1·0). Then a well-supported node (node G, BS = 89, BP = 0·99) included a lineage corresponding to *P. carinii* from *Rattus* (lineage 9, BS = 75, BP = 0·89) and a well-supported group (node H, BS = 99, BP = 1·0) encompassing *Pneumocystis* from *Berylmys* (lineage 10, BS = 98, BP = 0·99), *Bandicota* (lineage 11, BS = 98, BP = 0·99) and *Rattus* (*P. wakefieldiae*, lineage 12, BS = 95, BP = 1·0). The ML and BI tree topologies were congruent for all the main internal nodes except one (node D). Branching topologies within each of the 12 main lineages are poorly-resolved and only two monophyletic sub-lineages specific to one host species (i.e. a sublineage within lineage

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7 including *Pneumocystis* from *Niviventer fulvescens* and a sublineage within lineage 8 including *Pneumocystis* from *Mus caroli*) were retrieved in both ML and BI trees.

The tree topology of the concatenated dataset excluding the hypervariable regions of mtSSU rRNA recovered the same 12 main lineages but differed in some internal nodes (nodes B, D, F, and I) that are weakly-supported in both trees (Supplementary Fig. S1).

Genetic distance among the main Pneumocystis *lineages*

The percentage of net genetic distance is 11·6% (15·7% after complete deletion of gaps) between *P. carinii* and *P. wakefieldiae*, 10·9% (16.5%) between *P. carinii* and *P. murina* and 12·3% (18·7%) between *P. wakefieldiae* and *P. murina* (Table 5). Similar or slightly lower levels of genetic distance were observed between lineages 4, 5, 6, 7 and these *Pneumocystis* species while the genetic distances among *Pc* f. sp. *rattus-tertii* (lineage 1), *Pc* f. sp. *rattus-quarti* (lineage 2), *Pneumocystis* from *C. badius* (lineage 3) and all other lineages were much higher (Table 5). Low levels of genetic distance were observed among lineage 10 (*Pneumocystis* from *Berylmys*), lineage 11 (*Pneumocystis* from *Bandicota*) and *P. wakefieldiae* (Table 5).

Phylogenetic congruence between Pneumocystis *and Rattini species in Southeast Asia*

Both ParaFit (ParaFitGlobal = 11·73, P value = 0·009) and PACo (m^2 = 8·15, P value = 0·004) provided evidence for significant global congruence between the topologies of *Pneumocystis* and Southeast Asian Rattini trees. However, only six of the 19 host–parasite associations were significant according to ParaFit1 values ($P \leqslant 0{\cdot}05$) (Fig. 3A).

Jane inferred three co-speciation events, 0 duplication, two host switches, 10 losses and 13 failures to diverge (=solution 1) between *Pneumocystis* and Rattini phylogenies under the four tested cost schemes (Fig. 3B). However, an alternative solution of similar cost (two co-speciation events, 0 duplication, three host switches, nine losses and 13 failures to diverge = solution 2) was also suggested under cost scheme 3 (Fig. 3B). For both solutions and whichever the cost scheme applied, the number of co-speciation events inferred by Jane was always significantly greater than expected by chance.

According to the solution 1, the first co-speciation event occurred between lineage 5 and lineage 7, followed by a host switch occurring from lineage 7, which led to a co-speciation event between lineage 10 and *P. carinii*. A second host switch occurred from lineage 10 and led to a co-speciation event between lineage 11 and *P. wakefieldiae*. The solution 2 inferred the first co-speciation event between lineage 5 and lineage 7 followed by host switch from lineage 7 leading to co-speciation between lineage 11 and *P. carinii*. Then two host switches occurred from lineage 11 to lineage 10 and from lineage 11 to *P. wakefieldiae*. All loss events occurred within the genus *Rattus* for both solutions (Fig. 3B).

