Pneumocystis diversity as a phylogeographic tool

S Derouiche[1,2], M Deville[3], ML Taylor[4], H Akbar[1,2], J Guillot[3], LE Carreto-Binaghi[4], M Pottier[1], EM Aliouat[1,2], CM Aliouat-Denis[1,2], E Dei-Cas[2,5], C Demanche[1,2/+]

[1]Department of Parasitology, Faculty of Biological and Pharmaceutical Sciences [5]Department of Parasitology-Mycology, Faculty of Medicine, University Hospital Center, Lille North of France University, rue du Pr. Laguesse 3, BP83, 59006 Lille, France [2]EA3609, IFR142, Pasteur Institute of Lille, Lille, France [3]Parasitology-Mycology Service, UMR BIPAR, National School Veterinary of Alfort, Maisons-Alfort, France [4]Laboratorio de Inmunología de Hongos, Department de Microbiología-Parasitología, Facultad Medicina, Universidad Nacional Autónoma de México, México, DF, México

Parasites are increasingly used to complement the evolutionary and ecological adaptation history of their hosts. Pneumocystis *pathogenic fungi, which are transmitted from host-to-host via an airborne route, have been shown to constitute genuine host markers of evolution. These parasites can also provide valuable information about their host ecology. Here, we suggest that parasites can be used as phylogeographic markers to understand the geographical distribution of intra-specific host genetic variants. To test our hypothesis, we characterised* Pneumocystis *isolates from wild bats living in different areas. Bats comprise a wide variety of species; some of them are able to migrate. Thus, bat chorology and migration behaviour can be approached using* Pneumocystis *as phylogeographic markers. In the present work, we find that the genetic polymorphisms of bat-derived* Pneumocystis *are structured by host chorology. Therefore,* Pneumocystis *intra-specific genetic diversity may constitute a useful and relevant phylogeographic tool.*

Key words: bats - co-evolution - ethology - host specificity - phylogeography - *Pneumocystis*

Recent research suggests that parasites may help to resolve the evolutionary and ecological history of their hosts. In some cases, parasites provide an additional source of information, since parasite data can better reconstruct the common history of host and their parasite (Nieberding & Olivieri 2007). For this reason, some parasites have been recently used as phylogeographic markers (Nieberding et al. 2004, Taylor et al. 2005, Criscione et al. 2006, Nieberding & Olivieri 2007). The *Pneumocystis* species have been shown to be a powerful tool in phylogenetic studies (Demanche et al. 2001, Guillot et al. 2001, 2004, Hugot et al. 2003, Aliouat-Denis et al. 2008). These eukaryotic micro-organisms, discovered by Carlos Chagas in 1909, had been described previously as enigmatic protists but are now recognised as major fungal pathogens able to provoke severe pneumonitis in severely weakened mammals. *Pneumocystis* pneumonia (PcP) is considered one of the most serious fungal respiratory infections to occur in immunocompromised patients, especially in human immunodeficiency virus-infected individuals (Dei-Cas 2000). Numerous aspects of the biology of these fungi still need to be clarified, notably their life cycle, which remains hypothetical (Yoshida 1989, Dei-Cas 2000). Neither the life cycle

stage responsible for the infection nor the eventual environmental infection sources have been identified. It has been shown however, that host-to-host *Pneumocystis* transmission via an airborne route can occur not only between immunosuppressed laboratory rodents (Hughes 1982, Soulez et al. 1991, Walzer et al. 1977) but also between non-immunocompromised hosts, which may constitute a dynamic reservoir to the *Pneumocystis* species (Dumoulin et al. 2000, Chabé et al. 2004). Indeed, healthy hosts are able to radically eliminate the pathogens from their lungs but, as long as they remain infected, they can transmit *Pneumocystis* by an airborne route to immunocompetent hosts or to weakened members of the population, which may then develop PcP (Chabé et al. 2004, Aliouat-Denis et al. 2008).

