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## HIGHLIGHTS

- Advances in genomics of *Pneumocystis* species unlocked new areas of research
- Slow genome decay and limited expansions of specific gene families and introns
- Adaptation influenced by self-fertility, host specificity, and transmission mode
- Establishment of culture *in vitro* needed to unravel the forces driving evolution

1 **Revised version:** cleaned.

2

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## 4 **Genomics and evolution of *Pneumocystis* species**

5

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## 1 **ABSTRACT**

2 The genus *Pneumocystis* comprises highly diversified fungal species that cause severe  
3 pneumonia in individuals with a deficient immune system. These fungi infect exclusively  
4 mammals and present a strict host species specificity. These species have co-diverged with their  
5 hosts for long periods of time (> 100 MYA). Details of their biology and evolution are  
6 fragmentary mainly because of a lack of an established long-term culture system. Recent  
7 genomic advances have unlocked new areas of research and allow new hypotheses to be tested.  
8 We review here new findings of the genomic studies in relation with the evolutionary trajectory  
9 of these fungi and discuss the impact of genomic data analysis in the context of the population  
10 genetics. The combination of slow genome decay and limited expansion of specific gene families  
11 and introns reflect intimate interactions of these species with their hosts. The evolutionary  
12 adaptation of these organisms is profoundly influenced by their population structure, which in  
13 turn is determined by intrinsic features such as their self-fertilizing mating system, high host  
14 specificity, long generation times, and transmission mode. Essential key questions concerning  
15 their adaptation and speciation remain to be answered. The next cornerstone will consist in the  
16 establishment of a long-term culture system and genetic manipulation that should allow  
17 unravelling the driving forces of *Pneumocystis* species evolution.

1 Main text : 7482 words

2

### 3 **BACKGROUND**

#### 4 **History**

5 *Pneumocystis* species form a group of opportunistic fungi that cause severe pulmonary infections  
6 in mammals with a deficient immune system. These organisms infect exclusively mammals.  
7 They were first described by Chagas (Chagas, 1909), and wrongly classified as special forms of  
8 trypanosomes. They were later identified as a *bona fide* separate species by the Delanoë couple  
9 at the Pasteur Institute in Paris (Delanoë and Delanoë, 1912). Their taxonomic classification  
10 remained then elusive because of a phenotypic resemblance with the protists. The issue was  
11 resolved using molecular phylogeny based on sequencing ribosomal DNA, which clearly  
12 indicated their fungal nature (Edman et al., 1988).

13

#### 14 **Phylogeny and taxonomy**

15 *Pneumocystis* species belong to the subphylum of Taphrinomycotina within the Ascomycota  
16 (Eriksson, 1997; Sugiyama et al., 2006). The Taphrinomycotina subphylum is monophyletic and  
17 encompasses mostly plant-associated or soil-dwelling fungi (Liu et al., 2009). *Pneumocystis*  
18 closest relatives are *Schizosaccharomyces pombe* and *Taphrina deformans*, their common  
19 ancestor having diverged from the other Taphrinomycota members ca. 467 million years ago  
20 (MYA) (Beimforde et al., 2014).

21 Although all *Pneumocystis* species are ubiquitous, each mammal species can be infected  
22 with only one or two of them. Five species have been formally described so far based on the  
23 requirements of the International Code of Botanical Nomenclature (ICBN): *Pneumocystis*

1 *jirovecii* in *Homo sapiens* (Frenkel, 1999), *Pneumocystis carinii* in *Rattus norvegicus* (Frenkel,  
2 1999), *Pneumocystis wakefieldiae* also in *Rattus norvegicus* (Cushion et al., 1993; Cushion et al.,  
3 2004), *Pneumocystis murina* in *Mus musculus* (Keely et al., 2004a), and *Pneumocystis oryctologi*  
4 in Old World rabbits (*Oryctolagus cuniculus*; Dei-cas et al., 2006). Antigenic and DNA based  
5 studies suggest the presence of distinct species also in macaques, ferrets, bats, shrews, horses,  
6 pigs, and dogs (Banerji et al., 1994; Peters et al., 1994; Christensen et al., 1996; English et al.,  
7 2001; Guillot et al., 2004).

8 *P. jirovecii* is the only species known to infect humans and has never been detected in  
9 any other animals. *P. carinii* is the best studied species because of the availability of protocols  
10 for experimental or natural infections in laboratory rats. *P. wakefieldiae* was reported either  
11 mixed with *P. carinii* (Cushion et al., 1993; Cushion, 1998; Cushion et al., 2004; Chabé et al.,  
12 2010), or alone (Palmer et al., 2000). The two latter species are different in terms of  
13 electrophoretic karyotypes, gene localization on the chromosomes, sequence identity (4-7%  
14 nucleotide divergence in seven orthologs; Cushion, 1998; Cushion et al., 2004), antigenic  
15 profiles (Vasquez et al., 1996), and major surface glycoproteins (MSG) expression (Schaffzin  
16 and Stringer, 2000). They might be competing against each other for resources when present  
17 together within the same rat (Icenhour et al., 2006a).

18

### 19 **Species divergence**

20 According to the evolutionary rates of several genomic loci, the radiation of the *Pneumocystis*  
21 genus occurred ca.100 MYA (Keely et al., 2003a; Keely et al., 2004a), which roughly overlaps  
22 with the radiation of the mammalian species (Holmes, 1991; dos Reis et al., 2015). *P. murina*  
23 would have diverged from *P. carinii* between 51 and 71 MYA (Keely et al., 2003a), while *P.*

1 *carinii* and *P. wakefieldiae* diverged between 15 and 22 MYA (Cushion et al., 2004; Fischer et  
2 al., 2006). The neat superposition of multiple *Pneumocystis* species phylogenetic trees with those  
3 of their respective hosts supports a co-evolution of these organisms (Guillot et al., 2001).  
4 Therefore, a plausible co-speciation scenario is that each species became physically separated  
5 from the other species, the hosts acting as barriers that led to the accumulation of genetic  
6 differences and the gradual reproductive isolation over time. The absence of gene flow or mating  
7 among the different species has been inferred based on linkage disequilibrium analysis consistent  
8 with an ancient reproductive isolation (Mazars et al., 1997; Keely et al., 2004a; Keely and  
9 Stringer, 2009). Furthermore, no evidence of hybridization was detected between *P. carinii* and  
10 *P. wakefieldiae*, even during co-infection of the same rat (Cushion, 1998; Cushion et al., 2004).  
11 However, caution is warranted because the absence of gene flow was inferred from a small set of  
12 conserved markers, which may have not allowed detecting all genetic events. Consequently,  
13 whole genome sequencing studies are necessary to validate these findings.

14

## 15 **Life cycle**

16 The life cycle of *Pneumocystis* organisms is still hypothetical and mostly derived from  
17 microscopic and molecular studies on *P. carinii* (Figure 1). As fungal organisms with an obligate  
18 parasitic behavior, the cycle would occur only inside host's lungs, and begin with the inhalation  
19 of infectious asci. Once inhaled, each ascus would release first eight ascospores which will  
20 evolve to what is known as trophic forms that bind to the type I pneumocytes of the alveolar  
21 epithelium. The cycle would then alternate between asexual multiplication of metabolically  
22 active trophic cells by binary fission, and sexual reproduction upon mating of two trophic cells  
23 that would culminate by the production of asci containing eight ascospores (Figure 2). Trophic

1 cells are amoeboid in shape and represent generally 90-98% of the populations in the infected  
2 lungs (Aliouat-Denis et al., 2009). These forms are mononuclear, 2-8  $\mu\text{m}$  in diameter (Dei-Cas et  
3 al., 2004), and mostly haploid (Stringer and Cushion, 1998; Wyder et al., 1998; Martinez et al.,  
4 2011). Multiploid forms are rare and possibly caused by asymmetrical or post-mating divisions  
5 (Martinez et al., 2011). Trophic cell surface is composed of a single layer of electron dense  
6 material containing glycoproteins, but possibly no  $\beta$ -glucans. Indeed, the enzymes responsible  
7 for the synthesis of  $\beta$ -glucans and the associated endo-1,3- $\beta$  glucanase are expressed almost  
8 exclusively in asci (Nollstadt et al., 1994; Kottom and Limper, 2000; Kutty et al., 2015). The  
9 presence of structural carbohydrate polymers of glucans in asci increases the physical strength of  
10 the cell wall, which might facilitate the survival outside the host. The doubling times are  
11 relatively long compared to free-living yeasts ( $\sim$ 2 hours) and range from 1.5 to 10.5 days  
12 depending on the species (Aliouat et al., 1999; Keely et al., 2003b). The presence of a sexual  
13 cycle was initially supported by the ultrastructural observations of synaptonemal complexes  
14 (Matsumoto and Yoshida, 1984) and the expression of one pheromone receptor at the surface of  
15 *P. carinii* trophic cells (Vohra et al., 2004). Recent comparative genomic studies suggest that  
16 *Pneumocystis* species use primary homothallism (self-fertility) based on the genes number and  
17 arrangement on the chromosomes as a fusion of Plus and Minus mating type loci (Almeida et al.,  
18 2015). Thus, each strain would be able to produce asci on its own, without the need to find a  
19 compatible partner. Asci would be expelled by infected hosts and be the infectious stages  
20 because their specific inactivation or removal blocks the transmission chain (Cushion et al.,  
21 2010; Martinez et al., 2013). Consistently, recent analyses suggested that *Pneumocystis* sexuality  
22 is obligatory within host's lungs in order to complete the cell cycle and produce asci that are  
23 necessary for airborne transmission to new hosts (Richard et al., 2018). Furthermore, the

1 necessity of asci for transmission has been demonstrated by inhibition of the sexual cycle using  
2 echinocandins (Cushion et al., 2010), and by the fact that only purified asci could transmit the  
3 disease (Martinez et al., 2013). Recently, activation of sex-related genes upon treatment with  
4 echinocandins in RNA-seq analyses also suggested that sexuality is obligate (Cushion et al.,  
5 2018).

## 1 **Transmission**

2 *Pneumocystis jirovecii* pneumonia is a major public health problem with >400,000 cases per year  
3 worldwide and a mortality rate possibly as high as 80% if untreated (Brown et al., 2012).

4 Epidemiological data for *Pneumocystis* species in animal populations are scarce, but  
5 investigations in shrews and rats suggest a pervasive low level of infections (Laakkonen, 1998;  
6 Chabé et al., 2010).

7 *Pneumocystis* organisms are transmitted via the air from infected individuals to new hosts  
8 (Hughes, 1982), including between individuals within hospitals (de Boer et al., 2011), but also  
9 possibly via the transplacental route (Céré et al., 1997; Sanchez et al., 2007; Montes-Cano et al.,  
10 2009). The current hypothesis is that infections occur over short distance among infected and  
11 susceptible individuals (Chabé et al., 2011). The transfer of parasites from animals to humans is  
12 no longer considered as a valid hypothesis based on the strict host species specificity (Chabé et  
13 al., 2011). Consistently, no convincing evidence of an environmental source of *Pneumocystis* has  
14 been found so far, which strongly suggests that mammals constitute the only reservoir of these  
15 fungi. Furthermore, the erosion of metabolic capabilities evidenced by the genome sequencing  
16 studies suggests that these organisms are unable to live outside their hosts (see below, losses of  
17 metabolic machinery section). Finally, they apparently complete their whole cell cycle within  
18 host's lungs since sexuality occurs therein. Healthy infected hosts colonized by the organism are  
19 believed to contribute greatly to the transmission and circulation process (Chabé et al., 2004;  
20 Peterson and Cushion, 2005; Le Gal et al., 2012; Alanio and Bretagne, 2017).