Discussion

Species boundaries in Pneumocystis *infecting murid rodents*

Due to the complexity of the *in vitro* culture of *Pneumocystis* organisms, the Phylogenetic Species Concept (PSC) has been widely used to recognize distinct species of this fungal parasite (Stringer *et al.* 2001; Keely *et al.* 2004). A phylogenetic species is an independent evolutionary lineage having a unique combination of DNA sequences (Taylor *et al.* 2000). The phylogenetic concordance of multiple unlinked genes indicates a lack of genetic exchange and the evolutionary distinctiveness of these lineages

Table 5. Net genetic distance (percentages) among the main *Pneumocystis* lineages recovered in the phylogenetic tree (Fig. 2) computed under Jukes–Cantor model with complete deletion of gaps (above the grey cells) or with pairwise-deletion of gaps (below the grey cells)

	1	2	3	4	5	6	7	8	9	10	11	12
Lineage 1 – *Pc* f. sp. *rattus-tertii* (from Danish wild *R. norvegicus*)		3	12·8	20·1	28·2	13·7	12	28·2	28·2	20·1	20·1	19·5
Lineage 2 – *Pc* f. sp. *rattus-quarti* (from Danish wild *R. norvegicus*)	7·5		9·4	16·4	28·2	10	15·5	24·1	24·1	16·4	16·4	15·8
Lineage 3 – *Pneumocystis* from *C. badius*	25·5	19·7		20·1	24·1	13·7	23·2	28·2	24·1	20·1	20·1	19·4
Lineage 4 – *Pneumocystis* from *M. agrestis*	22·5	24·8	24·6		9·4	4.1	9·5	12·8	12·8	16·4	16·4	15·8
Lineage 5 – *Pc* f. sp. *rattus-secundi* (from Danish wild *R. norvegicus*) and *Pneumocystis* from *R. nitidus*	26·8	31·9	27·7	9·6		14·7	16·5	16·4	20·1	20·1	20·1	20·1
Lineage 6 – *Pneumocystis* from *A. sylvaticus*	23·3	23·3	26·2	8.7	11·1		7·5	11	11	14·7	14·7	14·1
Lineage 7 – *Pneumocystis* from *M. surifer*, *L. herberti*, *N. fulvescens*	23	24·1	25·5	7·5	11·1	8·9		16·5	12·8	9·4	9.4	9.5
Lineage 8 – *P. murina*	23·4	23·6	23·7	10·2	10	8	8·1		**16·5**	19·2	19·2	**18·7**
Lineage 9 – *P. carinii*	28·1	28	28·2	13·5	13	9	10·1	**10·9**		16·4	16·4	**15·7**
Lineage 10 – *Pneumocystis* from *Berylmys* sp.	25·4	24·1	27·4	11·3	11·2	12·1	10·9	10·4	10·4		0	0
Lineage 11 – *Pneumocystis* from *Bandicota* sp.	27·1	26·4	30·2	12·8	14	13·4	11·3	11·5	11·9	4·9		0
Lineage 12 – *P. wakefieldiae*	24·1	23·6	27	11	14·1	10·7	10·9	**12·3**	**11·6**	5·7	5·4	

Distances among recognized *Pneumocystis* species (*P. murina*, *P. carinii* and *P. wakefieldiae*) are in bold.

