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Characterizing *Pneumocystis* in the Lungs of Bats: Understanding *Pneumocystis* Evolution and the Spread of *Pneumocystis* Organisms in Mammal Populations

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Bats belong to a wide variety of species and occupy diversified habitats, from cities to the countryside. Their different diets (i.e., nectarivore, frugivore, insectivore, hematophage) lead Chiroptera to colonize a range of ecological niches. These flying mammals exert an undisputable impact on both ecosystems and circulation of pathogens that they harbor. *Pneumocystis* species are recognized as major opportunistic fungal pathogens which cause life-threatening pneumonia in severely immunocompromised or weakened mammals. *Pneumocystis* consists of a heterogeneous group of highly adapted host-specific fungal parasites that colonize a wide range of mammalian hosts. In the present study, 216 lungs of 19 bat species, sampled from diverse biotopes in the New and Old Worlds, were examined. Each bat species may be harboring a specific *Pneumocystis* species. We report 32.9% of *Pneumocystis* carriage in wild bats (41.9% in Microchiroptera). Ecological and behavioral factors (elevation, crowding, migration) seemed to influence the *Pneumocystis* carriage. This study suggests that *Pneumocystis*-host association may yield much information on *Pneumocystis* transmission, phylogeny, and biology in mammals. Moreover, the link between genetic variability of *Pneumocystis* isolated from populations of the same bat species and their geographic area could be exploited in terms of phylogeography.

Pneumocystis species are mostly airborne-transmitted, highly host-specific opportunistic microfungi responsible for severe pneumonia in a wide range of mammalian species (3). In humans, an unexpectedly high number of clustered cases of *Pneumocystis* pneumonia (PcP) occurring in 1981 revealed the AIDS pandemic (14). Nowadays, PcP is still quite frequently diagnosed in HIV-positive patients but also in patients with other causes of immunodeficiency, such as organ transplantation or anticancer therapy (5, 35, 62, 78). Still, *Pneumocystis jirovecii* remains a leading cause of high mortality in HIV patients, even after the introduction of highly active antiretroviral therapy (HAART) (11, 64). Moreover, new data have recently emerged implicating low burdens of *Pneumocystis* organisms as a cause of symptom worsening in patients with chronic obstructive pulmonary disease (42). Also *Pneumocystis* organisms may temporarily cause asymptomatic infection in immunocompetent hosts, hence constituting a potential infection source (16). Thus, immunocompetent *Pneumocystis* carriers could transmit the infection to immunocompromised individuals (16, 30, 32).

Pneumocystis organisms constitute a huge genus consisting of a large number of host-species-specific organisms found in different mammals (3, 17, 20, 21, 26, 27, 41, 49). Indeed, the narrow host specificity of *Pneumocystis* species emerged clearly from the failure of cross species infection among laboratory animals (1, 38) or between humans and SCID mice (33). Surveys in domestic, synanthropic, or wild species showed that mammals may harbor one or several host-specific *Pneumocystis* species or strains (3, 17, 27, 31, 41, 46).

Whether *Pneumocystis* organisms are able to survive or multiply in the environment remains an unanswered question. It is known that they can be transmitted by aerial route (16, 32, 45, 85) and likely by transplacental route, at least in some mammal species (15, 29, 63, 79). The unavailability of continuous *in vitro* culture systems has hindered research aiming at clarifying the role of each life cycle stage in *Pneumocystis* proliferation and transmission (44). However, ultrastructure (25, 99), short-term culture (2, 19), and cell sorting approaches (56, 57) led to quite heuristic life cycle hypotheses. Furthermore, using PcP animal models, molecular strategies and the exploring of *Pneumocystis* occurrence in wild mammals resulted in major advancements, like the notions of strong host species specificity (1, 27, 31, 33, 38, 41) and coevolution (3, 27, 31, 46).

Bats (Chiroptera) are widely distributed across various ecosystems and constitute one of the largest groups of mammals, second in number of species after the order Rodentia and first in number of individuals (69). We thus attempted to explore how *Pneumocystis* organisms can circulate and adapt within this singular clade

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of flying mammals. Indeed, about one-fourth of all the living mammalian species consists of bats, a group comprising almost 1,100 species throughout the world. Body weight of bats varies widely: from 2 g (*Craseonycteris thonglongyai*; hog-nosed or bumblebee bat) to 1,200 g (*Pteropus vampyrus*; large flying lemur) (10). The order of Chiroptera is divided into two suborders (66): megachiropters (megabats) and microchiropters (microbats). Megabats were reported to live in the Old World and microbats in the New and Old Worlds.

Chiropters occupy diversified habitats, from cities to the countryside, and exhibit the largest variety of diets among all mammalian orders (66). Most are insectivorous (about 700 species), but a significant number of bats feed almost exclusively on fruits (about 230 species), nectar or pollen (about 50 species), small vertebrates (7 species), or blood of mammals or birds (3 species) (66). Their different diets led Chiroptera to colonize diverse ecosystems, ranging from deserts to temperate forest or rainforests, of every continent of the world except Antarctica (9, 69). In some islands, for instance in New Zealand, the only native mammals are bats (22, 36, 43). Bats roost in caves, abandoned or occupied buildings, mines, tree canopies, and hollows and under leaves, bark, or rocks (9, 51). Some bat species are living solitary, whereas others form huge colonies comprising millions of individuals (9). Bats are long-lived mammals: the small 7-g Brandt's bat, *Myotis brandtii*, can live up to 38 years in the wild (98). Furthermore, some species of bats are able to migrate over long distances.

Interestingly, humans share many properties with bats: occupying diverse habitats, living in social organizations, being long-lived, able to perform long migrations, and harboring *Pneumocystis* (41). Bats may therefore represent interesting models to understand the circulation of the human-specific species *P. jirovecii* in human populations. Furthermore, bats have an unusual physiology. For instance, due to their large lungs and naked flight membranes, they are exposed to marked heat losses (66) that could influence the biology of bat-derived *Pneumocystis* organisms and reveal unknown adapting mechanisms of these singular parasitic fungi to their hosts.

The present study is based on the molecular detection of *Pneumocystis* in the lungs of bats sampled from diverse biotopes in New and Old Worlds. It explores the *Pneumocystis* infection frequency in chiropters and *Pneumocystis* genetic diversity in relation with environmental and behavioral conditions. The results suggest that wild bat populations constitute a good model for approaching the circulation of *Pneumocystis* organisms in mammal populations, host specificity, and coevolution events.

MATERIALS AND METHODS

Samples. A total of 216 bat specimens from the New World (Mexico, Guyana, Argentina) and from the Old World (France) were examined for the presence of *Pneumocystis* in their lungs. A total of 155 wild microbats belonging to 17 species were collected in different areas of Mexico (88 specimens), French Guyana (13 specimens), Argentina (16 specimens), and France (38 specimens). A total of 61 megabat specimens belonging to 2 species (*Rousettus aegyptiacus* and *Pteropus rodricensis*) were sampled from two colonies held in the same enclosure in La Palmyre Zoological Park (France). The founders of these colonies came from Northern Africa and Rodrigues Island (southwest Indian Ocean, 19°43′00′′S and 63°25′00′′E), respectively. Nineteen and 20 *Pteropus rodricensis* individuals were, respectively, transferred in 1993 and 1994 out of Jersey Zoological Park (Jersey Island) to La Palmyre Zoological Park. The colony housed in Jersey was founded by 10 individuals captured in Rodrigues Island in 1967 and 1977. The population of *Rousettus aegyptiacus* in La Palmyre was founded in 1994, with 120 individuals from various locations.

After euthanasia, the lungs were removed and immediately stored at −20°C in sterile cryotubes until used. In all cases, national rules regulating bat species protection have been respected.

Size of bat colonies. For the genus *Tadarida*, colony size was assessed by counting bat individuals. As no exact assessment of colony size was available for the other species, a semiquantitative estimation was performed on the basis of direct observational appreciation of the number of colony members. Such a method led to defined scores: big colony, >200 individuals; medium colony, 31 to 200 individuals; and small colony, 10 to 30 individuals.

DNA extraction. DNA extraction from lung tissue samples was performed using a DNeasy tissue kit (Qiagen, Courtabœuf, France) by following the manufacturer's procedure with some modifications. Part of the lung tissue (25 mg) was lysed overnight in an incubator at 56°C with permanent rotation. In order to concentrate *Pneumocystis* DNA, the column was rehydrated with 100 μl of elution AE buffer. DNA was stored at −20°C. A negative control was systematically included in each series of DNA extraction.

DNA amplification and analyses. The presence of *Pneumocystis* DNA in lungs was assessed by nested PCR at the mtLSU rRNA and mtSSU rRNA loci (91, 96), mitochondrial genes encoding rRNA. Primer sequences and PCR cycling conditions are shown in Table 1.