21

## 1 **Host specificity and biotrophy**

2 The strict host species specificity of the *Pneumocystis* species means that the fungal cells can  
3 only infect or survive in the host in which they were isolated in the first place. This view is  
4 mainly supported by the systematic failure of cross-infection experiments involving severely  
5 combined immuno-deficient animals and nude rats (Aliouat et al., 1993; Furuta et al., 1993;  
6 Gigliotti et al., 1993; Aliouat et al., 1994; Atzori et al., 1999; Durand-Joly et al., 2002). The  
7 selective activation of trophic cells by their host seems to trigger the formation of cytoplasmic  
8 projections by *Pneumocystis* cells, the filopodia (Aliouat-Denis et al., 2008). Accordingly, *P.*  
9 *carinii*, the species naturally infecting rats, is unable to form filopodia and infect when  
10 inoculated in mice, whereas *P. murina*, the natural parasite of mice, produce filopodia and high  
11 parasite loads under the same conditions (Aliouat-Denis et al., 2008). The function of the  
12 filopodia remains elusive but these structures display ultrastructural differences that are species  
13 specific, and that might account for some aspects of the host specificity.

14 Another aspect of this host specificity is that *Pneumocystis* species are most probably  
15 obligate biotrophs (Cushion et al., 2007; Cushion and Stringer, 2010; Hauser, 2014; Ma et al.,  
16 2016a). The way fungal parasites scavenge nutrients from their host is an active research field  
17 and three modes are broadly recognized: (i) biotrophy, where the parasite acquires nutrients from  
18 a living cell, (ii) necrotrophy, where host cells are killed to release nutrients, and (iii)  
19 saprotrophy, where the organism feeds on dead or decaying organic material. Biotrophs do little  
20 damage to host cells and lack virulence factors (van der Does and Rep, 2007). *Pneumocystis*  
21 perfectly fits to the biotrophy definition because they cause no apparent cell death and lack any  
22 experimentally verified fungal virulence factors such as glyoxylate cycle, secondary metabolism,  
23 and secreted effectors (Cushion et al., 2007; Cissé et al., 2012; Cissé et al., 2014; Ma et al.,

1 2016a). This implies that they rely entirely on their host for their survival and thus have evolved  
2 close relationships that rendered them host species specific.

3         The physiological characteristics of the hosts are key determinants of parasite adaptation  
4 (Poulin et al., 2006). For example, micromammals are small bodied with short lifespans, high  
5 reproduction rates, and high population densities, whereas these distinctive features are reversed  
6 in large mammals such as humans. The co-evolution theory predicts that parasitic species  
7 infecting micromammals exhibit a weaker host specificity compared those adapted to long-lived  
8 hosts with more stable population densities (Poulin et al., 2006). This prediction has been  
9 validated in fish parasites, among which strong host specificity is favored in stable resources  
10 found in hosts with a large body size (Sasal et al., 1999; Desdevises et al., 2002). As far as  
11 *Pneumocystis* is concerned, humans are infected by only one species whereas rats can be co-  
12 infected by two (Cushion et al., 1993 and 2004; Icenhour et al., 2006a; Golab, 2009). The  
13 number of *Pneumocystis* species able to infect rodents might even be more important, as shown  
14 by the recent discovery of multiple lineages shared among species and genera of the Southeast  
15 Asian murid species (Latinne et al., 2017). These findings might indicate a relaxation of the strict  
16 host specificity in small mammals harboring *Pneumocystis*, although additional supporting data  
17 are needed to fully challenge the concept of widespread strict host specificity.

18         Co-phylogenetic studies of *Pneumocystis* species and their hosts suggest that the host  
19 specificity evolved as a continuous trait resulting from a long-lasting co-evolution (Demanche et  
20 al. 2001; Guillot et al., 2001; Hugot et al., 2003). Strict host specificity is rare in animal  
21 pathogens but widespread in plant fungal pathogens (Parker and Gilbert, 2004; Restrepo et al.,  
22 2014). In the latter, the ecological adaptation often results in a pronounced specialization to  
23 particular hosts (Clay and Kover, 1996). In these systems, host specificity acts as a reproductive

1 isolating mechanism because it favors higher rate of mating between individuals on the same  
2 host and reduced gene flow among populations from different hosts (Giraud, 2006). A rapid  
3 divergence of the virulence factors, the pathogen “effector repertoire”, is often associated with  
4 the emergence of host specificity (Schulze-Lefert and Panstruga, 2011). The hypothesis of the  
5 latter authors states that changes in pathogen host range is driven by variation in the pathogen  
6 effector repertoire. This description fits the lineage specific expansion of the MSG superfamily  
7 in *Pneumocystis* species (Ma et al., 2016a; Ma et al., 2016b; Schmid-Siegert et al., 2017), which  
8 suggests that these proteins might account for some aspects of the host specificity.

9  
10

## 1 GENOME ORGANIZATION

### 2 Genomic data acquisition

3 The quest for genome sequence data began with the successful cloning of *P. carinii* genomic  
4 fragments (Tanabe et al., 1988). Pulse field gradient gel electrophoreses have been then  
5 instrumental for karyotypic characterization of *Pneumocystis* genomes and evidenced 12 to 20  
6 chromosomes according to the species totaling ca. 8 Mb (Hong et al., 1990; Yoganathan et al.,  
7 1989; Stringer and Cushion, 1998). Differences in karyotype profiles determined that the species  
8 infecting humans and rats are genetically distinct (Stringer et al., 1993). Significant genome size  
9 variations among species have been reported, *e.g.* that of ferret *Pneumocystis* would be ca. 1.7  
10 times bigger than that of *P. carinii* (Stringer and Cushion, 1998). A draft of *P. carinii* genome  
11 covering ca. 70% of genome was generated in 2006 using a clone-based Sanger sequencing  
12 approach from infected laboratory rats (Slaven et al., 2006). In 2012, the first draft of *P. jirovecii*  
13 genome was obtained from a single bronchoalveolar lavage of a patient with pneumonia (Cissé et  
14 al., 2012). This assembly encompasses 358 contigs capturing 90 to 95% of the genome, but the  
15 repetitive subtelomeric and centromeric regions could not be resolved. The centromeres have not  
16 been discovered yet in *Pneumocystis*, whereas the subtelomeric regions were resolved using  
17 Sanger sequencing of cosmids (Keely et al. 2005), and more recently assembled using  
18 sequencing generating long reads (Ma et al., 2016a; Ma et al., 2016b; Schmid-Siegert et al.,  
19 2017) (see below, chromosomal ends section). Chromosomal level assemblies of *P. jirovecii*, *P.*  
20 *carinii*, and *P. murina* were recently published revealing genome sizes ranging from 7.4 to 8.3  
21 Mb (Table 1; Ma et al., 2016a). It became evident that the genomes of *Pneumocystis* species had  
22 undergone an important reduction relative to *S. pombe* (7.5 to 8.3 Mb versus 12.5 Mb).

23

## 1 Nuclear Genome content

2 The analysis of the *Pneumocystis* genome assemblies validated the presence of single copy  
3 ribosomal DNA reported previously for *P. jirovecii* (Giuntoli et al. 1994; Stringer, 1996; Tang et  
4 al., 1998; Nahimana et al., 2000a). This is similar to *Taphrina deformans* (Cissé et al., 2013), but  
5 contrasts with most fungi which harbors commonly tens or hundreds of copies of the locus.  
6 Figure 3 shows the genome compositions of *Pneumocystis* species compared to related fungi.  
7 These data highlight the contraction of the protein coding regions as compared to free-living  
8 yeasts, which reflect massive gene losses. Figure 3 also evidences the expansions of the MSG  
9 superfamily, of introns, as well as of the cumulative length of the intergenic regions (IGR). We  
10 previously reported that IGR in *P. jirovecii* occupy a larger genome fraction as compared to free  
11 living yeasts *Saccharomyces cerevisiae* and *S. pombe* despite a significantly smaller genome  
12 (Cissé et al., 2014). This observation holds when we re-evaluate here IGRs in the newly  
13 published full-length genomes of *P. jirovecii*, *P. carinii*, and *P. murina* (Ma et al. 2016a). This  
14 strongly suggests that genome streamlining in *Pneumocystis* species is driven by gene deletions  
15 rather than reduction of IGRs. This observation seems counterintuitive because genome  
16 reduction is almost always associated to a reduction of introns and IGRs in parasites (Keeling  
17 and Slamovits, 2005). Alternatively, large IGRs might favor chromosomal re-arrangements by  
18 increasing the number of possible breakpoints, as hypothesized in fungal microsporidian  
19 parasites (Slamovits et al., 2004; Keeling and Slamovits, 2005). The other characteristics of these  
20 genomes are discussed in the following sections.

21

## 1 **Chromosomal ends**

2 Subtelomeres in microbial parasites are often enriched with multi-copy surface glycoprotein  
3 gene families (Deitsch et al. 2009). These genomic regions are prone to (i) gene silencing that  
4 can be used for mutually exclusive expression, (ii) enhanced mutagenesis, and (iii) ectopic  
5 recombinations facilitated by the formation of clusters of telomeres at the nuclear periphery  
6 (Barry et al., 2003). These regions correspond to an important proportion of the *Pneumocystis*  
7 genomes (ca. 5%), and harbor a superfamily including five to six families of highly polymorphic  
8 multi-copy proteins called major surface glycoproteins (MSG) that are believed to be crucial for  
9 the fungus' lifestyle (Ma et al., 2016a; Schmid-Siegert et al., 2017). These *msg* genes exist only  
10 in *Pneumocystis* species and all species of *Pneumocystis* have their own repertoire, which  
11 suggest they have been acquired in a common ancestor, although their origin is not known. The  
12 absence of homology of these MSGs outside *Pneumocystis* lineages might indicate a transfer  
13 from an unknown species or a gene co-option. *msg* families have been first described and studied  
14 in *P. carinii* (Kovacs et al., 1993; Sunkin et al., 1994; Sunkin et al., 1996; Keely et al., 2005;  
15 Keely and Stringer, 2009), and subsequently analyzed in *P. jirovecii* and *P. murina* (Haidaris et  
16 al., 1998; Kutty et al., 2008; Ma et al., 2016a; Schmid-Siegert et al., 2017). Important differences  
17 exist among *Pneumocystis* species in terms of *msg* gene copy numbers, 60 to 140 copies per cell,  
18 and protein divergence (Ma et al., 2016a). Moreover, one MSG family is present only in *P.*  
19 *jirovecii* (*msg*-IV or -B), whereas another one is present only in *P. carinii* and *P. murina* (MSR  
20 family, *i.e.* MSG-related). MSGs are believed to be involved in antigenic variation (Stringer,  
21 2007). MSGs would also mask glucans at the asci surface from the immune recognition (Kutty et  
22 al., 2016). The antigenic diversity seems to be created via intra-family recombination of *msg*  
23 genes encoding different isoforms, creating mosaic genes, as well as through increased

1 mutagenesis (Kutty et al., 2008; Keely and Stringer, 2009; Schmid-Siegert et al., 2017). The  
2 expression of the most abundant MSG family (*msg-I* or –A1) that is present in all species is  
3 subject to mutually exclusive expression of a single isoform in each cell by using a single copy  
4 transcription promoter (the upstream conserved sequence, UCS) (Edman et al., 1996; Kutty et  
5 al., 2001; Sunkin et al., 1996; Wada et al., 1995). The UCS ends by the conserved recombination  
6 joint element (CRJE) which is also present at the beginning of each *msg-I* gene and may serve as  
7 recombination breakpoint (Stringer, 2007). The CRJE would be larger in *P. wakefieldiae* (ca.  
8 330 bps) than in *P. murina* (132), which in turn is larger than in *P. carinii* and *P. jirovecii* (28  
9 and 33, respectively) (Keely et al., 2007). On the other hand, at least in *P. jirovecii*, members of  
10 the other five families possess each their own promoter (Schmid-Siegert et al., 2017), but their  
11 expression patterns remain to be characterized. Recently, one family has been shown in *P.*  
12 *murina* to be expressed only in ascospores within asci and young trophic forms (Bishop et al.,  
13 2018).