Recently, we also know that *Pneumocystis* species consist of a heterogeneous group of host specific fungal parasites that have colonised a wide range of mammalian hosts. Host species-related signatures have been reported in *Pneumocystis* populations using genomic, karyotypic, isoenzymatic and antigenic markers (Dei-Cas et al. 1998, Aliouat-Denis et al. 2008). Cross-infection experiments showed that *Pneumocystis* organisms inoculated into non-specific hosts do not induce the infection (Aliouat et al. 1993, 1994, Gigliotti et al. 1993, Durand-Joly et al. 2002) and are rapidly and radically eliminated from the lungs (Aliouat-Denis et al. 2008). Indeed, on the basis of morphological, phylogenetic and experimental data, it has been demonstrated that *Pneumocystis* constitutes a highly diversified biological group, with numerous species (Dei-Cas et al. 1998, 2006), which are strongly host-specific and well adapted to live inside the lungs of a diverse range of mammals (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003, Aliouat-Denis et al. 2008).
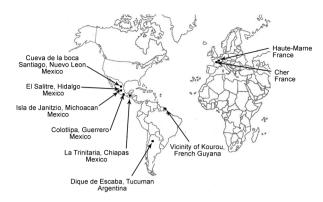
It is likely that there are as many *Pneumocystis* species as there are mammalian host species, though some mammals may be parasitised by more than one host-specific *Pneumocystis* species (Aliouat-Denis et al. 2008). The close host specificity of the numerous described or still undescribed *Pneumocystis* species led to the hypothesis of co-evolution. Consistently, several studies compared host and *Pneumocystis* phylogenies and reported a strong congruence between their phylogenetic trees (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003). Co-phylogeny seems to be the evolutionary pattern for *Pneumocystis* species, which have dwelt in the lungs of mammals for more than 100 million years (Demanche et al. 2001, Guillot et al. 2001, Hugot et al. 2003, Keely et al. 2003, Aliouat-Denis et al. 2008). Since *Pneumocystis* species and variants constitute interesting tools to understand the phylogeny and taxonomy of their hosts, we speculated about any related possibilities they could offer. Parasites, as proxies for host genealogy, may also be useful at the phylogeographic scale (Nieberding & Olivieri 2007).

Phylogeography is a field of research that analyses the geographical distribution of genealogical lineages and can be used to detect processes such as population subdivision, speciation events, ecological adaptation or migration routes (Emerson & Hewitt 2005). In this study, we analysed wild bat species and their corresponding *Pneumocystis*. Bats display a large biodiversity, a global distribution and a flying capacity that allow some of them to migrate over short or long distances. Bats also represent a large reservoir for emerging and re-emerging pathogens (Hance et al. 2006). Thus, the bat model seems ideally suited to assess our hypothesis of using *Pneumocystis* diversity as a potential phylogeographic tool.

MATERIALS AND METHODS

Bats - A total of 104 lung tissue samples were obtained from wild bats in Old or New World areas. Bats were captured from December 2004-July 2007. Of the samples from *Tadarida brasiliensis*, 79 were collected in Tucuman (Argentina) (16) and in four states of Mexico (63) (Table I, Figure). Nine samples of *Pipistrellus pipistrellus* from two French regions (Table I) were obtained through the Museum d'Histoire Naturelle de Bourges (France). A total of 16 samples of *Glossophaga soricina* were obtained

Geographical repartition of wild bat samples.

from French Guyana (in the vicinity of Kourou) (12) and Mexico (4) (Table I). Lung tissues were frozen immediately following necropsy and stored at -20°C.

DNA extraction - DNA extraction of all the samples was carried out using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions for tissue DNA extraction. Negative controls were included in the extraction procedure to monitor for contamination and extracted DNA was stored at -20°C prior to amplification.