Negative controls were included in each experiment of PCR amplification, to monitor for eventual contamination. When nonspecific bands were detected, amplification products of the expected size (about 250 to 300 bp) were extracted from a 2% agarose gel (run in Tris-borate-EDTA buffer) using a PCR purification kit (QIAEX II gel extraction kit; Qiagen, Courtabœuf, France). When a unique band of expected size was present, amplified products were directly sent for sequencing to GenoScreen (Pasteur campus, Genopole of Lille, France). Sequencing from both ends using sets of internal primers was performed on an automated DNA sequencer (3730XL DNA Analyser; Applied Biosystems). Amplification and sequencing of each sample were repeated at least twice. The mtLSU and mtSSU sequences were aligned with already known *Pneumocystis* sequences using the computer program CLUSTAL X (version 1.63b, December 1997) (89). Then, alignments were refined by visual optimization using the software Se-Al version 2.0a11 Carbon (75). The aligned sequences were converted to distance matrix (percentage of differences). The BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) program allowed the comparison of our query sequence with the sequences available from databases in order to confirm sequence novelty and absence of contamination. To infer phenetic relationships among *Pneumocystis* isolates of our data set, an improved version of the neighbor-joining algorithm based on a simple model of sequence data (BIONJ) (37) analyses using PAUP 4.0b9 software was run (87). Evaluation of statistical confidence in nodes was based on 1,000 bootstrap replicates in BIONJ (34).

Sensitivity of the PCR. Amplification products of mtLSU and mtSSU rRNA from *Pneumocystis carinii* were cloned by using a TOPO TA cloning kit (Invitrogen). Fifteen separate colonies were selected from the transformant plates and examined for each positive sample. DNA extraction was performed with a QIAprep miniprep kit (Qiagen). To check that the insert contained in the plasmid was the expected sequence, we used the external primer set of each nested PCR mtLSU ribosomal DNA (rDNA) and mtSSU rDNA locus (Table 1). A range of dilution containing from 10^6 copies to 1 copy of the targeted gene was performed. To determine the PCR sensitivity, the presence of a *Pneumocystis carinii* gene fragment within plasmids was assessed by nested PCR at the mtLSU rRNA and mtSSU rRNA loci as described above. The amplification products were visualized on a 2% agarose gel containing ethidium bromide.

Statistical analysis. Statistical analyses were performed using SAS (version 9; SAS Institute). Variables are described as counts (proportions). A generalized linear mixed-model approach (with PROC

TABLE 1 Primer sequences and cycling conditions of *Pneumocystis* nested PCR assays at either the mtSSU rDNA or mtLSU rDNA locus (modified from references 97 and 91)

Loci or cycling step	Sequence or cycling conditions	
	mtSSU rRNA	mtLSU rRNA
Loci		
External primer pair		
Forward	pAZ112-10F/R1: 5′-GGG AAT TCT AGA CGG TCA CAG AGA TCA G-3′	pAZ102-H: 5′-GTG TAC GTT GCA AAG TAC TC-3′
Reverse	pAZ112-10R/R1: 5′-GGG AAT TCG AAC GAT TAC TAG CAA TTC C-3′	pAZ102-E: 5′-GAT GGC TGT TTC CAA GCC CA-3′
Internal primer pair		
Forward	pAZ112-13/R1: 5′-GGG AAT TCG AAG CAT GTT GTT TAA TTC G-3′	pAZ102-X/R1: 5′-GGG AAT TCG TGA AAT ACA AAT CGG ACT AGG-3′
Reverse	pAZ112-14/R1: 5′-GGG AAT TCT TCA AAG AAT CGA GTT TCA G-3′	pAZ102-Y/R1: 5′-GGG AAT TCT CAC TTA ATA TTA ATT GGG GAG C-3′
Cycling step		
1st round		
Denaturation	30 s, 94°C	30 s, 94°C
Annealing	1 min, 55°C	1 min, 50°C
Elongation	1 min, 65°C	1 min, 65°C
No. of cycles	40	30
2nd round (performed with 5% [vol/vol] of the first-round mix)		
Denaturation 1	30 s, 94°C	30 s, 94°C
Annealing 1	1 min, 52°C	1 min, 55°C
Elongation 1	1 min, 65°C	1 min, 72°C
No. of cycles	10	30
Denaturation 2	30 s, 94°C	
Annealing 2	1 min, 63°C	
Elongation 2	1 min, 65°C	
No. of cycles	30	

GLIMMIX) was used to investigate the association between *Pneumocystis* carriage and potential predictors. Models were built with a binomial error distribution and the logit link function, and capture site was included as a random factor. Covariates with a level of significance of <0.1 in the univariate analyses were entered in multivariate models, the variables being then considered significant with a P value of <0.05.

Nucleotide sequence accession numbers. *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequences were deposited in GenBank under accession numbers JQ039397, JQ061293 to JQ061303, and JQ061304 to JQ061318.

RESULTS

Detection of *Pneumocystis* DNA in the lungs of bats. A sample was considered to be positive for the presence of *P. carinii* DNA when a specific band was amplified by PCR at the expected size either at the mtLSU rRNA or mtSSU rRNA locus or at both loci and then sequenced. Two hundred sixteen lung samples from 19 bat species were examined (Tables 2 and 3). None of the tested lung tissue samples gave a positive amplification after the first PCR round at either locus. At the second round, PCR amplification was positive in 71 animals (32.9%) belonging to 12 bat species: one megabat (*Rousettus aegyptiacus*) and 11 microbats (Table 3) species. Most positive lung samples were from neotropical bat specimens: 6/13 from French Guyana, 36/88 from Mexico, and 8/16 from Argentina. In metropolitan France, 14/38 lung samples from wild bats were found to be positive. In captive animals (North African bats housed in La Palmyre Zoological Park in metropolitan France), *Pneumocystis* DNA was detected in 6/61 lung samples. On the whole, a positive amplification at both mtSSU rDNA and mtLSU rDNA was obtained in 48/216 (22.2%) animals belonging to 9 bat species.

The sensitivities of the nested PCR mtLSU and the nested PCR mtSSU indicate, respectively, a limit of detection of 10 copies and 1 copy. However, our experimental results indicate that the ratios of mtLSU-positive/mtSSU-negative samples and mtLSU-negative/mtSSU-positive samples are, respectively, 33.14% and 37.2% when *P. rodricensis* flying foxes were excluded. The sensitivity of *Pneumocystis* detection is not the same at both loci. Moreover, the *Pneumocystis* sequences are variable and primers hybridize differently according to the species of bats analyzed. Consequently, the rate of the *Pneumocystis* carriage is probably underestimated in this study.

Potential influence of host suborder and geography. The frequency of *Pneumocystis* DNA detection was significantly higher in suborder Microchiroptera (41.9%) than in suborder Megachiroptera (9.8%) ($P < 0.0001$). However, only two species of megabats could be examined in the present work: Egyptian fruit bat (*R. aegyptiacus*) and Rodrigues Island flying fox (*Pteropus rodricensis*). The sampled specimens of these two species lived in the same enclosure since 1994 in La Palmyre Zoological Park. *Pneumocystis* DNA was detected in 35.3% of the 17 Egyptian fruit bats, but none of the 44 flying foxes was found to be positive.

With regard to geography, the frequencies of *Pneumocystis* carriage in bats from the New World and Old World were 43.6% and

TABLE 2 Rate of *Pneumocystis* carriage in bat species and ecological data

Origin	Bat species	Rate of *Pneumocystis* carriage[a]	Size of bat colonies[b]	Migratory	Day roost	Mating system[c]	Close physical contact	Reference(s) or source
Wild microchiropters of the New World	*Tadarida brasiliensis*	36/79 (45.6)	Big	Yes	Caves	MM/MF	Yes	10, 59
	Artibeus hirsutus	3/5 (60)	Small	No	Caves	Harem	ND	72, 88
	Mormoops megalophylla	0/3 (0)	Big	No	Caves	Harem	No	*Mormoops megalophylla*, unpublished abstract compiled and edited by the Heritage Data Management System, Arizona Game and Fish Department, and reference 88
	Myotis californicus	1/1 (100)	Small	No	Caves	ND	ND	88
	Pteronotus parnellii	1/3 (33.3)	ND[d]	No	Caves	ND	ND	88
	Natalus stramineus	1/8 (12.5)	Big	No	Caves	ND	No	88, 90
	Pteronotus davyi	0/1 (0)	ND	ND	Caves	ND	ND	
	Glossophaga soricina	9/16 (56.5)	Small	No	Caves	Harem	Yes	60, 66
	Carollia perspicillata	0/1 (0)	Small	Yes	Tree-dwelling, forest	Lek	Yes	59
Wild microchiropters of the Old World	*Nyctalus noctula*	6/20 (30)	Medium	Yes	Tree-dwelling, forest	Lek	Yes	10, 60, 59
	Pipistrellus pipistrellus	3/9 (33.3)	Medium	Yes	Anthropophilic	Harem	Yes	59
	Eptesicus serotinus	2/2 (100)	Medium	No	Anthropophilic	ND	ND	60
	Nyctalus leisleri	0/1 (0)	Small	Yes	Tree-dwelling, forest	Lek	Yes	86
	Myotis daubentoni	0/1 (0)	Medium	Yes	Tree-dwelling, forest	MM/MF	Yes	6, 60
	Myotis myotis	0/2 (0)	Big	Yes	Caves	MM/MF	Yes	10, 60, 59
	Plecotus austriacus	1/1 (100)	Medium	No	Anthropophilic	ND	Yes	80
	Plecotus auritus	2/2 (100)	Small	No	Tree-dwelling, forest	MM/MF	Yes	10, 60, 59
Captive megachiropters of the Old World	*Rousettus aegyptiacus*	6/17 (35.3)	Medium	No	Zoological park			
	Pteropus rodricensis	0/44 (0)	Medium	No	Zoological park			

[a] Number of *Pneumocystis* PCR-positive samples/total number of tested samples (%).
[b] Bat colony size was scored as follows: small, 10 to 30 individuals; medium, 31 to 200 individuals; big, >200 individuals.
[c] MM/MF, multi-male/multi-female.
[d] ND, not done.