14

## 15 **Introns**

16 Introns are extremely abundant in *Pneumocystis* genes and are as many as several tens per gene  
17 with a mean of five, and more than 40% of genes are interrupted by at least four introns (Stringer  
18 and Cushion, 1998; Ma et al., 2016a). Their presence can be equally explained by massive gains  
19 in *Pneumocystis* most recent common ancestry, or retention of ancestral elements that would  
20 have been lost in some Taphrinomycotina lineages such as *Schizosaccharomyces*. The introns are  
21 short (average length of 48 nucleotides), have a strong adenine and thymine bias, and present  
22 typical donor, acceptor and branch site patterns (Slaven et al., 2006). *Pneumocystis* introns  
23 cannot be processed by *S. pombe* and *S. cerevisiae* spliceosomes because of the divergence in  
24 intron-exon boundaries and branching sites within the introns (Thomas et al., 1999). RNA-seq

1 data indicate that intron retention affects ca. 45% of all introns (Ma et al., 2016a). *Pneumocystis*  
2 species contain self-splicing group I introns that are absent in higher eukaryotes such as humans  
3 (Liu et al., 1994), which renders them a prime target for the development of new drugs. These  
4 latter introns catalyze their own excision from RNA transcripts, a reaction that is inhibited by the  
5 drug pentamidine that is used against *Pneumocystis* (Liu and Leibowitz, 1993).

6         Given the important genome reduction at the *Pneumocystis* genus level, the presence of a  
7 high intron density per gene suggests a selective constraint to conserve them. Intron loss is  
8 dominant in fungi (Stajich et al., 2007), and this tendency is even more pronounced in some  
9 parasites such as microsporidia (Keeling et al., 2010). The intron history is highly flexible within  
10 the Taphrinomycota, with the plant-associated *Neolecta* having a high intron density similar to  
11 *Pneumocystis* (Nguyen et al., 2017), and the intron-poor free-living yeast *S. pombe* (Wood et al.,  
12 2002). The non-sense-mediated mRNA decay machinery is conserved in *Pneumocystis* species  
13 (Ma et al., 2016a). Under neutral scenario (no advantage) and widespread intron retention, most  
14 of the introns would produce non-functional transcripts tagged for destruction. This would be an  
15 incredible waste of resources in absence of another function. The latter could consist in  
16 alternative splicing increasing transcript diversity and regulating gene transcription or mRNA  
17 stability. Consistently, the *P. carinii* inosine 5'-monophosphate dehydrogenase pre-mRNA is  
18 differentially spliced, which was suggested to reflect changes in environmental stresses (Ye et  
19 al., 2001). These considerations suggest that introns might be neutral elements involved in many  
20 cellular processes via a greater proteome diversity, possibly including acting as a favorable  
21 substrate to facilitate shifts in lifestyle (*i.e.* parasite transition from one host species to another, or  
22 from plant to animal).

23

## 1 Mitogenomes

2 The mitochondrial genomes of *P. carinii* (Sesterhenn et al., 2010; Ma et al., 2013), *P. jirovecii*  
3 (Cissé et al., 2012; Ma et al., 2013), and *P. murina* (Ma et al., 2013) have been sequenced. The  
4 mitogenome sizes range from 24 to 35-kb with a substantial size variability among isolates in all  
5 species. *P. carinii* and *P. murina* mitogenomes end with single-stranded loop sequences that  
6 would allow forming linear concatemers and protecting the ends of the molecule. The presence  
7 of these repeats might account for the variable size of *P. carinii* mitogenomes. *P. jirovecii*  
8 mitochondrial genome is circular since it lacks inverted terminal repeat allowing circulation. The  
9 significance of circularity versus linearity is unknown. Related Taphrinomycota of the genera  
10 *Schizosaccharomyces*, *Taphrina*, and *Neolecta* have circular genomes (Bullerwell et al., 2003;  
11 Cissé et al., 2013; Tsai et al., 2014; Nguyen et al., 2017), which might indicate that the circular  
12 form is ancestral. Interestingly, *P. carinii* and *P. murina* mitogenomes are highly co-linear  
13 whereas *P. jirovecii* mitogenome presents some re-arrangements, similarly to the nuclear  
14 genomes (see below Chromosomal re-arrangement section). The gene content is highly  
15 conserved among the three *Pneumocystis* species, although there is a substantial nucleotide  
16 divergence among species (27 to 31%) (Ma et al., 2013). These mitogenomes encode ca. 17  
17 genes commonly found in mitochondrial fungal genomes such as ATP synthases, cytochrome *c*  
18 oxidases, NADH dehydrogenases, and the full repertoire of at least 20 transfer RNAs.  
19 Reports investigating the dynamics of the mitochondrial genes during infection have revealed  
20 that mitogenomes would be very plastic in terms of copy number variations (Valero et al., 2016),  
21 and of genetic diversity including heteroplasmy (Alanio et al., 2016). The subsequent sections of  
22 this review focus on nuclear genomes.

23

## 1 **EVOLUTION**

2 Comparison of the gene families and pathways present in *Pneumocystis* genomes to those in  
3 selected fungi has revealed numerous losses / contractions and relatively few expansions (Table  
4 2). The hypothetical evolutionary history of *Pneumocystis* species derived from these  
5 observations is represented in Figure 4 and discussed in the following sections.

6

### 7 **Losses in the metabolic and cellular machineries**

8 Massive gene losses suggest that *Pneumocystis* species are auxotroph for essential nutrients,  
9 which might explain the recurrent failures of *in vitro* culturing attempts. The lost pathways  
10 include basic components of metabolic machinery such as the synthesis of amino acids or  
11 carbohydrates (Table 2). The loss of purines catabolism seems unique to *Pneumocystis* (Chitty  
12 and Fraser, 2017). *Pneumocystis* species are able to synthesize fecosterol and episterol but lack  
13 enzymes to convert them into ergosterol. Consequently, their membranes contain cholesterol  
14 instead of ergosterol, which probably explains their resilience to azole treatment. *Pneumocystis*  
15 organisms are also able to synthesize a unique class of sterols, the “pneumocysterols” (Kaneshiro  
16 et al. 1994; Kaneshiro et al. 1999; Florin-Christensen et al. 1994; Giner et al., 2002). It  
17 interesting to note the early steps of the sterol biosynthetic pathway leading to the formation of  
18 pneumocysterol and episterol are conserved in *Pneumocystis* species, and only the final steps  
19 toward ergosterol/cholesterol production are missing (Joffrion et al., 2010). This is exemplified  
20 by the fact that key enzymes for the formation of ergosterol (*i.e.* *erg3*, *erg4* and *erg5*) are not  
21 identifiable within the genomes. Analysis of the sterol biosynthesis machinery suggest that these  
22 species may be able to synthesize ergosterol/cholesterol precursors such as zymosterol, fecosterol

1 and episterol. Thus, the sterol pathway may have been re-routed and branch to form unique  
2 sterols, the pneumocystisterols.

3 Overall, these observations are consistent with the idea that losses of metabolic genes correlate  
4 with an increased dependency of the parasite on its host. Therefore, nutrients need to be  
5 scavenged from the host, which often mechanistically involves large batteries expanded  
6 transporters (*e.g.* as observed in microsporidia [Cuomo et al., 2012]). This is not the case in  
7 *Pneumocystis* since transporters families are also greatly reduced (Cissé et al., 2014; Ma et al.,  
8 2016a). For instance, the amino acid permeases and transporters that can respectively carry  
9 amino acids and oligopeptides are greatly reduced relatively to other Taphrinomycota (one copy  
10 of general amino acid permease versus 21 copies in *S. pombe*). Transmembrane proteins such as  
11 those of the major facilitator superfamily, sugar transporters, or more specific transporters (*e.g.*  
12 efflux pumps) are significantly reduced in *Pneumocystis*. The reduction of the transporters  
13 battery might be compensated by the use of highly selective transporters for critical compounds.  
14 The recent discovery of the import of *myo*-inositol in *Pneumocystis* cells via a low affinity but  
15 highly selective system supports this idea (Cushion et al., 2016). Unfortunately, high affinity  
16 transporters cannot be identified solely by computational means. Alternatively, simple diffusion  
17 across the membrane may occur, as evidenced in *P. carinii* for amino acids uptake using *in vitro*  
18 experiments (Basselin et al, 2001a; Basselin et al, 2001b). Basic cellular machinery is also  
19 affected by the loss of several fungal specific transcription factor families and the RNA  
20 interference machinery (Table 2).

21

## 1 **Evolutionary basis of gene loss**

2 Gene loss is a common trend in parasitic and symbiotic species, which often harbor a small sized  
3 genome (Keeling and Slamovits, 2005; Wolf and Koonin, 2013). The driving factors are often  
4 unknown or specific to the lifestyle of the species under study. A central question in evolutionary  
5 biology is whether gene loss is neutral or adaptive. In *Pneumocystis* species, there are footprints  
6 of both processes and we discuss here a few examples.

7         The neutral theory is usually sufficient to explain gene loss in parasites (O'Malley et al.,  
8 2016). Organisms with narrow host niche such as *Pneumocystis* are predicted to have small sized  
9 populations with increased **genetic drift** (bold: see glossary) (Papkou et al., 2016). The main  
10 mechanisms for gene loss are pseudogenization and sudden DNA deletions. Pseudogenization  
11 consists in the accumulation of deleterious mutations in non-essential genes ultimately leading to  
12 the loss (Kuo and Ochman, 2009; Wernegreen, 2015). The proportion of pseudogenes in *P.*  
13 *jirovecii* is low and equivalent to that present in free-living yeasts (0.02 pseudogene per protein-  
14 coding gene [Cissé et al., 2014]). This observation might indicate that pseudogenization is not  
15 the main driver of gene loss in this species. The following considerations do not undermine this  
16 observation but suggest that caution must be exercised: (i) this rate of pseudogenization is valid  
17 only for *P. jirovecii* and for the single isolate which genome was sequenced (Cissé et al., 2012),  
18 and (ii) only genes including stop codons were considered, that is, other types of gene  
19 inactivation were not considered (*e.g.* untranslated RNA genes or unfixed mutations). Gene loss  
20 can also be result of deletions independent of selection such as the movement of transposable or  
21 integrated viruses (reviewed by Albalat and Canestro, 2016).

22         The adaptive theory of gene loss implies a selective advantage and has been  
23 demonstrated to have occurred in many pathogenic lineages, for example for the Allergen 1 in

1 *Cryptococcus neoformans* (Jain et al., 2009), and for the *de novo* biosynthesis of nicotinic acid  
2 genes in *Candida glabrata* (Domergue et al., 2005). In *Pneumocystis*, the loss of chitin might  
3 have been lost to allow avoiding recognition from the host immune system (Ma et al., 2016a).  
4 The gene families and pathways cited in Table 2 are missing in the three *Pneumocystis* genomes  
5 available (Ma et al 2016a), which suggests that these losses occurred before the radiation of the  
6 genus. An unexpected consequence is that the observed gene losses might not reflect the current  
7 selective forces, and therefore might not be relevant for the host specificity.