DNA amplification and purification - Detection of *Pneumocystis* DNA was based on nested PCR, which amplified a portion of the mitochondrial large subunit (mtLSU) and small subunit (mtSSU) of the rRNA genes. Nested PCR at the mtLSU rRNA locus used the external primers published by Wakefield et al. (1990): pAZ102-H (5' -GTG-TAC-GTT-GCA-AAG-TAG-TC-3') and pAZ102-E (5' -GAT-GGC-TGT-TTC-CAA-GCC-CA-3'). The internal primers were pAZ102-X (5' -GTG-AAA-TAC-AAA-TCG-GAC-TAG-G-3') and pAZ102-Y (5' -TCA-CTT-AAT-ATTAAT-TGG-GGA-GC-3') (Wakefield 1996). Nested PCR at the mtSSU rRNA locus used the following primers: pAZ112-10F (5' -GGG-AAT-TCT-AGA-CGG-TCA-CAG-AGA-TCA-G-3') and pAZ112-10R (5' -GGG-AAT-TCG-AAC-GAT-TAC-TAG-CAA-TCC-C-3'); then pAZ112-13RI (5' -GGG-AAT-TCG-AAG-CAT-GTT-GTT-TAA-TTC-G-3') and pAZ112-14RI (5' -GGG-AAT-TCT-TCA-AAG-AAT-CGA-GTT-TCA-G-3') (Tsolaki et

TABLE I

Number of positive samples per bat species and geographical location (mitochondrial large subunit and small subunit of the rRNA loci merged)

			Mexico					Museum of Natural History, Bourges			
	Argentina	French Guyana	Chiapas	Michoacan	Guerrero	Hidalgo	Nuevo Leon	Cher	Haute-Marne	Total positive samples	Total samples
Glossophaga soricina	-	6/12	-	-	3/4	-	-	-	-	9	16
Pipistrellus pipistrellus	-	-	-	-	-	-	-	2/8	1/1	3	9
Tadarida brasiliensis	8/16	-	2/10	2/6	-	5/20	19/27	-	-	36	79

al. 1998). The Hot Master® Taq DNA Polymerase (Eppendorf) was used for amplification of both loci. For mtLSU rRNA gene amplification, the thermocycling conditions for the first PCR round were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 50°C and extension for 1 min at 65°C, for 30 cycles. The second round of PCR was performed with 10% of the first-round mix and the thermocycling conditions were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 65°C, for 30 cycles. For mtSSU rRNA gene amplification, the thermocycling conditions for the first PCR round were as follows: each cycle consisted of denaturation for 30 s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 65°C, for 40 cycles. The second round of PCR was performed with 10% of the first-round mix and the thermocycling conditions were as follows: the first ten cycles consisted of denaturation for 30 s at 94°C, annealing for 1 min at 52°C and extension for 1 min at 65°C, and the following 30 cycles consisted of denaturation for 30 s at 94°C, annealing for 1 min at 63°C and extension for 1 min at 65°C. Negative controls were included in each experiment to monitor for possible contamination. Amplification products were purified on a 1.5% agarose gel (Trisborate-EDTA buffer) and extracted using the QIAEX II Gel Extraction kit (QIAGEN) when non-specific bands were detected. In the absence of smear or non-specific bands, the amplification products were directly purified using the Montage® PCR Centrifugal Filter Devices kit (Millipore). Purified DNA was stored at -20°C prior to the sequencing reaction.

Cloning of amplified and purified products - When the amplified DNA produced a band of low intensity in the agarose gel, cloning in the bacterial system was performed using the TOPO TA Cloning® kit (Invitrogen) and the One Shot® Top 10 Chemically Competent *Escherichia coli* (Invitrogen). Plasmid DNA was then extracted with the Perfectprep® Plasmid Mini kit (Eppendorf), amplified with the thermocycling conditions of the first round PCR at the locus mtLSU rRNA and purified with the Montage® PCR Centrifugal Filter Devices kit (Millipore).