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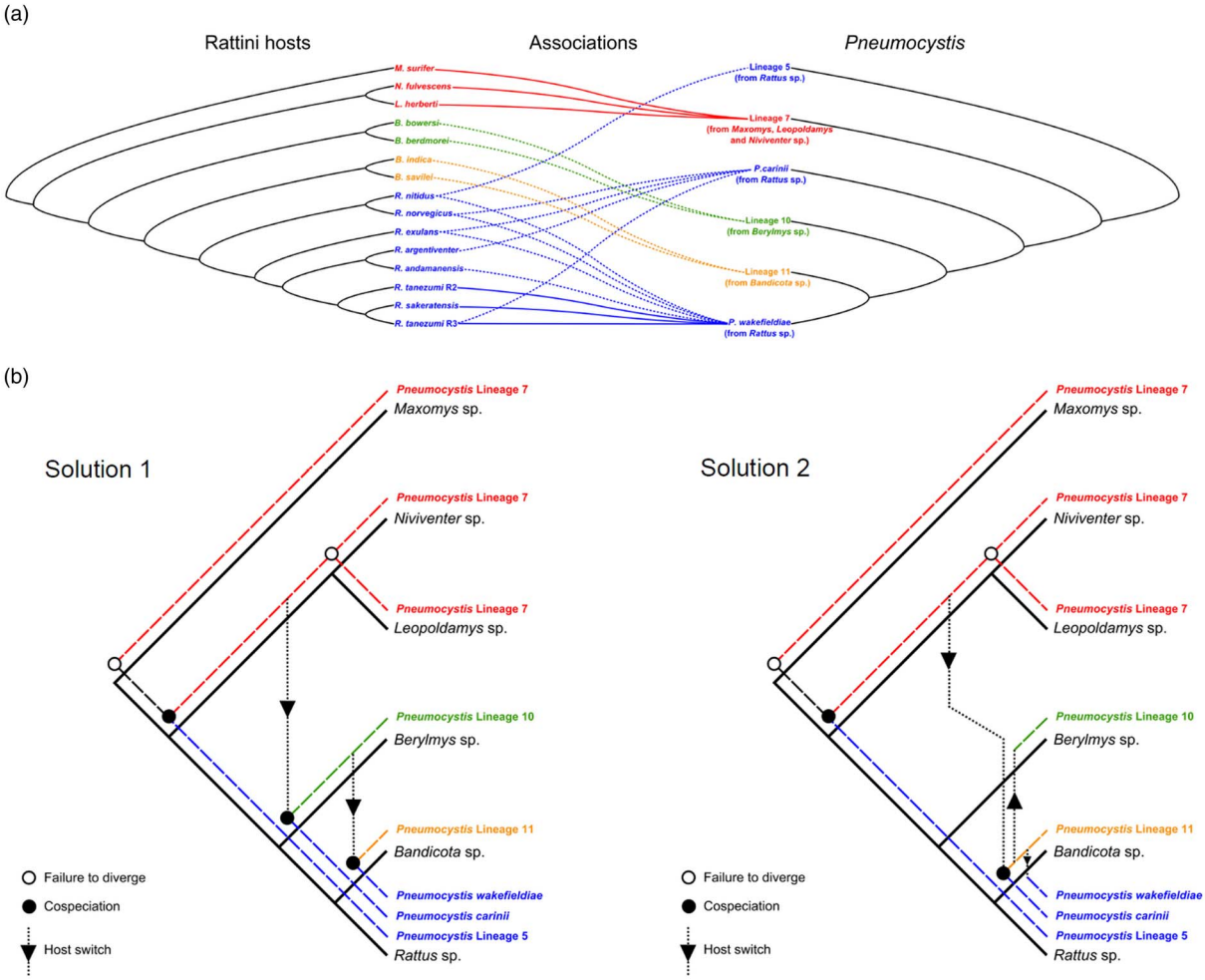

Fig. 3. Phylogenetic congruence between *Pneumocystis* and Rattini phylogenies. (A) Tanglegram depicting the co-phylogenetic pattern among *Pneumocystis* and Rattini. Lines connecting taxa indicate Rattini-*Pneumocystis* associations. Plain lines correspond to significant associations as indicated by ParaFit ($P \leqslant 0{\cdot}05$, 999 permutations) while dotted lines correspond to non-significant associations. (B) Reconstructions of the two optimal solutions recovered from Jane analysis under different cost schemes. Plain black lines and dashed coloured lines represent the host and *Pneumocystis* trees, respectively. The Rattini tree is limited to the genus level for clarity of presentation.

and therefore allows the recognition of distinct fungal species (Giraud *et al.* 2008). Standard loci should be used for this purpose and Stringer *et al.* (2001) suggested that one of these loci should be mtLSU rRNA. The level of genetic distance between lineages is also useful to delimit species boundaries among *Pneumocystis* (Keely *et al.* 2004; Dei-Cas *et al.* 2006). Stringer *et al.* (2001) indicated that 'if the genetic distance at the mtLSU rRNA locus between two *Pneumocystis* organisms equals or exceeds that seen between *P. carinii* and *P. carinii* f. sp. *ratti* (the older name of *P. wakefieldiae*), the two test organisms are recognizable as different species, even if they are found in the same host species.… A lower divergence calls for more analysis'.