8

### 9 **Chromosomal re-arrangement**

10 The chromosome level assemblies revealed that an important chromosomal re-arrangement  
11 occurred among *Pneumocystis* species (Ma et al., 2016a). The re-arrangement, however,  
12 followed the species tree, that is, the macrosynteny is broken between rodents infecting  
13 *Pneumocystis* (*P. carinii* and *P. murina*) and the humans infecting species (*P. jirovecii*), whereas  
14 *P. carinii* and *P. murina* genomes are highly collinear. Nevertheless, the gene order is conserved  
15 in syntenic regions among the three species (>92% of the genes), and ca. 83% of gene families  
16 are orthologous, with 4 to 30% of divergence at the nucleotide sequence level. The high gene  
17 conservation among the three species suggest that re-arrangements occurred mostly in the  
18 intergenic regions (IGR). In fungi, IGRs are often enriched in regulatory functions such as signal  
19 transduction or binding sites of transcription factors (Noble and Andrianopoulos, 2013).  
20 Chromosomal translocations impact gene expression as well as long-distance gene-to-gene  
21 contact via chromatin interactions, and thus might be involved in speciation (Rieseberg, 2001;  
22 Bakloushinskaya, 2016). Protein evolution is also faster in re-arranged chromosomes than  
23 collinear chromosomes because re-arrangements reduce homologous recombination and

1 facilitate positive selection (Rieseberg, 2001). A key question here is whether chromosomal re-  
2 arrangements are involved in the adaptation of each *Pneumocystis* species to its host. Future  
3 studies are required to probe an eventual role of these re-arrangements in *Pneumocystis*  
4 evolution.

## 6 **Loss of complex multicellularity**

7 The recent sequencing of the *Neolecta irregularis* genome revealed that the Taphrinomycotina  
8 last common ancestor was probably multicellular (Nguyen et al., 2017). These findings suggest  
9 that *Pneumocystis* organisms evolved from a plant-associated or soil-adapted multicellular  
10 organism. The shift in cell morphology to single celled organisms is associated with the deletion  
11 of an ancestral morphogenic kit that included many cell differentiation and cell-to-cell signaling  
12 genes. These losses are not specific to *Pneumocystis* and were observed in a wide range of  
13 unrelated yeasts (Nguyen et al., 2017; Nagy et al., 2014; Nagy, 2017), which suggests a  
14 convergent evolution. The transition from a hyphal to yeast form takes place in many fungal  
15 lineages and is often triggered by a thermal stimulus (Köhler et al., 2017), CO<sub>2</sub> levels (Hall et al.,  
16 2010), or pH (Davis, 2009), and is directly linked to the ability to invade hosts. Notable  
17 examples include the dimorphic human pathogenic fungi *Histoplasma*, *Blastomyces*,  
18 *Coccidioides*, and *Paracoccidioides* (Beaman et al., 1981; Medoff et al., 1987; Inglis et al.,  
19 2013).

20 The ancestral morphogenic kit for complex multicellularity (fruiting bodies) is lost in  
21 *Pneumocystis*. However, *Pneumocystis* species are able to produce biofilms (Cushion et al.,  
22 2009), which is an undifferentiated form of aggregative multicellularity often seen in bacteria  
23 (Claessen et al., 2014). Inversely, the yeast *Saitoella complicata* grows primarily by budding

1 (Goto et al., 1987), despite having the cellular machinery for the production of fruiting bodies  
2 (Nguyen et al., 2017). Comparative genomics and epigenomics would be extremely valuable to  
3 explore the molecular process underlying the loss of the multicellular phenotype. These  
4 considerations highlight the fact that phenotypes cannot be explained solely by gene loss and  
5 gain balance, and that other subtle mechanisms need to be considered.  
6

## 1 **POPULATION GENETICS**

### 2 **Strain typing**

3 Given the high homogeneity of genomic sequences at the nucleotide sequence level among *P.*  
4 *carinii* isolates, strain typing for this species relied on chromosomes' size analyses which  
5 allowed identifying numerous different karyotypic forms (Lundgren et al., 1990; Cushion, 1998;  
6 Wakefield, 1998a; Nahimana et al., 2001). On the other hand, the low but significant  
7 heterogeneity in many genomic loci among *P. jirovecii* isolates allowed using multilocus  
8 sequence typing (Wakefield, 1998b). The latter method represents nowadays the most used  
9 technique for *P. jirovecii* strains identification. The discrimination power of eight distinct loci  
10 has been validated and extensively used for epidemiological studies of *P. jirovecii* pneumonia  
11 (Maitte et al., 2013). Genotypes identification is performed by PCR of multiple loci followed by  
12 direct DNA sequencing (Sanger), restriction fragment length polymorphism, single-strand  
13 conformation polymorphism, type-specific oligonucleotide hybridization, tandem repeats  
14 number analysis, or high-throughput amplicon sequencing (Hauser et al., 1997; Hauser et al.,  
15 1998; Lee et al., 1993; Lu et al., 1995; Ma et al., 2002; Alanio et al., 2016; Esteves et al., 2016).

16

### 17 **Genetic diversity**

18 The conclusions drawn from the studies concerning *Pneumocystis* genetic diversity were often  
19 contradictory. Low levels of genetic diversity as defined by Shannon diversity and Simpson  
20 indexes (Shannon, 1948; Simpson 1949) have been reported at the *P. jirovecii* and *P. carinii*  
21 internal transcribed spacers of the nuclear rDNA operon using PCR-based Sanger sequencing  
22 (Palmer et al., 2000; Beser et al., 2011). On the other hand, moderate to important levels of  
23 diversity measured in term of DNA polymorphisms in *P. jirovecii* using multilocus sequence

1 typing have been reported (Matos and Esteves, 2010; Jarboui et al., 2013; Sun et al., 2015;  
2 Alanio et al., 2017). The lack of whole genome sequence data, differences in sampling strategies,  
3 differences in interpretation, as well as the likely frequent *in vitro* formation of PCR chimeras  
4 (Beser et al., 2007), make difficult the reconciliation of these conclusions.

5         Moreover, sexual recombination could explain partly these conflicting conclusions.  
6 Indeed, sexual reproduction is one of the main mechanisms to generate genetic diversity in fungi.  
7 It is believed to favor adaptation in fluctuating conditions while purging deleterious alleles  
8 (Heitman, 2010). *Pneumocystis* are probably homothallic species (Almeida et al., 2015; see life  
9 cycle section), and self-fertilization favors mating by avoiding the search of a compatible  
10 partner, a strategy thought to be favorable to and adopted by several human pathogens such as  
11 *Cryptococcus* and *Candida* species (Heitman, 2010). Sexual reproduction is based on classical  
12 Mendelian segregation, which supports both cross- and self-fertilization (Buscaglia et al., 2015).  
13 *Pneumocystis* would be able to perform both clonal and sexual propagation with various degrees  
14 of inbreeding or outcrossing. These variations in the multiplication process could explain the  
15 conflicting patterns of genetic diversity reported.

16         Polymorphism rates change substantially across loci and chromosomes in various  
17 species, including fungi, plants, and animals (Ellegren and Galtier, 2016). Genetic diversity is  
18 influenced by three main forces: mutation, demography (migration and **bottlenecks**), and  
19 selection (**selective sweeps** or **clonal interference**). Demography and selection create  
20 differences in the effective population size, whilst variations in mutation rate may create  
21 differences in the level of genetic diversity according to the geographical location. Neutral  
22 mutation rates in eurotiomycetes are typically between  $1 \times 10^{-8}$  and  $1 \times 10^{-9}$  substitutions per site  
23 per year (Kasuga et al., 2002), and a rate of  $1.2 \times 10^{-10}$  for the 18S rDNA has been used to

1 estimate *Pneumocystis* species divergence (Keely et al., 2003a). However, the genome-wide  
2 mutation rates for these species are unknown and expected to fluctuate greatly among genomic  
3 regions. For example, subtelomeric regions harboring MSGs have high substitution rates (Keely  
4 and Stringer, 2009; Schmid-Siegert et al., 2017), whereas ribosomal regions display a normal  
5 rate (Fischer et al., 2006). Moreover, given their likely variations according to the host, the  
6 mutation rates for each species must be determined independently. Care must be taken inferring  
7 these rates because recombination can be mutagenic and its impact as well as other confounder  
8 effects need to be addressed.

9         The size of the populations of *Pneumocystis* species are not known, but they are expected  
10 to be small because of their narrow host ranges. *P. jirovecii* would have a small population size  
11 relative to the species infecting micro-mammals, thus reflecting the small size of human  
12 populations relative to those of rodents. Variations in population size over time affect the genetic  
13 diversity, *e.g.* a strong population **bottleneck** creates a loss of allele diversity due to increased  
14 **genetic drift**. Using non-recombining neutral loci, realistic mutation rates, and appropriate  
15 molecular clock models, past population history can be traced back using coalescent theory  
16 based applications such as skyline plot methods (Drummond et al., 2005; Heled and Drummond,  
17 2008). These demographic reconstructions would provide key metrics such as ancestral  
18 population sizes and evolutionary rates.

19         Interestingly, the strongest prediction of genetic diversity in many species is the life  
20 history, not the population history (Ellegren and Galtier, 2016). This means that there is a strong  
21 correlation between phenotypic traits (*e.g.* mating system, generation times) and the genetic  
22 diversity. For example, homothallism is expected to have long term evolutionary cost fitness  
23 because selfing populations experience reduced recombination rates and size, which ultimately

1 reduce the strength of purifying selection and increase genetic drift (Charlesworth and Wright,  
2 2001; Hill and Robertson, 1966; Otto and Lenormand, 2002; Pollak, 1987). The homothallism  
3 used by *Pneumocystis* species is also often associated to higher probability to experience  
4 population **bottlenecks** via founder effects and linked selection (Jarne, 1995; Charlesworth and  
5 Wright, 2001). There is a complex interplay between demographic, selective factors, and genetic  
6 diversity. Alternative scenarios, such as purifying selection purging deleterious alleles, which is  
7 known as “background selection” (Charlesworth, 1994), need also to be considered. In  
8 conclusion, many factors may have influenced genetic diversity of *Pneumocystis* species, which  
9 remains unclear.

10

### 11 **Population structure**

12 The population structure of *Pneumocystis* species is also controversial. Indeed, data support an  
13 absence of strong subdivision in *P. jirovecii* (Parobek et al., 2014) and *P. carinii* (Palmer et al.,  
14 2000), whereas other data support possible geographical clusters in *P. jirovecii* (Esteves et al.,  
15 2016; Alanio et al., 2017). Importantly, Matos and Esteves (2010) noted that the infections are  
16 not necessarily clonal and recombination between multi-locus genotypes is possible. All these  
17 inferences are based on a relatively small number of markers (*e.g.* ITS, mitochondrial large  
18 subunit rDNA), and need to be validated at the genome scale using appropriate Bayesian  
19 methods based on unlinked multi-allelic genotypes, such as STRUCTURE (Pritchard et al.,  
20 2000). In the meantime, interesting clues can be extracted from the biological cycle. The  
21 question is whether the fluctuation of the population structure is caused by variations in spore  
22 dispersal or in sexual recombination. The asci are 4-6  $\mu\text{m}$  in size, which is small enough to be  
23 airborne dispersed efficiently over long distances. The asci cell wall is enriched with

1 glycoproteins, melanin,  $\beta$ -glucans, and mannans without outer chain (Kottom and Limper, 2000;  
2 Icenhour et al., 2003; Icenhour et al., 2006b; Ma et al., 2016a), which might allow them to resist  
3 desiccation and UV irradiation usually fatal to many fungal spores (Golan and Pringle, 2017;  
4 Latgé, 2007). The viability of spores for extended periods of time is supported by the detection  
5 of *P. jirovecii* mRNA in hospital air samples (Latouche et al., 2001; Maher et al., 2001). Their  
6 resistance to physical assaults is suggested by their detection in air spora trapped in rural  
7 locations (Wakefield, 1996).