Sequencing of purified DNA and sequence analysis - The sequencing reaction was carried out from both ends with the sets of internal primers (or primers included in the kit TOPO TA Cloning®, Invitrogen, for the cloned products) and the Big Dye Terminator® v3.1 kit (Applied Biosystems). The labelled products were directly sent to GenoScreen (Pasteur Institute of Lille) and analysed on an automated DNA sequencer (3730XL DNA Analyser®, Applied Biosystems). Sequences were first aligned with the software Clustal X v2.0 (Thompson et al. 1997) and then the alignment was improved manually using the software Se-Al v2.0a11 Carbon (Rambaut 2002). The aligned sequences were converted into a distance matrix (% of differences) using the Phylogenetic Analysis Using Parsimony Program (PAUP v4.0b10, Swofford 2001).

RESULTS AND DISCUSSION

Three species of bats were included in this study: *T. brasiliensis*, *P. pipistrellus* and *G. soricina*. *Pneumocystis* DNA (mtLSU and mtSSU rRNA loci merged) was detected in 48 out of 104 samples (Table I). Amplified *Pneumocystis* mtLSU and mtSSU rRNA gene fragments showed host species-specific DNA sequences. In other words, all samples from *T. brasiliensis* had a similar and unique DNA sequence of *Pneumocystis* mtLSU and mtSSU rRNA gene fragments. The same was true for *Pneumocystis* DNA samples from *G. soricina* and *P. pipistrellus*. These observations confirm the high specificity of *Pneumocystis* organisms for their hosts. Previous studies had reported high diversity in the *Pneumocystis* genus and to date at least one host-specific *Pneumocystis* sequence has been reported from each studied mammalian host species (Mazars et al. 1997, Wakefield 1998, Demanche et al. 2001, Guillot et al. 2001, Aliouat-Denis et al. 2008).

Interestingly, DNA sequences from the *Pneumocystis* organisms harboured within one host species showed some genetic polymorphism in accordance with the geographical origin of the samples. For *G. soricina* samples, the mtLSU rRNA sequences of *Pneumocystis* from French Guyana (6 identical sequences) and Mexico (2 identical sequences) were different at one base position (guanine or adenine) (Table II). Since there is low variability at the mtLSU rRNA locus from *G. soricina*-derived *Pneumocystis,* our results should be confirmed by sequencing and comparison of other loci.

For the *P. pipistrellus* samples, some polymorphism was also observed in the *Pneumocystis* mtSSU rRNA sequences which diverged in less than 1% of the sequence of the *P. pipistrellus* population (Table III) according to the geographical origin (one sequence from Cher and one sequence from Haute-Marne, France). *P. pipistrellus* bats undergo long-distance migrations (Fleming & Eby 2003); thus, mt rRNA polymorphisms could characterise two strains of *P. pipistrellus*-derived *Pneumocystis* specifically attached to two different migratory colonies of this bat species.

Comparable polymorphism was observed in the samples of *T. brasiliensis,* another long-distance migratory species. Two mtLSU and three mtSSU rRNA polymorphic sequences of *T. brasiliensis*-derived *Pneumocystis* were obtained. At the mtLSU rRNA locus, one unique sequence type was found in samples from the Argentinian colony (7 identical sequences), whereas a different sequence type was found in all Mexican samples (7 identical sequences). These two groups of sequences show only 1% of divergence from each other (Table II). At the mtSSU rRNA locus, one unique sequence type was found in samples from Argentina (7 identical sequences), another one in samples from Nuevo Leon (Mexico) (1 sequence) and another one in samples from Chiapas, Hidalgo and Michoacán (Mexico) (6 identical sequences). These sequences showed 0.9-1.6% divergence (Table III). Results in Nuevo Leon should obviously be confirmed with additional samples, since current results are based on only one *Pneumocystis* sequence. In future

TABLE II

Matrix of distance for *Pneumocystis* mitochondrial large subunit of the rRNA sequences of three bats species
and two outgroup species (*P. murina* from the mouse and *P. oryctolagi* from the rabbit)