20.2%, respectively. However, the presence of *Pneumocystis* DNA in bats from the Old World rose to 36.8% when *P. rodricensis* flying foxes were excluded (Table 4). No significant statistical association could be established between the presence of *Pneumocystis* DNA and Old or New World geographic location of the animals ($P = 0.81$) (Table 5). Consistently, no significant difference was found between microchiropters from the New (43.6%) and Old (35.6%) Worlds.

Impact of ecological and behavioral factors. Neither wild/captive state nor host phylogeny influenced *Pneumocystis* DNA detection significantly, with the obvious exception of *P. rodricensis*, in which *Pneumocystis* DNA was not found (Tables 2 and 3). In contrast, the size of the bat colony influenced the frequency of *Pneumocystis* DNA detection significantly ($P = 0.04$; Table 5). Thus, the probability of picking a *Pneumocystis*-infected bat was 3-fold higher in small colonies than in large ones, irrespective of species (Table 5). Likewise, migration and crowding influenced the frequency of *Pneumocystis* DNA detection significantly (Tables 5 and 6). The statistical multivariate analyses showed that migration and crowding are independent predictive factors of *Pneumocystis* carriage (Table 6). The probability of picking a *Pneumocystis*-infected bat was 5-fold higher in sedentary colonies than in migratory ones and 33-fold higher in crowding colonies (close physical contact between the colony members) than in non-crowding colonies (without close physical contact) (Table 6). When we limit analyses to bats in close physical contact ($n = 132$: 19 sedentary and 113 migratory bats), migration is the sole predictive factor, nonmigratory bats having an odds ratio (OR) of 5.091 (1.147 to 22.597) ($P = 0.03$) with regard to the migratory bats.

Cave temperature (°C) and relative humidity (%) were not recorded inside the main chamber. Temperature and relative humidity outside the cave were evaluated by general climate of the geographical area of the cave. The climate, roosting habits, and mating system did not seem to influence *Pneumocystis* carriage (Table 5). The elevation and food regimen did not seem to influence it either, although significance is borderline ($P = 0.07$).

TABLE 3 *Pneumocystis* carriage in bats[a]

Origin	Bat species	Targeted loci	No. of *Pneumocystis* PCR-positive samples/total number of tested samples by sampling area										
			La Trinitaria cave, Chiapas, Mexico	Isla de Janitzio cave, Michoacán, Mexico	Juxtlahuacave, Colotlipa Guerrero, Mexico	El Salitre, cave, Hidalgo, Mexico	El Salitre, cave Santa Rosa, Morelos, Mexico	La Boca cave, Nuevo León, Mexico	Dique Escaba, Tucumán, Argentina	French Guyana	Natural History Museum Bourges, France	La Palmyre zoological park, France	All areas
Wild microchiropters of the New World	*Tadarida brasiliensis*	LSU	0/10	2/6		5/20		16/27	7/16				30/79
		SSU	2/10	2/6		4/20		17/27	7/16				32/79
		Carrier	2/10	2/6		5/20		19/27	8/16				36/79
	Artibeus hirsutus	LSU					2/5						2/5
		SSU					3/5						3/55
		Carrier					3/5						3/5
	Mormoops megalophylla	LSU			0/2			0/1					0/3
		SSU			0/2			0/1					0/3
		Carrier			0/2			0/1					0/3
	Myotis californicus	LSU						1/1					1/1
		SSU						1/1					1/1
		Carrier						1/1					1/1
	Pteronotus parnellii	LSU			0/2	0/1							0/3
		SSU			0/2	1/1							1/3
		Carrier			0/2	1/1							1/3
	Natalus stramineus	LSU			1/8								1/8
		SSU			0/8								0/8
		Carrier			1/8								1/8
	Pteronotus davyi	LSU			0/1								0/1
		SSU			0/1								0/1
		Carrier			0/1								0/1
	Glossophaga soricina	LSU			3/4					6/12			9/16
		SSU			2/4					6/12			8/16
		Carrier			3/4					6/12			9/16
	Carollia perspicillata	LSU								0/1			0/1
		SSU								0/1			0/1
		Carrier								0/1			0/1
Wild microchiropters of the Old World	*Nyctalus noctula*	LSU									5/20		5/20
		SSU									6/20		6/20
		Carrier									6/20		6/20
	Pipistrellus pipistrellus	LSU									1/9		1/9
		SSU									3/9		3/9
		Carrier									3/9		3/9
	Eptesicus serotinus	LSU									2/2		2/2
		SSU									2/2		2/2
		Carrier									2/2		2/2
	Nyctalus leisleri	LSU									0/1		0/1
		SSU									0/1		0/1
		Carrier									0/1		0/1
	Myotis daubentoni	LSU									0/1		0/1
		SSU									0/1		0/1
		Carrier									0/1		0/1
	Myotis myotis	LSU									0/2		0/2
		SSU									0/2		0/2
		Carrier									0/2		0/2

	Plecotus austriacus	LSU											0/1
		SSU											1/1
		Carrier											1/1
	Plecotus auritus	LSU											2/2
		SSU											1/2
		Carrier											2/2
Captive megachiropters of the Old World	*Rousettus aegyptiacus*	LSU										4/17	4/17
		SSU										6/17	6/17
		Carrier										6/17	6/17
	Pteropus rodriensis	LSU										0/44	0/44
		SSU										0/44	0/44
		Carrier										0/44	0/44
All areas	All species	Positive *Pneumocystis* samples/total samples	2/10	2/6	4/17	6/21	3/5	20/29	8/16	6/13	14/38	6/61	71/216
		Pneumocystis carriage (%)	20.0	33.3	23.5	28.6	60.0	69.0	50.0	46.2	36.8	9.8	32.9

[a] DNA lung samples were screened by nested PCR at either the mtLSU rRNA (LSU) or mtSSU rRNA (SSU) locus or both loci for the presence of *Pneumocystis*. Results of *Pneumocystis* carriage in bats are given according to bat species, targeted locus, and sampling area. LSU, samples that are positive at the LSU locus, and some of these samples can also be positive at SSU; SSU, samples that are positive at the SSU locus, and some of these samples can also be positive at LSU; Carrier, a bat lung sample was considered positive for *Pneumocystis* DNA when nested PCR was positive at either mtLSU rRNA or mtSSU rRNA or at both loci.

The case of *Tadarida brasiliensis*, influence of elevation. Focusing on a single species of bats allowed us to remove all the behavioral factors, to evaluate only the environmental factors. The largest number of sampled bats belonged to the species *Tadarida brasiliensis*. We have collected 79 specimens of *T. brasiliensis* in five locations from North and South America (Table 3, Fig. 1): four regions in Mexico (La Trinitaria cave Chiapas [1,460 m], Isla de Janitzio cave Michoacán [2,120 m], El Salitre cave Hidalgo [1,320 m], and La Boca cave Nuevo León [445 m to 600 m]) and one province in Argentina (Dique Escaba, Tucumán Province [650 m]). The *Pneumocystis* detection rate in *T. brasiliensis* was related to elevation ($P = 0.04$): in bats coming from locations situated lower than 800 m, the *Pneumocystis* DNA carriage rate was 5-fold higher than in bats coming from locations situated at an elevation superior to 800 m (Tables 7 and 8). On the other hand, the climate (humidity and heat) did not seem to have an impact on *Pneumocystis* DNA carriage.

***Pneumocystis* genetic diversity in bats.** The genetic diversity of *Pneumocystis* from bats is examined by analyzing mtLSU rRNA and mtSSU rRNA sequences. PCR products ranged from 252 to 333 bp (nested PCR at the mtLSU rRNA gene) and from 334 to 480 bp (nested PCR at the mtSSU rRNA gene). The sequences under analysis are part of the ribosome and are coded by the mitochondrial genome, and no introns were detected in the mitochondrial genome (82). Direct sequencing of PCR products revealed that each *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequence could be associated with a unique bat species (Tables 9 and 10; Fig. 2 and 3). Comparison of the mtLSU rRNA aligned sequences was carried out on 458 positions, including gaps, for a total of 14 taxa: 12 original sequences and two sequences already published, *Pneumocystis murina* (GenBank accession AF257179) and *Pneumocystis oryctolagi* (GenBank accession S42915), were chosen as outgroups. For the mtSSU rRNA locus, comparison of the aligned sequences was carried out on 530 positions, including gaps, for a total of 17 taxa: 15 original sequences and two sequences already published, *Pneumocystis* f. sp. *Lepus europaeus* (GenBank accession no. JF431106) and *P. oryctolagi* (47), were chosen as outgroups. Genetic divergence between *Pneumocystis* organisms isolated from different bat species, as assessed by *Pneumocystis* mtLSU rDNA or mtSSU rDNA sequence divergence, varied from 0.35 to 26.88% (Tables 9 and 10).