8         Dispersal of fungi can occur in two modes: (i) multiple sequential short-distance  
9 dispersal, and (ii) a single successful long-distance move of spores ultimately coinciding with  
10 optimal conditions for the growth of the fungus (Golan and Pringle, 2017). The former option  
11 produces a strong population subdivision, while the latter ends up with no or weak population  
12 structure because the same genotype(s) will be spread over large geographical distance. If long  
13 distance dispersal occurs on a global scale, it will result in a global population structure (Pringle  
14 et al., 2005). Rare long-distance dispersal would involve stochastic founding events, which can  
15 be revealed by population structures with an excess of rare alleles. Future studies combining  
16 genetic and geography are needed to fully access the population structures of *Pneumocystis*  
17 species.

18

### 19 **Clonal evolution or predominant sex/recombination?**

20 *P. jirovecii* infections are most often caused by multiple populations co-infecting the same  
21 individual (Hauser et al., 1997; Nahimana et al., 2000b; Palmer et al., 2000; Ma et al., 2002;  
22 Alanio et al., 2016). Multilocus genotypes (MLGs), which refer to a unique combination of  
23 alleles, can persist over long periods of time (4 to 9 years), and be observed across different

1 countries (Wakefield et al., 1994; Esteves et al., 2010). Recombination was also detected among  
2 MLGs (Esteves et al., 2010), which would explain the reported lack of strong population  
3 subdivision, at least in *P. jirovecii* (Parobek et al., 2014). Under **panmictic** population  
4 conditions, MLGs should not persist in the population because they will be disturbed consistently  
5 by recombination.

6 Buscaglia et al. (2015) proposed that “a highly structured (*i.e.* clonal) population  
7 indicates that the main mode of reproduction for such a species lacks genetic exchange (*i.e.* is  
8 primarily asexual) or sex occurs only rarely”. In *Pneumocystis*, MLGs do recombine which  
9 would indicate a limited global population structure (Esteves et al., 2010; Parobek et al., 2014).  
10 Thus, the definition proposed by Buscaglia et al. would suggest a widespread sexual  
11 reproduction in *Pneumocystis* species. However, some MLGs persist over time, which suggests  
12 that these species might be mostly clonal and only rarely engage to sexual events. This latter  
13 scenario would be consistent with the theory of predominant clonal evolution (Tibayrenc and  
14 Ayala, 2012; Tibayrenc and Ayala, 2014), which proposes that restrained recombination is not  
15 strong enough to disturb the pattern of clonal structure. The frequency of recombination events at  
16 the genome level is unknown in *Pneumocystis*, which currently prevents reaching definitive  
17 conclusions.

18

### 19 **Intra individual short-term evolution**

20 Infections are usually caused by multiple *P. jirovecii* strains acquired from different origins  
21 (infections *de novo* but also possibly re-activation of organisms). The balance between different  
22 strains will likely change over the course of the disease because of either drug treatment,  
23 pressures from the host immune system, and/or varying metabolism and fitness among the strains

1 present. Other pathogens such as *Candida* and *Cryptococcus* species evolve rapidly within their  
2 hosts by acquiring new mutations or changes in genomic heterozygosity associated with drug  
3 resistance (Ford et al., 2015; Chen et al., 2017). It is unclear if these mutations result from  
4 positive selection or DNA repair errors in *Cryptococcus* (Rhodes et al., 2017), although it is also  
5 possible that DNA repair errors are selected by positive selection. Competition among  
6 multiclonal parasite populations within the same host can, in theory, promote parasite diversity  
7 (Bashey, 2015). The full extent of *Pneumocystis* short-term evolution within their host is  
8 unknown. Interestingly, Alanio and colleagues used a set of markers to evidence changes in  
9 population composition during *P. jirovecii* infections (Alanio et al. 2016). Multiple strains  
10 infections are frequently found in pathogens and may have clinically relevant consequences  
11 (Balmer and Tanner, 2011). Different strains might have different susceptibility to treatment or  
12 evolve differently so that they may escape detection by the immune system or diagnostics tools.  
13 We anticipate that the characterization of multiclonal infections will have serious implications  
14 for the treatment and the management of *P. jirovecii* pneumonia. Experimental setups will  
15 become realistic when long-term *in vitro* culture method will become widely reproducible.  
16

## 1 **PERSPECTIVES AND CONCLUSIONS**

2 The research on *Pneumocystis* is still in its infancy mainly because of the lack of culture *in vitro*,  
3 but the availability of genomic data will help exploring the mysteries of their evolution. The next  
4 cornerstone will be the establishment of a long-term culture system and genetic manipulation.  
5 The upcoming expectation goes far beyond the *Pneumocystis* research community and will allow  
6 exploring key questions in evolutionary cell biology such as the evolution of parasitism and  
7 multicellularity. The study of *Pneumocystis* organisms has the unique interest that they are the  
8 only strictly mammalian-adapted fungal pathogens. Thus, determining the molecular basis of  
9 their adaptation and speciation are of uttermost importance. The key questions are: what are the  
10 determinants of the genome reduction? What are the molecular determinants of the host  
11 specificity and speciation? Why introns are so abundant and what are their function(s)? What are  
12 the impact of multiclonal infections and short-term evolution within host in the context of drug  
13 resistance and development of vaccines? How do natural populations of *Pneumocystis* evolve in  
14 different hosts?  
15

1

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7

1 **GLOSSARY**

2

3 **Background selection**

4 Reduction of genetic diversity at linked loci owing to selection against deleterious mutations.

5

6 **Bottleneck**

7 A sharp and rapid reduction in the size of a population.

8

9 **Clonal interference**

10 Phenomenon in population genetics of organisms with significant linkage disequilibrium (*i.e.*  
11 absence of recombination), especially in asexual organisms. It occurs when two (or more)  
12 different beneficial mutations arise independently in different individuals.

13

14 **Effective population size**

15 The size that a theoretical population evolving under a Wright-Fisher model would need to be in  
16 order to match aspects of the observed genetic data.

17

18 **Genetic drift**

19 Fluctuation of allele frequency among generations in a population owing to the randomness of  
20 survival and reproduction of individuals, irrespective of selective pressures.

21

22 **Haploid selfing**

23 Refers to true homothallic species. A species able to accomplish their entire sexual reproduction  
24 without the need of a partner.

25

26 **Heterozygosity**

27 Measure of the genetic diversity, which represents the presence of different alleles at one or more  
28 loci on homologous chromosomes. Often presented as a probability that two randomly sampled  
29 gene copies in a population carry distinct alleles.

30

31

1 **Linkage disequilibrium**

2 Nonrandom association of alleles at two loci often but not always due physical linkage. Such  
3 association is broken over time by recombination.

4 **Panmictic population**

5 Random mating among individuals in an idealized population.

6

7 **Selective sweep**

8 Elimination or reduction of genetic diversity in the neighborhood of a beneficial allele that  
9 increases in frequency in the population, typically after an environmental change.

10

11 **Selective sweeps**

12 Elimination or reduction of genetic diversity in the neighborhood of a beneficial allele that  
13 increases in frequency in the population, typically after an environmental change.

14

15

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18

## 1 LEGENDS FIGURES

### 2 **Figure 1** | Cell cycle

3 The whole cell cycle of *Pneumocystis* species would take place within the host's lungs, airborne  
4 asci ensuring transmission to new hosts. The cycle is thought to include two phases: sexual and  
5 asexual. The trophic forms tightly adhere to the host's alveolar epithelial pneumocytes type I,  
6 whereas asci are generally localized within the alveolar lumen. The ring shown in green might  
7 allow the formation of a rent upon contact with humidity and so the release of the ascospores.  
8 This ring may correspond to the parentheses-like structure visible on Figure 2. This Figure does  
9 not include new features relatively to models previously proposed.

10

### 11 **Figure 2** | Cluster of *P. jirovecii* asci

12 Cluster of *P. jirovecii* asci stained with Grocott's Methenamine silver (Churukian and Schenk,  
13 1977) within a patient's bronchoalveolar lavage. The structures darker than the rest of the wall  
14 on each ascus are the parentheses-like structure (picture from the Institute of Microbiology,  
15 Lausanne University Hospital).

16

### 17 **Figure 3** | Genome composition of *Pneumocystis* and related fungi

18 Protein coding genes, intergenic spaces, and intron positions were obtained from NCBI  
19 (<https://www.ncbi.nlm.nih.gov/>, last accessed 2018-03-20). Curated *Schizosaccharomyces*  
20 *pombe* and *Saccharomyces cerevisiae* intron data were extracted respectively from Pombase  
21 database (Wood et al., 2002; <https://www.pombase.org/downloads/intron-data>, last accessed  
22 2018-03-20) and *Saccharomyces* Genome database (Cherry et al., 1998;  
23 <https://www.yeastgenome.org>, last accessed 2018-03-20). Repeats include DNA transposons,  
24 retrotransposons, and simple low complexity repeats proportions as roughly estimated using

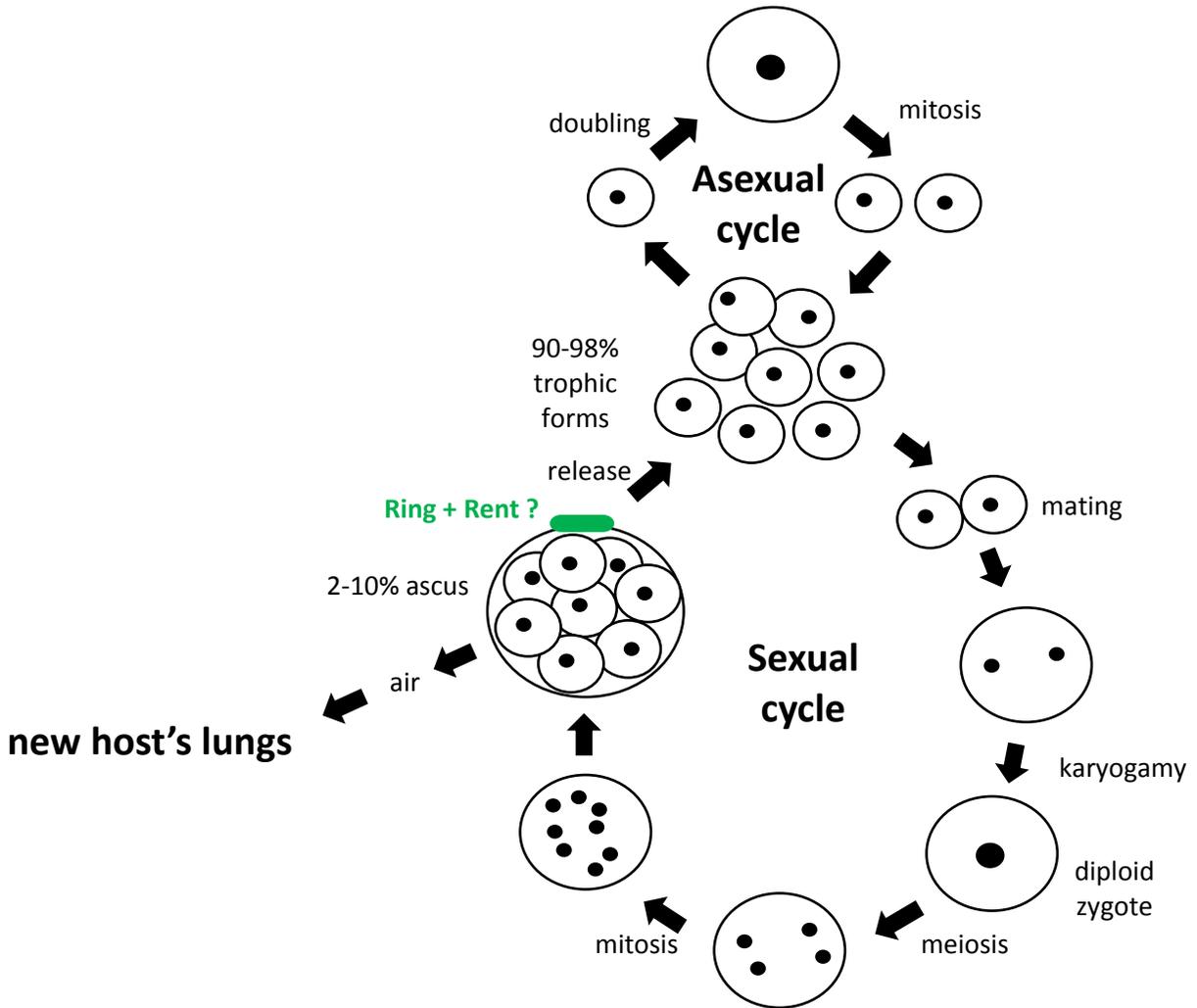
1 RepeatMasker (Smit et al., 2013) and RepBase database (Bao et al., 2015). The proportions of  
2 MSGs were calculated based on data from Ma et al. (2016a). Ribosomal DNA cassettes include  
3 each three genes (rDNA) and two internal transcribed spacers (ITS): 18S rDNA-ITS1-5.8 rDNA-  
4 ITS2-26S rDNA. *S. pombe* genome encodes roughly 140 copies of a cassette of a size of 5.8 kb.  
5 In *S. cerevisiae*, ca. 150 tandem copies of a 9.1 kb cassette are present (Venema and Tollervey,  
6 1999). In contrast, *Pneumocystis* species harbor each a single rDNA cassette of 11 kb.