Source of rDNA	% of divergence from					
	1	2	3	4	5	6
1. *Pneumocystis* from *G. soricina* (French Guyana)	-	-	-	-	-	-
2. *Pneumocystis* from *G. soricina* (Mexico)	0.48	-	-	-	-	-
3. *Pneumocystis* from *T. brasiliensis* (Mexico)	14.49	14.03	-	-	-	-
4. *Pneumocystis* from *T. brasiliensis* (Argentina)	14.48	14.02	0.92	-	-	-
5. *Pneumocystis* from *P. pipistrellus* (France)	16.48	16.00	12.57	12.05	-	-
6. *Pneumocystis murina*	23.13	22.66	22.58	22.50	23.57	-
7. *Pneumocystis oryctolagi*	19.90	19.40	22.46	22.95	21.69	18.70

TABLE III

Matrix of distance for *Pneumocystis* mitochondrial small subunit of the rRNA sequences of three bats species
and two outgroup species (hare and rabbit)

Source of rDNA	% of divergence from						
	1	2	3	4	5	6	7
1. *Pneumocystis* from *P. pipistrellus* (Haute-Marne)	-	-	-	-	-	-	-
2. *Pneumocystis* from *P. pipistrellus* (Cher)	0.28	-	-	-	-	-	-
3. *Pneumocystis* from *T. brasiliensis* (Nuevo Leon)	20.06	20.02	-	-	-	-	-
4. *Pneumocystis* from *T. brasiliensis* (Hidalgo-Chiapas-Michoacan)	20.00	19.98	0.94	-	-	-	-
5. *Pneumocystis* from *T. brasiliensis* (Argentina)	19.75	19.71	1.26	1.56	-	-	-
6. *Pneumocystis* from *G. soricina* (Mexico)	19.04	17.78	14.38	14.03	14.25	-	-
7. *Pneumocystis* f. sp. *Lepus europaeus*	26.13	25.29	26.37	26.69	26.84	21.00	-
8. *Pneumocystis oryctolagi*	20.12	19.16	21.57	21.90	21.43	18.42	8.83

studies, we should also use more polymorphic markers, like microsatellites, which offer a higher discriminating power, especially for characterising more accurately *Pneumocystis* species sub-populations linked to migratory colonies. For instance, microsatellites have already been used to characterise populations of the fungus *Histoplasma capsulatum* in the Americas (Carter et al. 2001).

Our results are in contrast with a 1994 study in which the frequently occurring one-base mutation of the mtLSU rRNA sequence could not be correlated with geography (UK, USA, Brazil and Zimbabwe) (Wakefield et al. 1994). This may be due to the fact that the portion of the mtLSU rRNA gene analysed in this study was relatively short and therefore other informative polymorphic characters may have been missed. In contrast to the Wakefield study, geography seemed to be a structuring factor to the distribution of four *Pneumocystis jirovecii* mtLSU rRNA genotypes isolated from HIV-infected patients in the USA (Beard et al. 2000). Likewise, a study in the UK showed a significant association between specific mtLSU rRNA genotypes of *P. jirovecii* and the patient's place of residence (Miller et al. 2005). Furthermore, several studies report that the relative proportions of *P. jirovecii* genotypes vary in accordance with the area from which

the patients originated. The frequency pattern of DNA sequences from *P. jirovecii* that combine the association of three gene sequences (mtLSU rRNA, Dihydropteroate synthase and internal transcribed spacer) in specimens from Lisbon (Portugal) and Seville (Spain)) show inverted proportions (Esteves et al. 2008). In short, geographical location could therefore have a structuring role in the distribution of *Pneumocystis* genotypes. In the present work, the association of genotypes with migratory bat colonies suggests that there is no inter-colony transmission of *Pneumocystis* and that bats from different colonies do not share migratory routes.