With regard to the *Pneumocystis* mtLSU rRNA locus, we found 12 sequences in the lungs of 9 bat species (Table 9, Fig. 2). In 6 bat species (*Myotis californicus*, *Nyctalus noctula*, *Artibeus hirsutus*, *Eptesicus serotinus*, *Pipistrellus pipistrellus*, *Plecotus auritus*), one specific *Pneumocystis* mtLSU rDNA sequence per species was detected (Fig. 2). In contrast, *Glossophaga soricina* and *Tadarida brasiliensis* species each harbored 2 variants of *Pneumocystis* mtLSU rDNA depending on the collection area (31). The mtLSU rDNA sequences of *Pneumocystis* detected in *G. soricina* from French Guyana and Mexico diverged by 0.35%, corresponding to one nucleotide difference (Table 9), and grouped together on a phenetic branch that is supported by a 100% bootstrap value (Fig. 2). Likewise, a relatively close order of divergence (0.78%) was found between *Pneumocystis* mtLSU rDNA sequences amplified from *T. brasiliensis* samples collected from either Argentinean or Mexican colonies (Tables 9 and 11, Fig. 1). Consistently, these sequences are included in the same group supported by a 100% bootstrap value in phenetic analysis (Fig. 2). Interestingly, mtLSU rDNA

TABLE 4 Ecological data on wild microchiropters and *Pneumocystis* carriage rates[a]

Characteristic	No. (%)		
	Whole sample ($n = 155$)	Noncarriers ($n = 90$)	Carriers ($n = 65$)
Bat colony size			
Big	92	55 (59.8)	37 (40.2)
Medium	33	21 (63.6)	12 (36.4)
Small	26	11 (42.3)	15 (57.7)
Migration			
Yes	113	68 (60.2)	45 (39.8)
No	41	21 (51.2)	20 (48.8)
World			
Old world	38	24 (63.2)	14 (36.8)
New world	117	66 (56.4)	51 (43.6)
Diet			
Insectivorous	133	80 (60.2)	53 (39.8)
Nectarivorous	16	7 (43.8)	9 (56.2)
Frugivorous	6	3 (50.0)	3 (50.0)
Day roost			
Tree-dwelling, forest	25	17 (68.0)	8 (32.0)
Cave	118	67 (56.8)	51 (43.2)
Anthropophilic	12	6 (50.0)	6 (50.0)
Mating system			
Multi-male/multi-female	84	46 (54.8)	38 (45.2)
Harem	33	18 (54.6)	15 (45.4)
Lek	22	16 (72.7)	6 (27.3)
Contact in the colony			
Crowding	132	75 (56.8)	57 (43.2)
Without contact	11	10 (90.9)	1 (9.1)
Climate			
1 = tropical	10	8 (80.0)	2 (20.0)
2 = warm temperate	49	34 (69.4)	15 (30.6)
3 = warm semi-arid	29	9 (31.0)	20 (69.0)
4 = subtropical	16	8 (50.0)	8 (50.0)
5 = equatorial	13	7 (53.9)	6 (46.2)
6 = temperate	38	24 (63.2)	14 (36.8)
Humidity			
No (climates 2, 3, 6)	116	67 (57.8)	49 (42.2)
Yes (climates 1, 4, 5)	39	23 (59.0)	16 (41.0)
Heat			
No (climates 2, 6)	87	58 (66.7)	29 (33.3)
Yes (climates 1, 3, 4, 5)	68	32 (47.1)	36 (52.9)
Elevation			
≤800 m (origins d, f, g, h)	96	48 (50.0)	48 (50.0)
>800 m (origins a, b, c, e, i)	59	42 (71.2)	17 (28.8)

[a] Bat colony size was scored as follows: small, 10 to 30 individuals; medium, 31 to 200 individuals; big, >200 individuals. Data from *P. rodricencis* were excluded from analysis. Origin: a, La Trinitaria cave, Chiapas, Mexico; b, Isla de Janitzio cave, Michoacán, Mexico; c, El Salitre cave, Hidalgo, Mexico; d, La Boca cave, Nuevo León, Mexico; e, Juxtlahuaca cave, Colotlipa Guerrero, Mexico; f, Dique Escaba, Tucumán, Argentina; g, French Guyana; h, Natural History Museum of Bourges, France; i, El Salitre cave, Santa Rosa, Morelos, Mexico.

TABLE 5 *Pneumocystis* carriage in bats: influence of ecological and behavioral factors (univariate analysis)[a]

Characteristic	OR	95% CI	*P*
Bat colony size			
Medium or big	1		
Small	3.259	1.038–10.230	0.04
Migration			
Yes	1		
No	2.959	0.997–8.779	0.05
Area			
Old World	1		
New World	1.213	0.170–8.659	0.81
Diet			
Insectivorous or frugivorous	1		
Nectarivorous	3.948	0.864–18.045	0.07
Day roost			
Tree-dwelling, forest	1		
Cavernicole	1.299	0.286–5.902	0.71
Anthropophilic	2.148	0.518–8.903	0.29
Mating system			
Lek	1		
Harem	1.905	0.512–7.080	0.33
MM-MF	1.827	0.477–6.897	0.37
Contact in the colony			
No	1		
Yes	11.387	1.154–112.354	0.038
Humidity			
Yes	1		
No	1.064	0.285–3.972	0.92
Heat			
No	1		
Yes	2.030	0.685–6.020	0.16
Elevation			
>800 m	1		
≤800 m	2.448	0.919–6.518	0.07

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

polymorphism was also detected in *R. aegyptiacus* (from the La Palmyre Zoo), with two *Pneumocystis* variants that diverged by a much higher percentage (19.91%; Table 9). Both Megachiroptera-derived *Pneumocystis* organism sequences were placed in a basal position according to the phenetic analysis (Fig. 2). All mtLSU rDNA sequences of *Pneumocystis* organisms from Microchiroptera were included in the same clade. In addition, *Pneumocystis* sequences derived from the New World bats and those from the Old World bats are mixed (Fig. 2).

Regarding the *Pneumocystis* mtSSU rRNA locus, we isolated 15 sequences from 11 bat species (Table 10, Fig. 3). In 8 bat species (*M. californicus*, *G. soricina*, *N. noctula*, *A. hirsutus*, *E. serotinus*, *Plecotus austriacus*, *P. auritus*, *Pteronotus parnellii*), we identified one specific *Pneumocystis* sequence per host species, while we detected two highly divergent mtSSU rRNA sequences in *R. aegyptiacus* (21.72%), two weakly divergent sequences in *P. pipistrellus*

TABLE 6 *Pneumocystis* carriage in bats: influence of ecological and behavioral factors (multivariate analysis)[a]

Characteristic	OR	95% CI	*P*
Migration			
Yes	1		
No	4.759	1.15–20.310	0.036
Contact in the colony			
No	1		
Yes	33.313	2.687–412.963	0.007

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

TABLE 7 *Pneumocystis* carriage in *Tadarida brasiliensis*: influence of climate and elevation

Characteristic[a]	No. (%)		
	Whole sample ($n = 155$)	Noncarriers ($n = 90$)	Carriers ($n = 65$)
Climate			
1 = tropical	10	8 (80.0)	2 (20.0)
2 = warm temperate	26	19 (73.1)	7 (26.9)
3 = warm semi-arid	27	8 (29.6)	19 (70.4)
4 = subtropical	16	8 (50.0)	8 (50.0)
Humidity			
No (climates 2, 3, 6)	53	27 (50.9)	26 (49.1)
Yes (climates 1, 4, 5)	26	16 (61.5)	10 (38.5)
Heat			
No (climates 2, 6)	26	19 (73.1)	7 (26.9)
Yes (climates 1, 3, 4, 5)	53	24 (45.3)	29 (54.7)
Elevation			
≤800 m (origins d, f)	43	16 (37.2)	27 (62.8)
>800 m (origins a, b, c)	36	27 (75.0)	9 (25.0)

[a] Origin: a, La Trinitaria cave, Chiapas, Mexico; b, Isla de Janitzio cave, Michoacán, Mexico; c, El Salitre cave, Hidalgo, Mexico; d, La Boca cave, Nuevo León, Mexico; f, Dique Escaba, Tucumán, Argentina.

(0.46%), and three lowly divergent sequences in *T. brasiliensis* (0.89% to 1.83%) (Tables 10 and 11). In the phenetic tree displaying mtSSU rDNA sequences, both sequences of macrochiroptera-derived *Pneumocystis* organisms are mixed with Microchiroptera-derived *Pneumocystis*, but the bootstrap values are not significant (Fig. 3). The *Pneumocystis* sequences derived from the same host species (*T. brasiliensis* and *P. pipistrellus*) clustered together with 100% bootstrap values (Fig. 3).