7  
8 **Figure 4** | Graphical overview of the hypothetical evolutionary history of *Pneumocystis* species  
9 *Pneumocystis* species divergence timing has been determined elsewhere (Keely et al., 2003a;  
10 2004a; Beimforde et al., 2014). Losses of multiple metabolic pathways, as well as contraction  
11 and expansion of specific gene families are presented (Table 2). Note that the timing and order of  
12 losses is unknown. The gain and loss of specific functions for *Pneumocystis* is inferred here to  
13 have occurred in the last most recent ancestor common of *Pneumocystis* species (MRCA)  
14 because the underlying genes are absent in the genomes of all *Pneumocystis* sequenced to date.  
15 The MSG superfamily emerged in *Pneumocystis* ancestry and displays a substantial level of  
16 lineage specific divergence (represented by blue triangles). Intron loads are similar among  
17 *Pneumocystis* species, which might suggest a common origin. The fission yeast clade diverged  
18 ~250 MYA ago (Rhind et al. 2011) and has lost most of the introns acquired from an intron rich  
19 ancestor (Roy et al., 2005; Stajich et al. 2007; Rhind et al., 2011). Although there is no dating  
20 estimates for the intron loss in fission clade, the absence of recent intron gains and the low rates  
21 of intron loss (Zhu and Niu, 2013) suggest that the majority of introns were lost before the  
22 diversification of the fission yeast clade. The colors of the lines representing the evolving species  
23 signify different nutritional modes (dark green, saprophytism; light green, gradual shift from  
24 saprophytism to the parasitism; yellow, animal parasitism). We assume that the MRCA of

1 Taphrinomycota subphylum was a multicellular or dimorphic saprotroph based on ancestral traits  
2 reconstruction (Schoch et al., 2009; Nguyen et al., 2017). The phylogenetic relationship  
3 presented here is consistent with published phylogenies (Liu et al., 2009; Sugiyama et al., 2006).  
4 RRM correspond to RNA binding proteins harboring an RNA recognition motif.

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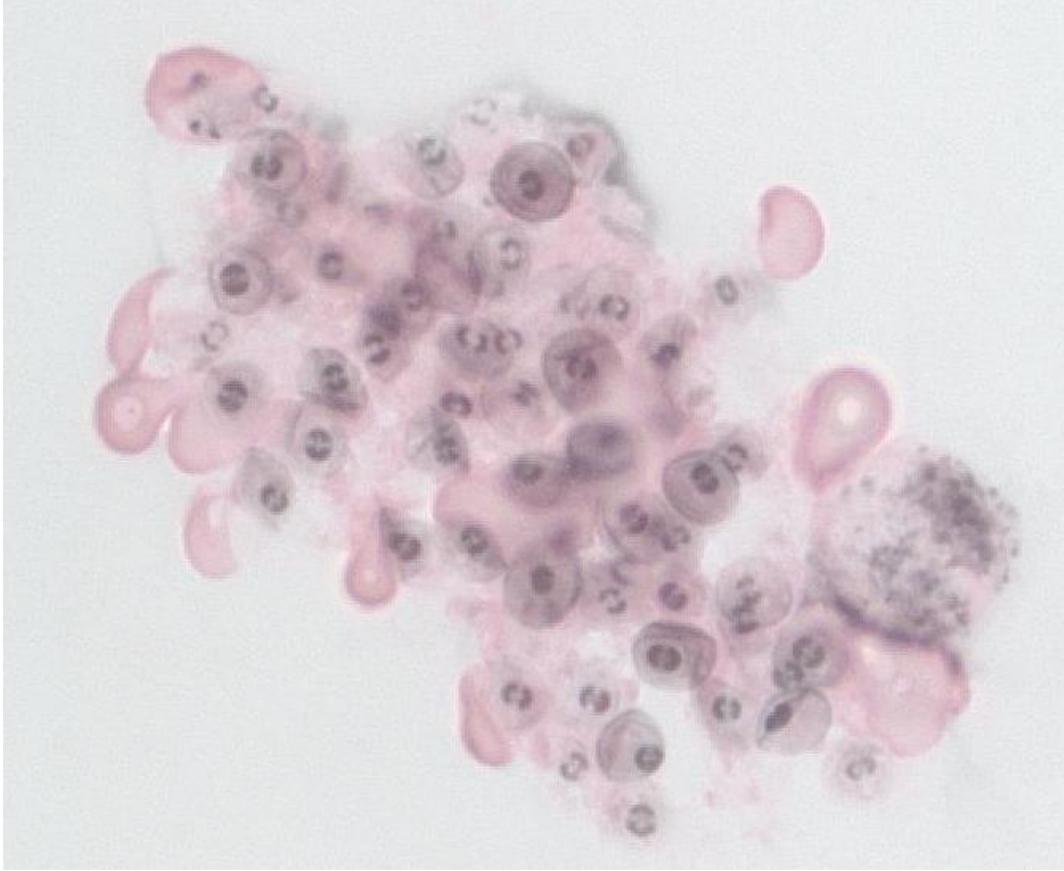
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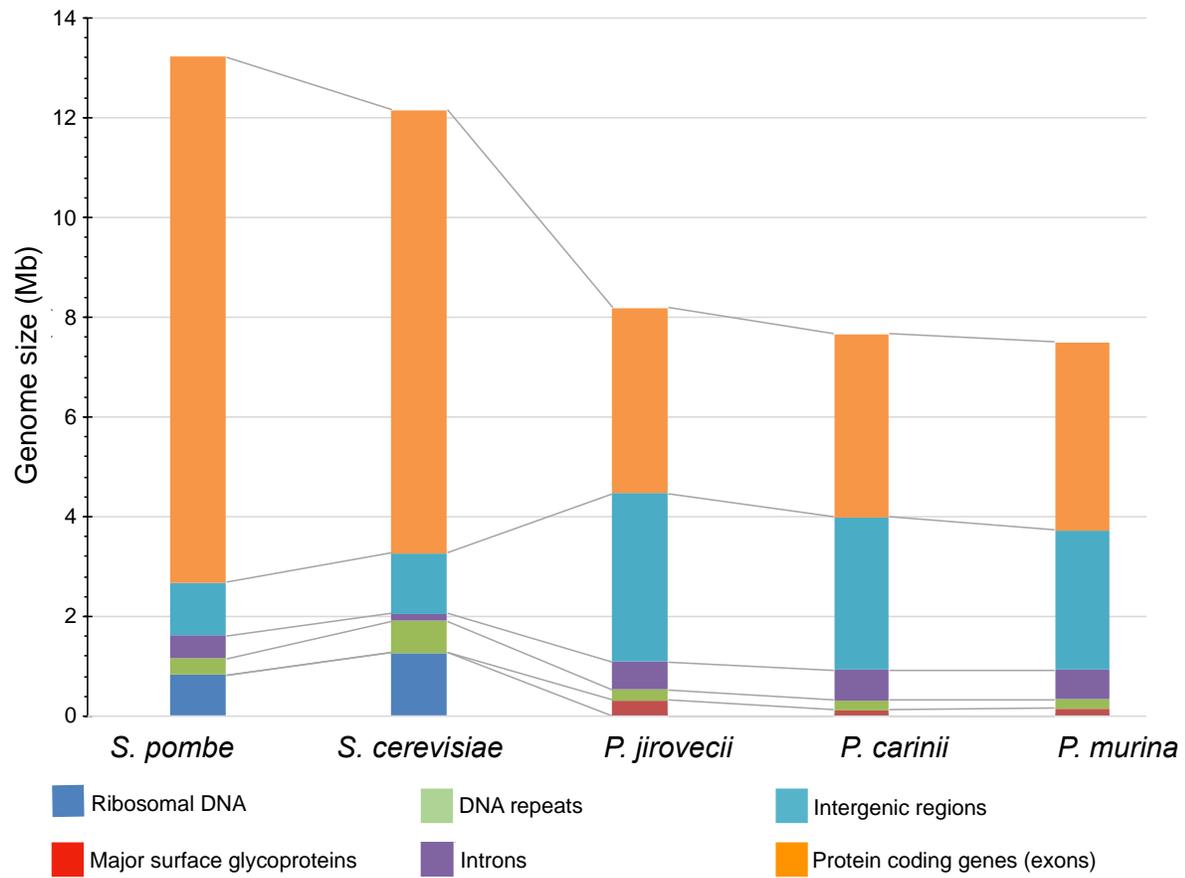
6 **Figure 1.**