On the whole, the present results based on two genetic markers (the mtLSU rRNA and mtSSU rRNA genes) widely used in *Pneumocystis* research, suggest that host species chorology could have a structuring effect on the distribution of *Pneumocystis* intra-specific variants. These could therefore be used as phylogeographic tools to approach the study of host species subpopulations. In fact, *T. brasiliensis* populations are organised into numerous colonies that could have different migratory behaviours. An attempt to clarify the migratory pathway of these different colonies was previously carried out by Russel et al. (2005). They studied bat mitochondrial DNA sequence but failed to correlate significant genetic

polymorphism to behaviourally distinct migratory colonies. *Pneumocystis* intra-specific genetic polymorphisms could perhaps help to differentiate the colonies of *T. brasiliensis* and help identify their migratory pathway.

Other studies have shown the usefulness of parasite phylogeny as a mirror to host genealogy and ecology (Nieberding & Olivieri 2007). Indeed, when parasite populations are more diversified than their host populations, gene trees of parasites can be used to infer recent migration events of infected hosts (Nieberding & Olivieri 2007). For instance, parasite genotypes identify source populations of migratory salmon more accurately than do fish genotypes (Criscione et al. 2006). Another example is the detailed study of the comparative phylogeography of the rodent *Apodemus sylvaticus* and one of its specific endoparasites, the nematode *Heligmosomoides polygyrus* (Nieberding et al. 2004, Nieberding & Olivieri 2007). Compared to the host tree, the parasite tree showed additional subgroups related to different geographic locations. Therefore, the phylogeographic pattern of the parasite suggested probable past differentiation and migration events that could not be detected through the study of the host itself (Nieberding & Olivieri 2007). A recent and similar study of the geographical distribution of *A. sylvaticus* was carried out with the same set of samples (Nieberding et al. 2004, Nieberding & Olivieri 2007), using *Pneumocystis* as the genetic marker (M Deville et al., unpublished observations). The same trend in phylogeographic history resulted: the host phylogeny showed two main geographical groups (Western Europe and Italy-Balkans) while the parasite phylogeny showed the same main groups but also many regional subgroups, suggesting past migration events of the host. Thus, these results support our hypothesis of using *Pneumocystis* as a potential phylogeographic tool. Furthermore, another pathogenic fungus, *H. capsulatum,* was also studied in *T. brasiliensis* bats (Taylor et al. 2005). Taylor et al. (2005) detected a pattern of molecular differences in accordance to the geography origin of *H. capsulatum* isolated from infected bats that could be used as a tool to monitor the distribution of the fungus and, thus, its host in the nature. These results support our data and suggest that such pathogenic fungi are a robust phylogeographic marker.

Finally, to be a good marker at a phylogenetic or phylogeographic scale, a parasite must share a common history with its host. One of the determining factors is a strong interaction between the host and the parasite. The strength of the host-parasite interaction depends on three parasitic traits: host specificity, presence or absence of intermediate hosts and presence or absence of a free-living phase (Nieberding & Olivieri 2007). The lack of a free-living phase increases the dependence of the parasite on its host, as parasite migration is then exclusively limited to host movements (Nieberding & Olivieri 2007). Thus, we can say that *Pneumocystis* species have a really intimate interaction with their hosts, since it has close host specificity and well documented co-evolution and co-speciation. In addition, *Pneumocystis* species do not have intermediate hosts and, most likely, no environmental stage. In fact, their very close host specificity and their fine adaptation to the alveolar microenvironment (Dei-Cas 2000, Dei-Cas et al. 2004, Aliouat-Denis et al. 2008) seems contradictory, or even incompatible, with any ability to multiply and survive outside their host. Furthermore, *Pneumocystis* populations seem to be more diversified than their host populations, as to be expected of parasites that can be used as proxies at the phylogeographic scale (Nieberding & Olivieri 2007). All these features provide further support to our hypothesis.

In conclusion, the genetic variability of *Pneumocystis* that we observed in bats from distinct species or from distinct geographical areas supports our hypothesis that *Pneumocystis* genetic polymorphisms provide useful and relevant phylogeographic markers of host chorology. Our results validate the use of *Pneumocystis* as a powerful tool in phylogenetic and phylogeographic studies.

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