If we apply this phylogenetic criterion to our dataset, several new species may be recognized among the *Pneumocystis* infecting murid rodents. We estimated the level of net genetic distance between *P. carinii* and *P. wakefieldiae*, between *P. carinii* and *P. murina* and between *P. wakefieldiae* and *P. murina* at around 11·6, 10·9 and 12·3%, respectively (pairwise gap deletion) (Table 5). The roughly similar or higher levels of genetic distance among lineages 1 (*Rattus*), 2 (*Rattus*), 3 (*Cannomys*), 4 (*Microtus*), 5 (*Rattus*), 6 (*Apodemus*), 7 (*Maxomys*, *Leopoldamys*, *Niviventer*), *P. murina* (*Mus*), *P. carinii* (*Rattus*) and *P. wakefieldiae* (*Rattus*) as well as the phylogenetic independence of all these lineages at both mtLSU rRNA and mtSSU rRNA (for the lineages for which mtSSU rRNA sequences were available) confirm the absence of gene flow among them and are indicative of phylogenetic species recognition. However, due to the particular and distinct evolutionary history of the mitochondrial genome, mitochondrial markers alone are not sufficient for the delineation of new *Pneumocystis* species. The use of nuclear genes is needed to confirm the phylogenetic concordance of mitochondrial and nuclear genes and validate the phylogenetic species status of these seven *Pneumocystis* lineages. Evidence of morphological or biological differences (e.g. ultrastructure, growth rate, etc.) among these lineages would also reinforce their taxonomic distinctiveness and allow us to describe new *Pneumocystis* species using the Morphological Species Concept (MSC) or the Biological Species Concept (BSC). However morphological and biological studies of *Pneumocystis* organisms require a large amount of fungal material, which cannot be obtained in wild rodents. Natural cases of severe *Pneumocystis* infection in wild rodents are scarce and *Pneumocystis* infections in wild rodents are mild compared with those of immunosuppressed laboratory animals that develop *Pneumocystis* pneumonia (Chabé *et al.* 2010). Inducing immunosuppression in wild rodents might be the answer, but this seems difficult to perform for wild specimens of animal species that cannot be routinely kept in laboratory facilities. The lower genetic distances among lineages 10, 11 and *P. wakefieldiae* preclude the recognition of *Pneumocystis* infecting *Berylmys* and *Bandicota* rodents as distinct

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phylogenetic species. The analysis of additional genes is needed to confirm their taxonomic status.

Pneumocystis *host specificity and diversity in Southeast Asian murid rodents*

Narrow specificity at the host-species level has usually been reported for *Pneumocystis* parasites based on genetic and phenotypic data and cross-infection experiments (Aliouat *et al.* 1993, 1994; Gigliotti *et al.* 1993; Demanche *et al.* 2001; Hugot *et al.* 2003; Akbar *et al.* 2012). However, the *Pneumocystis* monoxenism may not systematically occur at the host intra-generic level and several exceptions to this host-species specificity have been described in the literature when a single *Pneumocystis* species/lineage infected at least two closely related host species in primates (*Macaca mulatta* and *M. fascicularis*) (Guillot *et al.* 2004) and rodents (*Apodemus flavicollis* and *A. sylvaticus*) (Danesi *et al.* 2016; Demanche *et al.* 2017). Our study demonstrates that murid rodent *Pneumocystis* host specificity is mostly limited to the generic level rather than the species level as several mtLSU rRNA and mtSSU rRNA sequence types are shared among several host species belonging to the same genus (*Rattus*, *Berylmys*) or even among two well-differentiated Muridae genera (*Maxomys* and *Leopoldamys*) (Tables 3 and 4). Most of the *Pneumocystis* lineages/species retrieved in our phylogenetic tree infect several rodent species (lineage 5, *P. murina*, *P. carinii*, lineage 10, lineage 11 and *P. wakefieldiae*) or genera (lineage 7). The considerable temporal and geographical range of our sampling demonstrate that this *Pneumocystis* sharing across rodent species/genera is a long-term and large-scale process across the Indochinese region and is not limited only to a particular geographical location or to rodents sharing the same environment.