Geography seemed to have a structuring effect on *Pneumocystis* mtSSU rRNA polymorphism. Thus, the two *Pneumocystis* se-

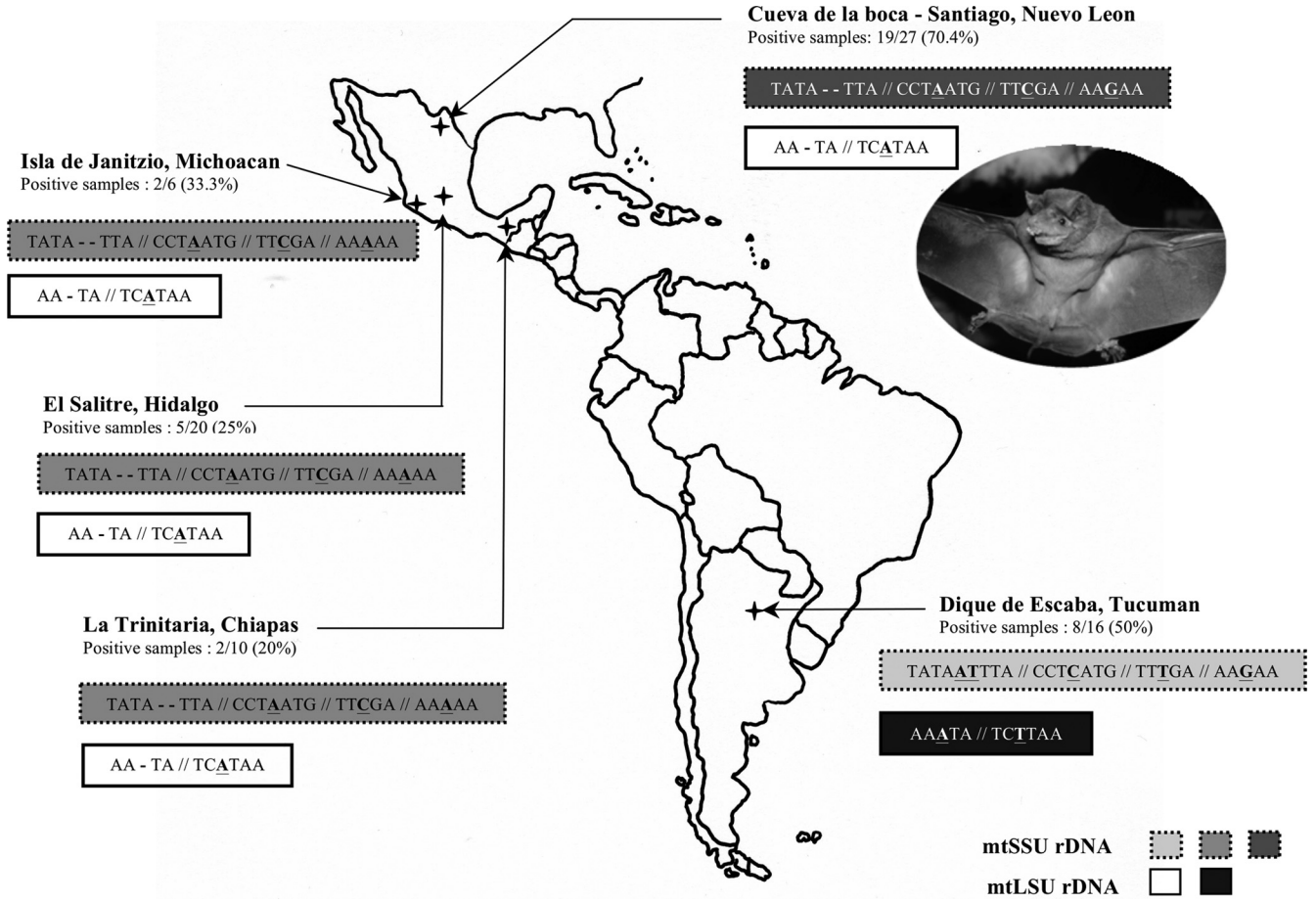

FIG 1 Genetic polymorphism of *Pneumocystis* isolates detected in the lungs of *Tadarida brasiliensis* bats sampled from North and South America. For each geographic location, mtLSU rDNA and mtSSU rDNA *Pneumocystis* polymorphic sequences are indicated and framed: two mtLSU rDNA variants and three mtSSU rDNA variants. The number of positive samples over total number of analyzed samples is also indicated below each location. The photo represents one male bat belonging to the species *Tadarida brasiliensis* and originating from Dique Escaba in Argentina.

TABLE 8 *Pneumocystis* carriage in *Tadarida brasiliensis*: influence of climate and elevation (univariate analysis)[a]

Characteristic	OR	95% CI	*P*
Humidity			
Yes	1		
No	1.450	0.074–28.587	0.73
Heat			
No	1		
Yes	2.521	0.176–36.122	0.36
Elevation			
>800 m	1		
≤800 m	5.049	1.096–23.252	0.04

[a] OR, odds ratio; CI, confidence interval. Significant results at $P = 0.05$.

quences isolated from *P. pipistrellus* (number reference P3 and P6) identified two Metropolitan French Departments (Haute Marne [51] and Cher [17], respectively) (Table 10). Likewise, the 3 *Pneumocystis* sequences isolated from *T. brasiliensis* identified 3 geographical areas: (i) Michoacán-Hidalgo-Chiapas (Mexico), (ii) Nuevo León (Mexico), and (iii) Tucumán (Argentina) (Fig. 1, Tables 10 and 11).

DISCUSSION

Detection of *Pneumocystis* DNA in the lungs of bats. The rate of the *Pneumocystis* carriage is probably underestimated in this study. Moreover, the abundance of *Pneumocystis* targets (mtLSU rRNA or mtSSU rRNA) in the bat samples studied here is generally low. Thus, chance can determine whether or not *Pneumocystis* gDNA is introduced in a given PCR. This random sampling could explain why some samples produced a positive amplification with one set of primers but not both.

Positive amplification requiring two PCR rounds is usually considered a case of *Pneumocystis* carriage (29, 30), i.e., healthy carriers harboring a low burden of *Pneumocystis* organisms. In the present work, the fact that single *Pneumocystis* PCR was negative in all cases suggests that no animal was heavily infected or developed pneumocystosis. In contrast, *Pneumocystis* carriage was a frequent event (about 33% of examined bats). Likewise, Laakkonen et al. reported high rates of *Pneumocystis* carriage (even if they used histological methods) in wild small mammals from California, but no animal showed histopathological changes typical of PcP (53). Indeed, PcP cases were only rarely reported in wild mammals (3).

Thus, mammals seem to usually develop mild though quite frequent subclinical *Pneumocystis* lung infections (17), suggesting that these microfungi develop efficient airborne circulation in natural ecosystems (3). Severe PcP would therefore be, as in humans, a rare event in the natural history of *Pneumocystis* infection (3).

This view is further consistent with the fine adaptation of *Pneumocystis* organisms to the alveolar microenvironment (8, 16, 23, 26), likely resulting from *Pneumocystis*-host species coevolution (46).

Consistently, it was experimentally shown that *Pneumocystis* organisms were able to replicate in the lungs of healthy hosts (16), which can subsequently transmit the microfungi by airborne route to susceptible or immunocompetent hosts (32, 39). These observations showed that healthy carriers could behave as a reservoir of *Pneumocystis* species, playing a critical role in the airborne circulation of *Pneumocystis* organisms in host populations (16, 17, 29, 30).

Prevalence of *Pneumocystis* colonization in bats. The reported frequency of *Pneumocystis* DNA detection varied markedly between bat species (Table 2). This finding was in accordance with previous studies, where important differences in *Pneumocystis* prevalence between mammal species have been reported (52, 53). In micromammals collected in France (58), rodent infection rates varied according to the species (e.g., 67% in *Apodemus sylvaticus*, 78% in *Eliomys quercinus*). In the same study, the *Pneumocystis* global prevalence was as high as 68%. In Thailand, 58% of the collected *Rattus norvegicus* specimens harbored *Pneumocystis* DNA (17), but infection rates in other rodents from this country

TABLE 9 Divergence matrix of *Pneumocystis* mtLSU rDNA sequences amplified from 9 bat species (GenBank accession no. JQ039397 and JQ061293 to JQ061303), one rodent (*P. murina* from *Mus musculus*, GenBank accession no. AF257179), and one lagomorph species (*P. oryctolagi* from *Oryctolagus cuniculus*, GenBank accession no. S42915)[a]

	% of divergence from:												
Host species	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *G. soricina* FG													
2. *G. soricina* JG	00.35												
3. *A. hirsutus* ES	09.64	08.87											
4. *T. brasiliensis* M	11.04	10.59	17.34										
5. *T. brasiliensis* A	10.35	09.92	17.57	00.78									
6. *M. californicus* LB	11.88	12.33	14.82	08.15	09.21								
7. *P. pipistrellus* B(P6)	14.11	13.12	14.37	12.15	12.72	07.42							
8. *N. noctula* B(NO19)	10.81	10.05	13.52	09.48	09.53	05.23	02.54						
9. *E. serotinus* B(S1)	11.22	10.57	13.84	06.91	07.91	02.00	06.83	04.76					
10. *P. auritus* B(OR3)	12.75	12.03	15.09	08.93	09.30	03.53	05.44	05.52	01.94				
11. *R. aegyptiacus* P(34)	15.60	15.19	20.58	18.30	18.40	20.19	23.19	17.68	18.22	20.14			
12. *R. aegyptiacus* P(13)	16.41	16.57	19.38	16.94	16.72	15.28	19.46	15.19	15.13	16.47	19.91		
13. *P. murina*	23.48	25.19	30.24	17.95	18.10	16.06	28.32	25.98	25.34	25.16	21.89	27.01	
14. *P. oryctolagi*	19.91	21.96	28.98	18.24	17.75	16.04	27.29	24.37	26.72	26.19	18.59	28.66	17.48

[a] Numbered column headings (1 to 13) correspond to numbered sources of host species DNA in the first column. Next to each bat species Latin name, the letter and number codes indicate geographic origin and reference number of samples, respectively. FG, French Guyana; B, Bourges, France; NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; LB, La Boca; JG, Juxtlahuaca Grotto, Mexico; ES, El Salitre, Mexico; P, Zoological Park of La Palmyre, France. Letters and numbers in parentheses indicate a particular sample.