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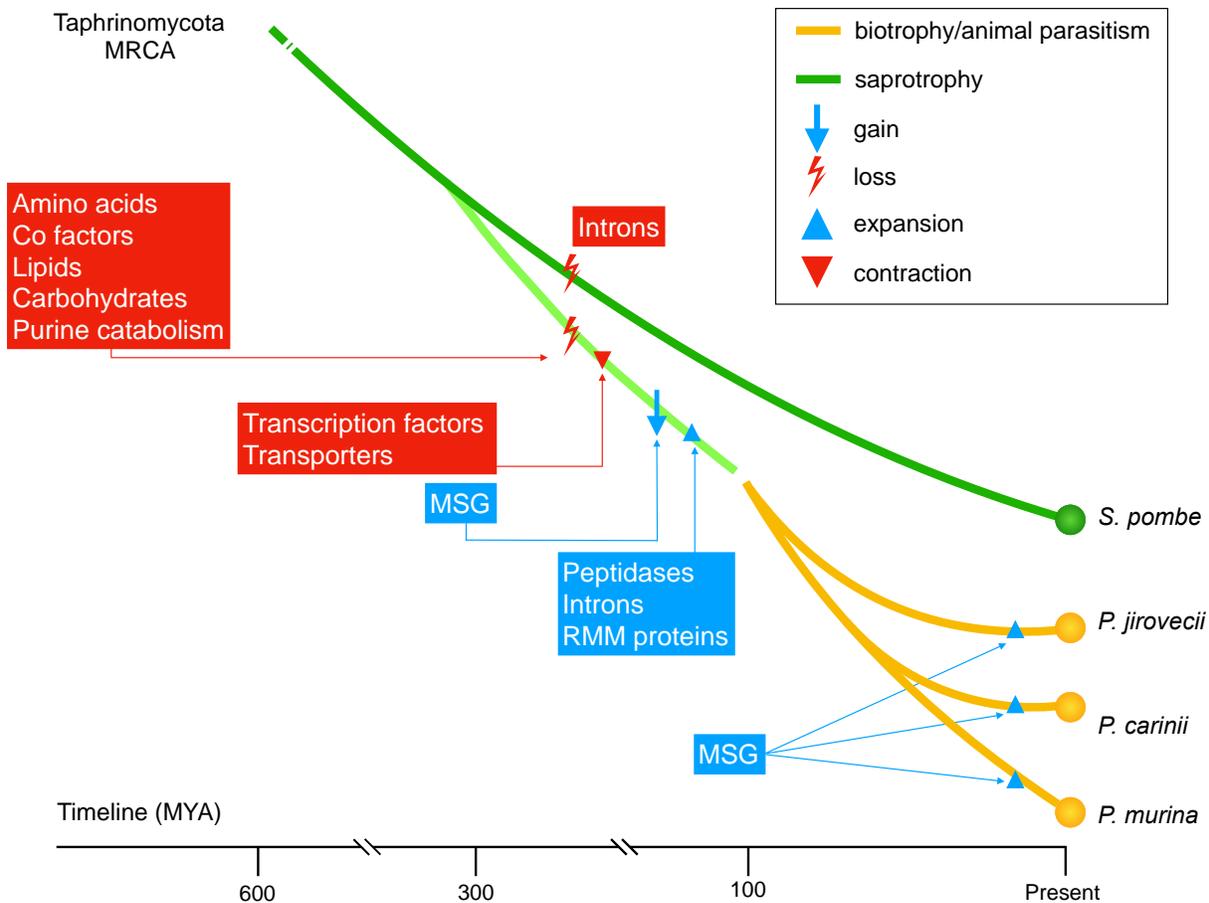
**Figure 2.**

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**Figure 3.**



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**Figure 4.**

**Table 1.** Features of *Pneumocystis* genomes <sup>a</sup>.

Species	Genome size Mb	%GC	Chromosomes no.	Protein coding genes no.
<i>P. jirovecii</i>	8.4	28.4	20	3,761
<i>P. carinii</i>	7.7	27.8	17	3,623
<i>P. murina</i>	7.5	27.0	17	3,646

<sup>a</sup> Genome data are from Ma et al. (2016a).

**Table 2.** Gene families expanded, contracted, and lost in the cellular and metabolic machineries of *Pneumocystis* species.

<b>Event</b>	<b>Gene family / Pathway</b>	<b>Reference</b>
<b>Expansion</b>		
	Major surface glycoproteins	Ma et al. 2016a; Schmid-Siegert et al. 2017
	S8 and M16 peptidases	Cissé et al., 2014 ; Ma et al. 2016a
	Proprotein convertase	Ma et al. 2016a
	Cystein rich CFEM_proteins	Ma et al. 2016a
	Kexin <sup>a</sup>	Ma et al. 2016a
<b>Contraction</b>		
	Transcription factors	Ma et al. 2016a
	Transporters	Cissé et al., 2012; Ma et al., 2016a
<b>Loss</b>		
	Co-factors coenzyme A, thiamine, biotin biosyntheses	Cissé et al., 2014; Ma et al., 2016a
	RNAi machinery <sup>b</sup>	Cissé et al., 2014
	Amino acids biosyntheses	Hauser et al., 2010; Cissé et al., 2012; Ma et al., 2016a

Steroids and <i>myo</i> -inositol biosyntheses	Porollo et al., 2014; Ma et al., 2016a
Inorganic sulfur and nitrogen assimilation	Cissé et al., 2014
Purines catabolism	Cissé et al., 2014
Nucleotide salvage pathways	Cushion et al., 2007; Cissé et al., 2012
Carbohydrate metabolism <sup>c</sup> , lipids <sup>d</sup> , and co-factors <sup>e</sup>	<u>Kaneshiro et al., 1999</u> ; Vestereng and Kovacs, 2004; <u>Cushion et al., 2007</u> ; Ma et al., 2016a

---

- <sup>a</sup> Kexin protease family are only expanded in *P. carinii*. It might be involved in the processing of MSGs at the cell surface (Lugli et al., 1999).
- <sup>b</sup> The RNA interference machinery includes the Dicer and Argonaute proteins.
- <sup>c</sup> The lost carbohydrate pathways are those of gluconeogenesis, glyoxylate cycle, chitin, and hyper-mannose glycosylation (outer chain N-mannans).
- <sup>d</sup> The lost lipids pathways are those of ergosterol, cholesterol, choline, ether lipids, sphingolipids, glycerol, and phosphatidylcholine.
- <sup>e</sup> Co-factors includes co enzyme A, and vitamins H and B1.

1 **Revised version:** modified or new text is underlined.

2

3

## 4 **Genomics and evolution of *Pneumocystis* species**

5

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7

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13

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## 1 **ABSTRACT**

2 The genus *Pneumocystis* comprises highly diversified fungal species that cause severe  
3 pneumonia in individuals with a deficient immune system. These fungi infect exclusively  
4 mammals and present a strict host species specificity. These species have co-diverged with their  
5 hosts for long periods of time (> 100 MYA). Details of their biology and evolution are  
6 fragmentary mainly because of a lack of an established long-term culture system. Recent  
7 genomic advances have unlocked new areas of research and allow new hypotheses to be tested.  
8 We review here new findings of the genomic studies in relation with the evolutionary trajectory  
9 of these fungi and discuss the impact of genomic data analysis in the context of the population  
10 genetics. The combination of slow genome decay and limited expansion of specific gene families  
11 and introns reflect intimate interactions of these species with their hosts. The evolutionary  
12 adaptation of these organisms is profoundly influenced by their population structure, which in  
13 turn is determined by intrinsic features such as their self-fertilizing mating system, high host  
14 specificity, long generation times, and transmission mode. Essential key questions concerning  
15 their adaptation and speciation remain to be answered. The next cornerstone will consist in the  
16 establishment of a long-term culture system and genetic manipulation that should allow  
17 unravelling the driving forces of *Pneumocystis* species evolution.

1 Main text : 7482 words

2

### 3 **BACKGROUND**

#### 4 **History**

5 *Pneumocystis* species form a group of opportunistic fungi that cause severe pulmonary infections  
6 in mammals with a deficient immune system. These organisms infect exclusively mammals.  
7 They were first described by Chagas (Chagas, 1909), and wrongly classified as special forms of  
8 trypanosomes. They were later identified as a *bona fide* separate species by the Delanoë couple  
9 at the Pasteur Institute in Paris (Delanoë and Delanoë, 1912). Their taxonomic classification  
10 remained then elusive because of a phenotypic resemblance with the protists. The issue was  
11 resolved using molecular phylogeny based on sequencing ribosomal DNA, which clearly  
12 indicated their fungal nature (Edman et al., 1988).

13

#### 14 **Phylogeny and taxonomy**

15 *Pneumocystis* species belong to the subphylum of Taphrinomycotina within the Ascomycota  
16 (Eriksson, 1997; Sugiyama et al., 2006). The Taphrinomycotina subphylum is monophyletic and  
17 encompasses mostly plant-associated or soil-dwelling fungi (Liu et al., 2009). *Pneumocystis*  
18 closest relatives are *Schizosaccharomyces pombe* and *Taphrina deformans*, their common  
19 ancestor having diverged from the other Taphrinomycota members ca. 467 million years ago  
20 (MYA) (Beimforde et al., 2014).

21 Although all *Pneumocystis* species are ubiquitous, each mammal species can be infected  
22 with only one or two of them. Five species have been formally described so far based on the  
23 requirements of the International Code of Botanical Nomenclature (ICBN): *Pneumocystis*

1 *jirovecii* in *Homo sapiens* (Frenkel, 1999), *Pneumocystis carinii* in *Rattus norvegicus* (Frenkel,  
2 1999), *Pneumocystis wakefieldiae* also in *Rattus norvegicus* (Cushion et al., 1993; Cushion et al.,  
3 2004), *Pneumocystis murina* in *Mus musculus* (Keely et al., 2004a), and *Pneumocystis oryctologi*  
4 in Old World rabbits (*Oryctolagus cuniculus*; Dei-cas et al., 2006). Antigenic and DNA based  
5 studies suggest the presence of distinct species also in macaques, ferrets, bats, shrews, horses,  
6 pigs, and dogs (Banerji et al., 1994; Peters et al., 1994; Christensen et al., 1996; English et al.,  
7 2001; Guillot et al., 2004).

8 *P. jirovecii* is the only species known to infect humans and has never been detected in  
9 any other animals. *P. carinii* is the best studied species because of the availability of protocols  
10 for experimental or natural infections in laboratory rats. *P. wakefieldiae* was reported either  
11 mixed with *P. carinii* (Cushion et al., 1993; Cushion, 1998; Cushion et al., 2004; Chabé et al.,  
12 2010), or alone (Palmer et al., 2000). The two latter species are different in terms of  
13 electrophoretic karyotypes, gene localization on the chromosomes, sequence identity (4-7%  
14 nucleotide divergence in seven orthologs; Cushion, 1998; Cushion et al., 2004), antigenic  
15 profiles (Vasquez et al., 1996), and major surface glycoproteins (MSG) expression (Schaffzin  
16 and Stringer, 2000). They might be competing against each other for resources when present  
17 together within the same rat (Icenhour et al., 2006a).

18

### 19 **Species divergence**

20 According to the evolutionary rates of several genomic loci, the radiation of the *Pneumocystis*  
21 genus occurred ca.100 MYA (Keely et al., 2003a; Keely et al., 2004a), which roughly overlaps  
22 with the radiation of the mammalian species (Holmes, 1991; dos Reis et al., 2015). *P. murina*  
23 would have diverged from *P. carinii* between 51 and 71 MYA (Keely et al., 2003a), while *P.*

1 *carinii* and *P. wakefieldiae* diverged between 15 and 22 MYA (Cushion et al., 2004; Fischer et  
2 al., 2006). The neat superposition of multiple *Pneumocystis* species phylogenetic trees with those  
3 of their respective hosts supports a co-evolution of these organisms (Guillot et al., 2001).  
4 Therefore, a plausible co-speciation scenario is that each species became physically separated  
5 from the other species, the hosts acting as barriers that led to the accumulation of genetic  
6 differences and the gradual reproductive isolation over time. The absence of gene flow or mating  
7 among the different species has been inferred based on linkage disequilibrium analysis consistent  
8 with an ancient reproductive isolation (Mazars et al., 1997; Keely et al., 2004a; Keely and  
9 Stringer, 2009). Furthermore, no evidence of hybridization was detected between *P. carinii* and  
10 *P. wakefieldiae*, even during co-infection of the same rat (Cushion, 1998; Cushion et al., 2004).  
11 However, caution is warranted because the absence of gene flow was inferred from a small set of  
12 conserved markers, which may have not allowed detecting all genetic events. Consequently,  
13 whole genome sequencing studies are necessary to validate these findings.