These results suggest that the host species (and genus in some cases) is not a barrier to *Pneumocystis* transmission in wild rodent populations, indicating a weaker host specificity of *Pneumocystis* species infecting Southeast Asian murid rodents than that of *Pneumocystis* infecting primates and bats (Demanche *et al.* 2001; Guillot *et al.* 2001; Akbar *et al.* 2012). We assume that the weaker host specificity of this *Pneumocystis* is possible because of physiological, cellular and/or immunological similarities among these closely related rodent species that diverged quite recently.

Rattus species are the only ones among the Southeast Asian Muridae rodents that we tested to be infected by several *Pneumocystis* species. Palmer *et al.* (2000) described five highly divergent *Pneumocystis* species/lineages infecting wild Danish *R. norvegicus*, sometimes in co-infection. We identified three of them [*P. carinii*, *P. wakefieldiae* and *Pc* f. sp. *rattus-secundi* (lineage 5)] in Southeast Asian *Rattus* specimens, each *Rattus* species hosting a maximum of two *Pneumocystis* species/lineages. According to the results of our species-specific PCR, *P. carinii* and *P. wakefieldiae* were mostly found alone but some instances of co-infection were also detected (10·7%). As the sensitivities of these species-specific PCR are similar (Chabé *et al.* 2010), these results revealed the higher prevalence of *P. wakefieldiae* (76.8%) in wild *Rattus* populations. As illustrated previously by Chabé *et al.* (2010), these findings are in discrepancy with studies performed on laboratory rats (*R. norvegicus*) where *P. wakefieldiae* was almost always found in co-infection with *P. carinii* and rarely alone (Cushion, 1998; Icenhour *et al.* 2006). In our study, *P. wakefieldiae* was found to infect all *Rattus* species except *R. argentiventer*. However, this could be due to the low number of *R. argentiventer* specimens tested in our study (15), examining a larger number of *R. argentiventer* is required before confirming the absence of *P. wakefieldiae* in this species. Moreover Sanger sequencing is not optimal for identifying *Pneumocystis* mixed infections and minority alleles might not have been detected using this method. The use of other methods able to detect a mixture of distinct sequences, such as next-generation sequencing, is needed to accurately investigate the *Pneumocystis* co-infection pattern among murid rodents.

With 66 described species, *Rattus* is among the most speciose mammal genera (Musser and Carleton, 2005). This genus likely originated in Southeast Asia, the centre of *Rattus* diversity, from where they dispersed to continental Asia and the Sahul region (Rowe *et al.* 2011). The origin of the genus is relatively recent, estimated at the Plio-Pleistocene boundary (around 2–3 Mya) and its diversification rate is more than 3 times higher than for other Murinae rodents (Rowe *et al.* 2011). This exceptionally high species richness of the genus *Rattus* is one of the hypotheses that may explain the higher diversity of *Pneumocystis* in *Rattus* compared with other Muridae genera. Several studies found a positive correlation between the taxonomic richness of hosts and that of their parasites, which may be explained by the role of both host availability and evolutionary co-diversification (Kamiya *et al.* 2014). The host social behavior has also been suggested as a variable that may explain parasite richness, with gregarious species living closely together having a higher parasite richness than solitary species (Desdevises *et al.* 2002). Several *Rattus* species are known to live communally with a strong hierarchical social system (Aplin *et al.* 2003) but the inter-specific interactions of *Rattus* species and the social behaviour of other Muridae genera remain poorly known, which currently prevents the further assessment of the relevance of this hypothesis.