TABLE 10 Divergence matrix of *Pneumocystis* mtSSU rDNA sequences amplified from 15 bats (GenBank accession no. JQ061304 to JQ061318) and 2 lagomorph species (*P. oryctolagi* [47]; *P.* f. sp. *Lepus europaeus*, GenBank accession no. JF431106)[a]

Host species	% of divergence from:															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *P. pipistrellus* B(P6)																
2. *P. pipistrellus* B(P3)	00.46															
3. *N. noctula* B(NO19)	07.21	06.52														
4. *E. serotinus* B	13.34	13.26	14.65													
5. *P. auritus* B(OR3)	12.64	12.01	14.15	02.71												
6. *M. californicus* NL	12.05	11.71	13.45	04.29	02.73											
7. *P. austriacus* B	12.34	11.57	14.23	03.24	00.49	03.06										
8. *T. brasiliensis* NL	17.25	14.63	14.58	13.31	13.43	13.06	13.53									
9. *T. brasiliensis* M	17.10	14.54	14.50	13.57	13.68	13.29	13.78	00.89								
10. *T. brasiliensis* A	18.06	15.05	15.76	13.64	14.00	13.23	13.71	01.53	01.83							
11. *G. soricina* JG	14.76	13.67	15.02	12.68	12.44	13.25	12.72	16.28	15.81	16.24						
12. *A. hirsutus* ES	13.91	13.46	14.71	12.01	11.66	11.34	11.75	15.36	14.92	15.77	08.41					
13. *P. parnellii* ES	13.83	14.04	14.64	13.71	14.33	13.36	13.74	16.93	16.45	16.62	08.81	08.45				
14. *R. aegyptiacus* P(12)	21.50	20.68	21.83	17.84	19.05	18.23	18.32	20.99	20.38	19.96	14.38	13.93	12.87			
15. *R. aegyptiacus* P(26)	23.85	23.19	24.38	23.31	23.36	22.06	26.88	21.84	21.34	21.29	15.59	12.58	16.58	21.72		
16. *P. oryctolagi*	25.12	25.25	25.79	23.97	23.34	23.60	23.48	23.32	23.50	24.15	18.50	16.33	20.22	23.02	24.77	
17. *P.* f. sp. *L. europaeus*	19.30	19.01	20.30	18.10	18.21	17.83	17.67	17.43	17.41	17.16	16.30	13.82	18.43	19.29	20.87	09.76

[a] Numbered column headings (1 to 16) correspond to numbered sources of host species DNA in the first column. Next to each bat species Latin name, the letter and number codes indicate geographic origin and reference number of samples, respectively. B, Bourges, France; NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; JG, Juxtlahuaca Grotto, Mexico; ES, El Salitre, Mexico; P, Zoological Park of La Palmyre, France. Letters and numbers in parentheses indicate a particular sample.

were quite divergent (M. Chabé and S. Morand, unpublished data). In primates, prevalence of *Pneumocystis* reached 33.6% in healthy macaques (*Macaca fascicularis*) maintained in partial release (30) and 26.5% in captive primates of 26 species taken as a whole (29).

All samples from megachiropters were collected from captive animals, and those from microchiropters were collected from wild animals. Thus, the significantly different *Pneumocystis* rates between megabats and microbats found in the present work may stem from bat taxonomy and/or from the captive/wild state of the

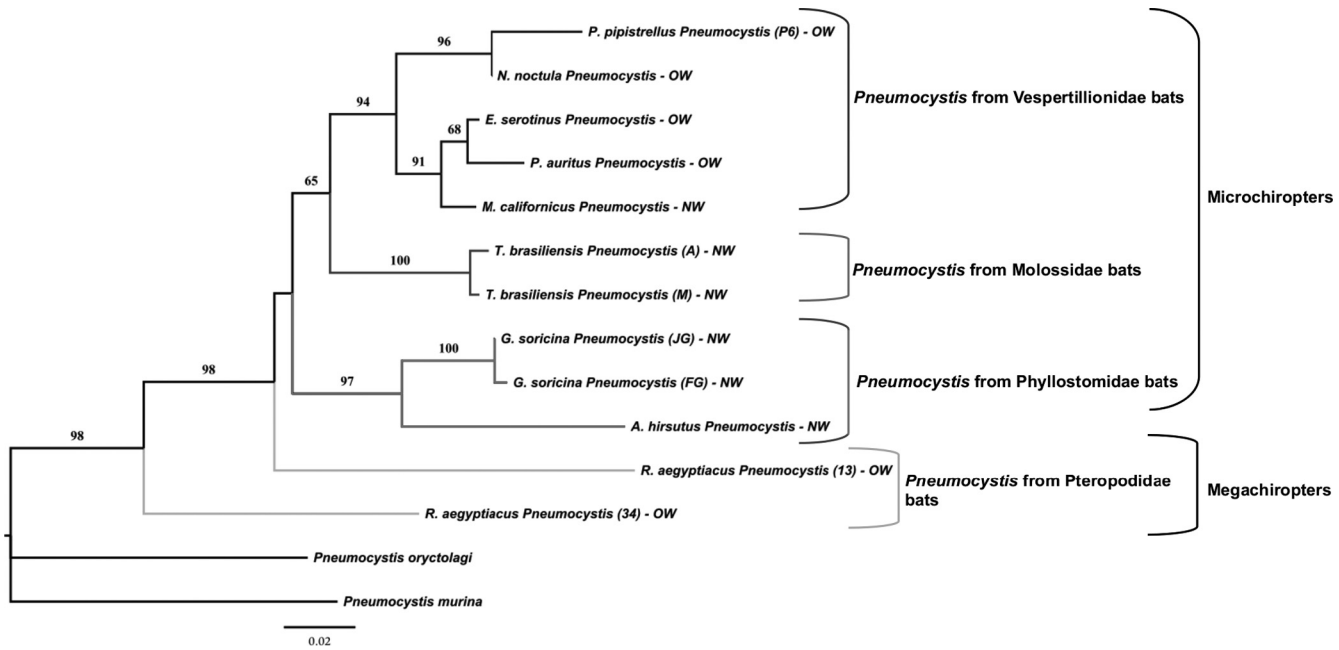

FIG 2 Phenetic relationships of *Pneumocystis* organisms from bat species inferred from mtLSU rDNA sequences. The phylogram presented resulted from bootstrapped data sets obtained by using BIONJ analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications. Bootstrap values below 50% are not displayed. Branch lengths correspond to the total nucleotide changes assigned to each branch by PAUP 4.0. *Pneumocystis* from rabbit (*P. oryctolagi*, GenBank accession S42915) and from mouse (*P. murina*, GenBank accession AF257179) were chosen as outgroups. Letter and number codes indicate geographic origin and reference number of samples, respectively. M, Michoacán, Mexico; A, Argentina; JG, Juxtlahuaca Grotto, Mexico; OW, Old World; NW, New World. Letters and numbers in parentheses indicate a particular sample (P6, 13, 34).