Pneumocystis *and Southeast Asian Rattini rodent evolutionary history*

Our co-phylogenetic analyses revealed a complex evolutionary history among *Pneumocystis* and their murid rodent hosts. Even if a significant global signal of co-speciation has been detected, co-speciation alone is not sufficient to explain the observed co-phylogenetic pattern. These results conflict with the traditional view of a prolonged process of co-evolution and co-speciation of *Pneumocystis* and their hosts. The most striking findings of this study are that the most basal Rattini genera (*Maxomys*, *Leopoldamys*, *Niviventer*) are infected by the less diversified *Pneumocystis* lineage (lineage 7 which failed to diverge) while the evolutionarily young genus *Rattus* host several paraphyletic and highly divergent *Pneumocystis* species/lineages, including the most basal ones (lineages 1, 2 and 5). These findings contradict two of the principles traditionally proposed to explain host–parasite evolution, the Fahrenholz's rule: 'parasite phylogeny mirrors that of its host' (Brooks, 1985) and Szidat's rule: 'evolutionarily primitive (basal) hosts harbour evolutionarily primitive (basal) parasites' (Brooks, 1979). According to our Jane's analysis, several host switches are the most likely reason to explain this incongruence between *Pneumocystis* and Rattini phylogenies. These host switches that were inferred in the deep *Pneumocystis* phylogeny are macro-evolutionary processes and they did not involve contemporary rodent and *Pneumocystis* species but their ancestors. From an ecological point of view, a host switch should be considered as the colonization of the new host by parasites. Simulation and empirical studies have shown that parasite species with various levels of ecological specialization are able to colonize new hosts, even those that are highly specialized (Hoberg and Klassen, 2002; Janz and Nylin, 2008; Araujo *et al.* 2015). Opportunity and compatibility (ecological fitting) are major determinants of host switch success but evolutionary changes (i.e. acquisition of novel genetic information) are not necessarily required (Agosta and Klemens, 2008; Hoberg and Brooks, 2008; Araujo *et al.* 2015). *Pneumocystis* ancestors may have come into

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contact with new rodent hosts through changes in rodent geographic distributions and/or ecological structure during past periods of environmental changes. They may also have been able to colonize distantly related hosts through 'stepping-stone process', sequentially colonizing several more closely related hosts (Araujo *et al.* 2015). Studying *Pneumocystis* diversity in murid rodents in other regions of the world would allow to better understand the historical biogeography of these host–parasite associations at the global scale. The use of nuclear genes in the future would also help to confirm these evolutionary hypotheses.

Estimation of *Pneumocystis* speciation time could be a useful tool to better understand the evolutionary history of this fungal parasite and confirm that speciation of both host and parasite occurred simultaneously when co-speciation events are inferred. Indeed, testing that reciprocal speciation is temporally plausible is essential to confirm true co-speciation events rather than host switches followed by parasite speciation ('pseudo co-speciation') (Percy *et al.* 2004; De Vienne *et al.* 2007, 2013). However, estimating divergence times in *Pneumocystis* is particularly difficult due to the lack of *Pneumocystis* fossil records (Leung, 2015). Several attempts have been made in the literature using fungi nucleotide substitution rates. Keely *et al.* (2004) estimated that *P. carinii* and *P. murina* diverged 30–40 Mya while Cushion *et al.* (2004) estimated that *P. carinii* and *P. wakefieldiae* diverged 15–22 Mya. However, these dates seem implausible as they are much older than the divergence times estimated for their rodent hosts: the *Mus*/*Rattus* split is estimated to be around 12 Mya, while the first divergences within the genus *Rattus* occurred around 2–3 Mya (Rowe *et al.* 2011; Fabre *et al.* 2013; Kimura *et al.* 2015).

Conclusion

This study revealed a complex evolutionary history among *Pneumocystis* and murid rodents, far from the traditional simple picture of strict host specificity and ancient co-evolution of *Pneumocystis* and their mammalian hosts. According to our results, the specificity of *Pneumocystis* infecting murid rodents is weaker than previously thought and mainly limited to the host genus level. *Pneumocystis* from wild rodents thus appears to be stenoxenous (narrow range of closely related hosts) rather than strictly monoxenous parasites (single host species). An important *Pneumocystis* diversity, which does not only result from a process of co-speciation but also from several host switches, has been evidenced within the genus *Rattus*. Several new *Pneumocystis* phylogenetic species could be recognized among the *Pneumocystis* lineages that we identified in murid rodents. Additional genetic and phenotypic data are now needed to confirm their taxonomic status.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017001883

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