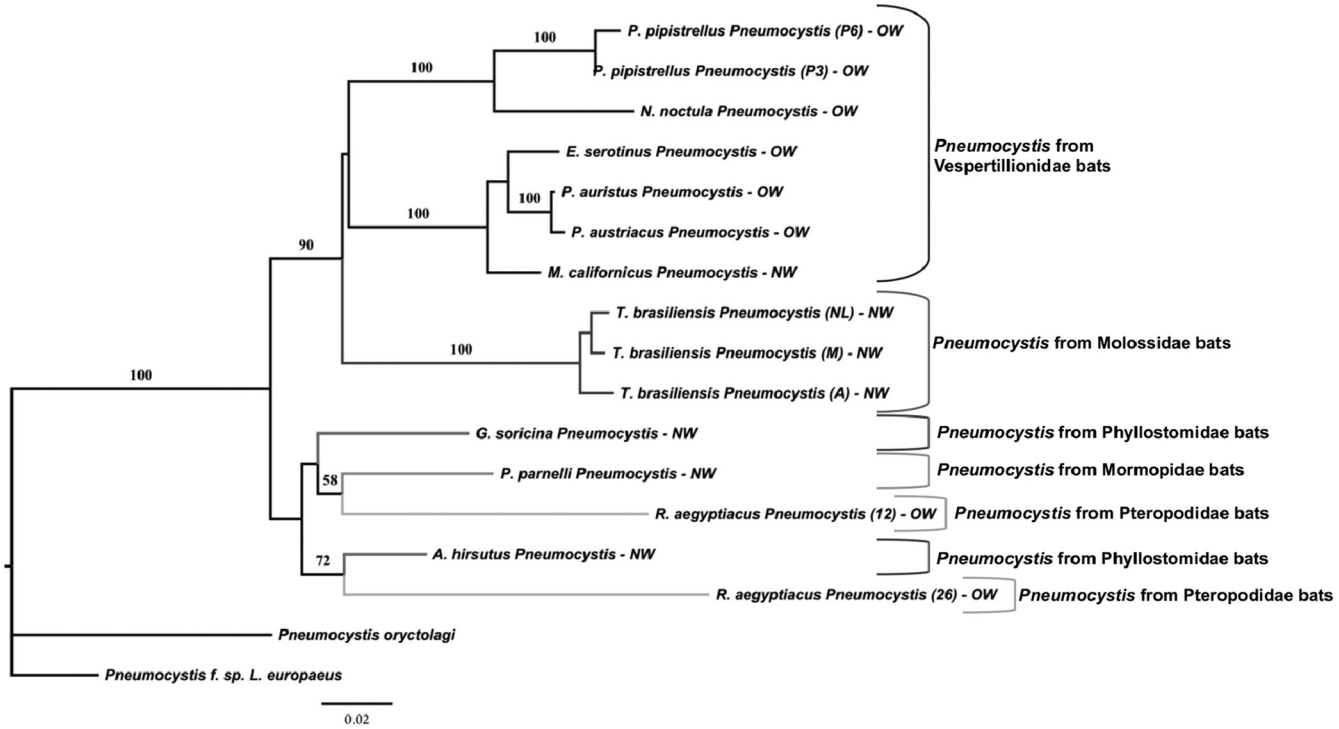

FIG 3 Phenetic relationships of *Pneumocystis* organisms from bat species inferred from mtSSU rDNA sequences. The phylogram presented resulted from bootstrapped data sets obtained by using BIONJ analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications. Bootstrap values below 50% are not displayed. Branch lengths correspond to the total nucleotide changes assigned to each branch by PAUP 4.0. *Pneumocystis* from rabbit (*P. oryctolagi* [47]) and from hare (*P.* f. sp. *Lepus europaeus*, GenBank accession JF431106) were chosen as outgroups. Letter and number codes indicate geographic origin and reference number of samples, respectively. NL, Nuevo León, Mexico; M, Michoacán, Mexico; A, Argentina; OW, Old World; NW, New World. Letters and numbers in parentheses indicate a particular sample (P3, P6, 12, 26).

animals as well. Within megachiropters, two species, namely, Egyptian roussette (*R. aegyptiacus*) and flying fox (*P. rodricensis*), were sampled, revealing strikingly different infection rates. *Pneumocystis* DNA was detected in 35.3% of Egyptian roussettes, while no *P. rodricensis* specimen was found to be carrying *Pneumocystis*. Egyptian roussette, a fruit bat species of the Old World, can be found throughout Africa (except in the desert regions of the Sahara) and Middle East, as far as Pakistan and Northern India. In this extensive distribution area, Egyptian roussettes exist as a relatively large wild population (12). In contrast, *P. rodricensis* is restricted to Rodrigues, the smallest of the Mascarene islands, and it is considered an endangered species mainly because of deforestation and cyclones that devastate the area from time to time. Only about 4,000 individuals are thought to exist (48). Rodrigues flying foxes seem to be the sole mammal species studied so far that revealed to be entirely negative for *Pneumocystis* DNA. A first hypothesis is that *Pneumocystis* DNA sequences at the studied loci would be so divergent that they could not be detected by our techniques. Another hypothesis is that the animals used to set up the captive population were totally free of *Pneumocystis* and thus suggests that *Pneumocystis* prevalence is very low or absent in wild Rodrigues flying fox populations or that the sampling of breeders that set up the colony was too small (40 individuals from Jersey Zoological Park). A third hypothesis is that *Pneumocystis* organisms had never colonized or had been lost by this insular host species. Furthermore, Rodrigues flying foxes and Egyptian rous-

TABLE 11 *Pneumocystis* polymorphism at the mtSSU and mtLSU rRNA loci in DNA lung samples from *Tadarida brasiliensis*[a]

Sample origin	mtSSUrRNA sequence	mtLSUrRNA sequence
Mexico		
Cueva de la Boca, Nuevo León	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**G**AA	AA-AT//TC**A**TAA
Isla de Janitzio, Michoacan	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
El Salitre, Hidalgo	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
La Trinitaria, Chiapas	TATA–TTA//CCT**A**ATG//TT**C**GA//AA**A**AA	AA-AT//TC**A**TAA
Argentina		
Dique de Escaba, Tucuman	TATA**AT**TTA//CCT**C**ATG//TT**T**GA//AA**G**AA	AA**A**AT//TC**T**TAA

[a] Results of *Pneumocystis* polymorphism in these bat specie are given according to sampling area. Bold nucleic acids indicate the mutations. *Pneumocystis* polymorphic sequences show two mtLSUrDNA variants (one from Mexico and the other from Argentina) and three mtSSUrDNA variants (two from Mexico and the third from Argentina).

settes are cohoused in the same enclosure in La Palmyre Zoological Park, and the cross-infection does not seem to occur between these 2 host species of megachiropters. This result confirms the strong host specificity in *Pneumocystis* strains (3, 27, 41).

If we exclude the absence of *Pneumocystis* DNA in Rodrigues flying foxes, the global infection rate is close between microchiropters (41.9%) and megachiropters (35.3%), suggesting a similar circulation pattern of *Pneumocystis* within both groups of either captive or wild chiropters. Likewise, *Pneumocystis* prevalence was found to be identical in bats from the New and Old Worlds. This may indicate similar intensity of circulation of *Pneumocystis* organisms in bat populations and a comparable pattern of *Pneumocystis* host-to-host transmission worldwide.

Crowding in the bat colony and the infection source issue. *Pneumocystis* rates were found to increase when the individuals of the colony are in close contact independently of colony size (Tables 5 and 6). For example, in *Natalus stramineus*, a cave-dwelling bat living in big noncrowding colonies, the *Pneumocystis* DNA carriage rate is low (12.5%) (Table 2). This could be explained by the fact that bats hang individually without contact with each other, keeping a distance of 5 to 50 cm between themselves (90). While in *Tadarida brasiliensis*, a cave-dwelling bat living in big crowding colonies, the carriage rate is high (45.6%) (Table 2). A recent study in human beings showed that the level of *Pneumocystis jirovecii* DNA in exhaled air from infected patients decreased with increased distance from the patients (18). Furthermore, it has been shown that *Pneumocystis* organisms were able to multiply transiently in the lungs of immunocompetent hosts and to transmit the infection to either susceptible or immunocompetent hosts by the airborne route (16, 32, 39). Crowding could therefore favor *Pneumocystis* host-to-host airborne transmission in bats or in other mammals. This observation raises the question of the *Pneumocystis* infection source: do *Pneumocystis* hosts contract the infection from carrier (or infected) hosts or from hypothetical environmental forms of development?

The high rate of *Pneumocystis* carriage found in *T. brasiliensis* (Tables 2 and 3) suggests a highly active interhost airborne transmission. An intensive circulation of *Pneumocystis* organisms was also reported within the members of a social organization of healthy macaques (30). The occurrence of clustered cases of PcP in hospitals (68) and the reported evidence of human-to-human *Pneumocystis* transmission in the community (76) further suggest that interhost transmission could also be highly active in humans. In addition, the fact that *Pneumocystis* organisms are able to dwell and replicate in the lungs of immunocompetent hosts points out the healthy carrier hosts as the infection source and reservoir for *Pneumocystis* species (16).

Another underlying factor which may affect transmission efficiency of *Pneumocystis* is the proportion of newly born animals in the colony. In human communities, infants could constitute a major reservoir for *Pneumocystis* organisms (67, 94), and some data collected in other mammals, like domestic or wild rabbits (26, 40), pigs (50), and macaques maintained in partial release (30), seem to strengthen this hypothesis. A survey scheduled just after the breeding period in a bat colony could provide data on the levels of infection of young bats and on their potential role in the transmission of *Pneumocystis* organisms within the colony.

Alternatively, other observations suggested that *Pneumocystis* infection could be contracted from undefined environmental sources (45). *Pneumocystis* DNA was identified in air and water samples (7, 13, 73, 96). Wakefield was able to detect DNA from rat and human-derived *Pneumocystis* in air samples from rural locations in the United Kingdom (96). Furthermore, outdoor activities, such as gardening, camping, or hiking, have been reported to be associated with PcP in HIV-infected adults (65). However, on the whole, the active airborne host-to-host circulation of *Pneumocystis* organisms and the widening of the parasite reservoir to immunocompetent hosts (16, 24, 39) render the hypothesis of *Pneumocystis* environmental forms of infection less and less plausible. The detection of *Pneumocystis* DNA in air sampled either from the room of PcP patients (7, 71) or from facilities housing laboratory animals with PcP (54, 70) could attest to *Pneumocystis* dissemination with the exhaled air of infected hosts and, therefore, potential transmission to other hosts (7, 70, 83).

In the case of *T. brasiliensis*, the existence of migratory and nonmigratory populations could render the circulation of *Pneumocystis* organisms in the population more complex (77). Thus, migratory bats could carry significantly less *Pneumocystis* organisms than sedentary bats. Nevertheless, it is possible that the increased physiological stress and immunocompromise associated with migration might increase fungal growth within bats and increase transmission (4). But new research has also shown that migration allows hosts to escape from infected habitats, reduces disease levels in successful migrants when infected animals do not migrate successfully, and may lead to the evolution of less-virulent pathogens (4). So, between intervals of habitat use, unfavorable conditions (such as a lack of hosts) could eliminate most parasites, resulting in hosts returning to these habitats after a long absence to encounter largely disease-free conditions (4). Furthermore, parasites that decline in response to host migration may include specialist pathogens, as well as those with transmission stages that can build up in the environment (4). Actually, we can assume that when migrating, bats are less exposed to high fungal loads because (i) crowding is less important, (ii) the period of reproduction is over, and (iii) changing roosts implies renewal of ambient air and, likely, exposure to lower numbers of *Pneumocystis* infective airborne forms.

Climatic factors and altitude. Temperature and humidity did not seem to impact the *Pneumocystis* DNA carriage in bats. However, the climatic factors used in our study represent global trends and did not necessarily reflect the actual conditions inside the caves. Samples from *T. brasiliensis* represent the biggest collection in the present study. In this species, *Pneumocystis* carriage rates ranged from 25% to 70% in five Latin American regions (Fig. 1). The factors accounting for such variability remain to be explored: location and sampling season but also direct exposure to weather changes versus living in a cave, where environmental conditions can be remarkably stable (66). Some studies explored the impact of climatic factors on *Pneumocystis* carriage in other host species. In Finland, an influence of seasonal changes on *Pneumocystis* carriage was reported in wild rodents and insectivores (52). In these host species, the highest prevalence of *Pneumocystis* organisms in lung samples was reported in late autumn (November), when the precipitation rate was high. The impact of environmental factors on *Pneumocystis* carriage was also evaluated in immunocompetent macaques (*Macaca fascicularis*) maintained in partial release (28). The number of macaques with detectable *Pneumocystis* DNA (assessed by nested PCR from deep nasal swab samples) was apparently correlated with mean precipitation rates (28). However, behavioral factors could also intervene. Actually, when it is rain-

ing, macaques group together, some against one another, or enter their shelters, which increases crowding and consequently the *Pneumocystis* host-to-host airborne transmission. Elsewhere, the detection rate of *Pneumocystis* DNA was higher in primates, which died (from any cause) during spring or summer (33.3%) rather than during the colder seasons (19.5%), though these differences did not reach statistical significance (29).

Regarding PcP, spontaneous pneumocystosis in domestic rabbits at weaning was usually found to be markedly less extensive in summer than in winter (74). A few studies reported seasonal variations in the occurrence of human cases of pneumocystosis (higher incidence of PcP with higher temperatures in London, Geneva, and Munich) (61, 84, 92). In contrast, surveys from Spain (93) or the United Kingdom (55) showed PcP incidence to peak in the winter months, as it occurs with other infectious respiratory diseases. In the Spanish survey, PcP incidence was negatively correlated with the mean temperature but not with rainfall activity or wind strength (93). These studies indicated that seasonal variation in PcP incidence may exist, albeit to different extents or tendencies. However, nonclimatic factors, such as human behavior or leisure activities, could also be associated with seasonal change and indirectly influence the PcP incidence (84). In bats, the present data suggest that host behavior (migration, crowding) as well as environmental factors such as climate or geographical area may influence *Pneumocystis* carriage. However, the roosting site did not seem to have a direct impact on the *Pneumocystis* DNA carriage, the elevation excepted. Altitude exposes the body to a set of constraints, the most important of which is hypoxia. The almost exponential decline of the atmospheric pressure is accompanied by a parallel decline of the oxygen pressure in the inspired air, which induces hyperventilation. Thus, altitude could directly impact on respiratory physiology and host defense mechanisms (95) and indirectly act on the development of *Pneumocystis* organisms in the alveolar microenvironment. These aspects were not explored in bats. In nonimmunocompromised laboratory rats, hypobaric hypoxia weakened host immune mechanisms and significantly impaired the surfactant composition. Such changes were not enough, however, to favor *Pneumocystis* growth or to inhibit *Pneumocystis* clearing from their lungs (95).

Genetic variability. For each bat species carrying *Pneumocystis* DNA, at least one novel sequence was amplified at both mtLSU rRNA and/or mtSSU rRNA loci, suggesting that each species of bats could be harboring specific species of *Pneumocystis* (Fig. 2 and 3). The *Pneumocystis* sequences amplified from bat lung samples were markedly different from those of other host species registered in the GenBank database. Furthermore, no cross-infection occurred between Rodrigues flying foxes and Egyptian roussettes, although they were cohoused in the same enclosure. Present data strengthened therefore the host specificity concept of *Pneumocystis* (1, 3, 38) and were consistent with the strong host specificity demonstrated in previous studies dealing with other mammals (27, 41). A first investigation of primate-derived *Pneumocystis* demonstrated that *Pneumocystis* phylogeny mirrors its host phylogeny, suggesting a long-range physiological and genetic adaptation process leading to cospeciation (46). *Pneumocystis* species may have evolved together with their hosts. Likewise, present data showed that genetic divergence in bat-derived *Pneumocystis* organisms parallels phylogenetic divergence existing among the corresponding host, also suggesting coevolution (27, 41).

Unexpectedly, in the megabat *R. aegyptiacus*, we found two sequences highly divergent from each other at both mtLSU rRNA and mtSSU rRNA loci (19.9% and 21.7%, respectively). Interestingly, this divergence was comparable to divergence existing between *Pneumocystis* organisms harbored by *M. californicus* and rabbits (16.0% at the mtLSU rRNA locus and 23.6% at the mtSSU rRNA locus), as well as between *G. soricina* and rabbits (19.9% at the mtLSU rRNA locus and 18.5%, at the mtSSU rRNA locus). The presence of two *Pneumocystis* species in *R. aegyptiacus* could therefore be hypothesized. Two species of *Pneumocystis*, i.e., *Pneumocystis carinii* and *Pneumocystis wakefieldiae* (21), have been described in the same rat species (*Rattus novergicus*), though divergence was lower (9.6% and 8.9% at mtLSU rRNA and mtSSU rRNA loci, respectively) (26). In the case of *T. brasiliensis*, *Pneumocystis* genetic polymorphism was more limited and apparently related with host infraspecific variants. We found three mtSSU rDNA sequences: two were located in Mexico (Hidalgo/Michoacán/Chiapas region and Nuevo León), and the third one was located in Tucumán (Argentina) (Fig. 1; Table 11). In contrast, one mtLSU rDNA sequence type was amplified from *T. brasiliensis* lung samples from Mexico, and another one was amplified from lung samples of *T. brasiliensis* from Argentina. Interestingly, *Pneumocystis* polymorphism seemed to be related with *T. brasiliensis* subspecies, which have been described on the basis of geographical distribution and morphology (81). As we have previously suggested (31), *Pneumocystis* strain polymorphism could be used as a phylogeographic tool to be applied to host natural populations.

Conclusions. *Pneumocystis* spp. form a group of parasitic microorganisms infecting a vast diversity of hosts in various ecosystems. A great number of mammalian species belonging to different orders of the clade Mammalia were found to be harboring *Pneumocystis* organisms. So far, all bat species (11 bat species belonging to 5 families of Chiroptera) examined in this study were found to harbor *Pneumocystis* DNA except for the flying fox species, *Pteropus rodricensis*. Interestingly, if confirmed, the absence of *Pneumocystis* organisms in 44 specimens of *P. rodricensis* is, as far as we know, the first report of a mammal population, in which no *Pneumocystis* organisms were detected. Globally, we found a high *Pneumocystis* carriage rate of 41.3% in bats. Social or behavioral factors (migration, breeding, crowding) may influence transmission of *Pneumocystis* within the colonies of bat species, while the environmental factors (such as the climate, the roosting habits, geographical place) do not seem to have of impact on carriage rate of *Pneumocystis*, with the exception of altitude.

In addition, genetic divergence existing among *Pneumocystis* DNA sequences isolated from different bat species illustrates the close host species specificity reported for *Pneumocystis* species (3, 38). A comprehensive phylogeny of *Pneumocystis* from bats is in progress, and comparison with bat phylogeny suggests coevolution (C. Demanche and C.-M. Aliouat-Denis, unpublished results) as it was reported in primates (27, 41, 46) and suggested in other mammalian groups (41). Finally, the link between genetic variability of *Pneumocystis* isolated from populations of the same bat species and their geographic localization could be exploited in terms of phylogeographical research (31).

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