14

## 15 **Life cycle**

16 The life cycle of *Pneumocystis* organisms is still hypothetical and mostly derived from  
17 microscopic and molecular studies on *P. carinii* (Figure 1). As fungal organisms with an obligate  
18 parasitic behavior, the cycle would occur only inside host's lungs, and begin with the inhalation  
19 of infectious asci. Once inhaled, each ascus would release first eight ascospores which will  
20 evolve to what is known as trophic forms that bind to the type I pneumocytes of the alveolar  
21 epithelium. The cycle would then alternate between asexual multiplication of metabolically  
22 active trophic cells by binary fission, and sexual reproduction upon mating of two trophic cells  
23 that would culminate by the production of asci containing eight ascospores (Figure 2). Trophic

1 cells are amoeboid in shape and represent generally 90-98% of the populations in the infected  
2 lungs (Aliouat-Denis et al., 2009). These forms are mononuclear, 2-8  $\mu\text{m}$  in diameter (Dei-Cas et  
3 al., 2004), and mostly haploid (Stringer and Cushion, 1998; Wyder et al., 1998; Martinez et al.,  
4 2011). Multiploid forms are rare and possibly caused by asymmetrical or post-mating divisions  
5 (Martinez et al., 2011). Trophic cell surface is composed of a single layer of electron dense  
6 material containing glycoproteins, but possibly no  $\beta$ -glucans. Indeed, the enzymes responsible  
7 for the synthesis of  $\beta$ -glucans and the associated endo-1,3- $\beta$  glucanase are expressed almost  
8 exclusively in asci (Nollstadt et al., 1994; Kottom and Limper, 2000; Kutty et al., 2015). The  
9 presence of structural carbohydrate polymers of glucans in asci increases the physical strength of  
10 the cell wall, which might facilitate the survival outside the host. The doubling times are  
11 relatively long compared to free-living yeasts (~2 hours) and range from 1.5 to 10.5 days  
12 depending on the species (Aliouat et al., 1999; Keely et al., 2003b). The presence of a sexual  
13 cycle was initially supported by the ultrastructural observations of synaptonemal complexes  
14 (Matsumoto and Yoshida, 1984) and the expression of one pheromone receptor at the surface of  
15 *P. carinii* trophic cells (Vohra et al., 2004). Recent comparative genomic studies suggest that  
16 *Pneumocystis* species use primary homothallism (self-fertility) based on the genes number and  
17 arrangement on the chromosomes as a fusion of Plus and Minus mating type loci (Almeida et al.,  
18 2015). Thus, each strain would be able to produce asci on its own, without the need to find a  
19 compatible partner. Asci would be expelled by infected hosts and be the infectious stages  
20 because their specific inactivation or removal blocks the transmission chain (Cushion et al.,  
21 2010; Martinez et al., 2013). Consistently, recent analyses suggested that *Pneumocystis* sexuality  
22 is obligatory within host's lungs in order to complete the cell cycle and produce asci that are  
23 necessary for airborne transmission to new hosts (Richard et al., 2018). Furthermore, the

1 necessity of asci for transmission has been demonstrated by inhibition of the sexual cycle using  
2 echinocandins (Cushion et al., 2010), and by the fact that only purified asci could transmit the  
3 disease (Martinez et al., 2013). Recently, activation of sex-related genes upon treatment with  
4 echinocandins in RNA-seq analyses also suggested that sexuality is obligate (Cushion et al.,  
5 2018).

## 1 **Transmission**

2 *Pneumocystis jirovecii* pneumonia is a major public health problem with >400,000 cases per year  
3 worldwide and a mortality rate possibly as high as 80% if untreated (Brown et al., 2012).

4 Epidemiological data for *Pneumocystis* species in animal populations are scarce, but  
5 investigations in shrews and rats suggest a pervasive low level of infections (Laakkonen, 1998;  
6 Chabé et al., 2010).

7 *Pneumocystis* organisms are transmitted via the air from infected individuals to new hosts  
8 (Hughes, 1982), including between individuals within hospitals (de Boer et al., 2011), but also  
9 possibly via the transplacental route (Ceré et al., 1997; Sanchez et al., 2007; Montes-Cano et al.,  
10 2009). The current hypothesis is that infections occur over short distance among infected and  
11 susceptible individuals (Chabé et al., 2011). The transfer of parasites from animals to humans is  
12 no longer considered as a valid hypothesis based on the strict host species specificity (Chabé et  
13 al., 2011). Consistently, no convincing evidence of an environmental source of *Pneumocystis* has  
14 been found so far, which strongly suggests that mammals constitute the only reservoir of these  
15 fungi. Furthermore, the erosion of metabolic capabilities evidenced by the genome sequencing  
16 studies suggests that these organisms are unable to live outside their hosts (see below, losses of  
17 metabolic machinery section). Finally, they apparently complete their whole cell cycle within  
18 host's lungs since sexuality occurs therein. Healthy infected hosts colonized by the organism are  
19 believed to contribute greatly to the transmission and circulation process (Chabé et al., 2004;  
20 Peterson and Cushion, 2005; Le Gal et al., 2012; Alanio and Bretagne, 2017).

21

## 1 **Host specificity and biotrophy**

2 The strict host species specificity of the *Pneumocystis* species means that the fungal cells can  
3 only infect or survive in the host in which they were isolated in the first place. This view is  
4 mainly supported by the systematic failure of cross-infection experiments involving severely  
5 combined immuno-deficient animals and nude rats (Aliouat et al., 1993; Furuta et al., 1993;  
6 Gigliotti et al., 1993; Aliouat et al., 1994; Atzori et al., 1999; Durand-Joly et al., 2002). The  
7 selective activation of trophic cells by their host seems to trigger the formation of cytoplasmic  
8 projections by *Pneumocystis* cells, the filopodia (Aliouat-Denis et al., 2008). Accordingly, *P.*  
9 *carinii*, the species naturally infecting rats, is unable to form filopodia and infect when  
10 inoculated in mice, whereas *P. murina*, the natural parasite of mice, produce filopodia and high  
11 parasite loads under the same conditions (Aliouat-Denis et al., 2008). The function of the  
12 filopodia remains elusive but these structures display ultrastructural differences that are species  
13 specific, and that might account for some aspects of the host specificity.

14 Another aspect of this host specificity is that *Pneumocystis* species are most probably  
15 obligate biotrophs (Cushion et al., 2007; Cushion and Stringer, 2010; Hauser, 2014; Ma et al.,  
16 2016a). The way fungal parasites scavenge nutrients from their host is an active research field  
17 and three modes are broadly recognized: (i) biotrophy, where the parasite acquires nutrients from  
18 a living cell, (ii) necrotrophy, where host cells are killed to release nutrients, and (iii)  
19 saprotrophy, where the organism feeds on dead or decaying organic material. Biotrophs do little  
20 damage to host cells and lack virulence factors (van der Does and Rep, 2007). *Pneumocystis*  
21 perfectly fits to the biotrophy definition because they cause no apparent cell death and lack any  
22 experimentally verified fungal virulence factors such as glyoxylate cycle, secondary metabolism,  
23 and secreted effectors (Cushion et al., 2007; Cissé et al., 2012; Cissé et al., 2014; Ma et al.,

1 2016a). This implies that they rely entirely on their host for their survival and thus have evolved  
2 close relationships that rendered them host species specific.

3         The physiological characteristics of the hosts are key determinants of parasite adaptation  
4 (Poulin et al., 2006). For example, micromammals are small bodied with short lifespans, high  
5 reproduction rates, and high population densities, whereas these distinctive features are reversed  
6 in large mammals such as humans. The co-evolution theory predicts that parasitic species  
7 infecting micromammals exhibit a weaker host specificity compared those adapted to long-lived  
8 hosts with more stable population densities (Poulin et al., 2006). This prediction has been  
9 validated in fish parasites, among which strong host specificity is favored in stable resources  
10 found in hosts with a large body size (Sasal et al., 1999; Desdevises et al., 2002). As far as  
11 *Pneumocystis* is concerned, humans are infected by only one species whereas rats can be co-  
12 infected by two (Cushion et al., 1993 and 2004; Icenhour et al., 2006a; Golab, 2009). The  
13 number of *Pneumocystis* species able to infect rodents might even be more important, as shown  
14 by the recent discovery of multiple lineages shared among species and genera of the Southeast  
15 Asian murid species (Latinne et al., 2017). These findings might indicate a relaxation of the strict  
16 host specificity in small mammals harboring *Pneumocystis*, although additional supporting data  
17 are needed to fully challenge the concept of widespread strict host specificity.

18         Co-phylogenetic studies of *Pneumocystis* species and their hosts suggest that the host  
19 specificity evolved as a continuous trait resulting from a long-lasting co-evolution (Demanche et  
20 al. 2001; Guillot et al., 2001; Hugot et al., 2003). Strict host specificity is rare in animal  
21 pathogens but widespread in plant fungal pathogens (Parker and Gilbert, 2004; Restrepo et al.,  
22 2014). In the latter, the ecological adaptation often results in a pronounced specialization to  
23 particular hosts (Clay and Kover, 1996). In these systems, host specificity acts as a reproductive

1 isolating mechanism because it favors higher rate of mating between individuals on the same  
2 host and reduced gene flow among populations from different hosts (Giraud, 2006). A rapid  
3 divergence of the virulence factors, the pathogen “effector repertoire”, is often associated with  
4 the emergence of host specificity (Schulze-Lefert and Panstruga, 2011). The hypothesis of the  
5 latter authors states that changes in pathogen host range is driven by variation in the pathogen  
6 effector repertoire. This description fits the lineage specific expansion of the MSG superfamily  
7 in *Pneumocystis* species (Ma et al., 2016a; Ma et al., 2016b; Schmid-Siegert et al., 2017), which  
8 suggests that these proteins might account for some aspects of the host specificity.

9  
10

## 1 GENOME ORGANIZATION

### 2 Genomic data acquisition

3 The quest for genome sequence data began with the successful cloning of *P. carinii* genomic  
4 fragments (Tanabe et al., 1988). Pulse field gradient gel electrophoreses have been then  
5 instrumental for karyotypic characterization of *Pneumocystis* genomes and evidenced 12 to 20  
6 chromosomes according to the species totaling ca. 8 Mb (Hong et al., 1990; Yoganathan et al.,  
7 1989; Stringer and Cushion, 1998). Differences in karyotype profiles determined that the species  
8 infecting humans and rats are genetically distinct (Stringer et al., 1993). Significant genome size  
9 variations among species have been reported, *e.g.* that of ferret *Pneumocystis* would be ca. 1.7  
10 times bigger than that of *P. carinii* (Stringer and Cushion, 1998). A draft of *P. carinii* genome  
11 covering ca. 70% of genome was generated in 2006 using a clone-based Sanger sequencing  
12 approach from infected laboratory rats (Slaven et al., 2006). In 2012, the first draft of *P. jirovecii*  
13 genome was obtained from a single bronchoalveolar lavage of a patient with pneumonia (Cissé et  
14 al., 2012). This assembly encompasses 358 contigs capturing 90 to 95% of the genome, but the  
15 repetitive subtelomeric and centromeric regions could not be resolved. The centromeres have not  
16 been discovered yet in *Pneumocystis*, whereas the subtelomeric regions were resolved using  
17 Sanger sequencing of cosmids (Keely et al. 2005), and more recently assembled using  
18 sequencing generating long reads (Ma et al., 2016a; Ma et al., 2016b; Schmid-Siegert et al.,  
19 2017) (see below, chromosomal ends section). Chromosomal level assemblies of *P. jirovecii*, *P.*  
20 *carinii*, and *P. murina* were recently published revealing genome sizes ranging from 7.4 to 8.3  
21 Mb (Table 1; Ma et al., 2016a). It became evident that the genomes of *Pneumocystis* species had  
22 undergone an important reduction relative to *S. pombe* (7.5 to 8.3 Mb versus 12.5 Mb).

23

## 1 Nuclear Genome content

2 The analysis of the *Pneumocystis* genome assemblies validated the presence of single copy  
3 ribosomal DNA reported previously for *P. jirovecii* ([Giuntoli et al. 1994](#); Stringer, 1996; Tang et  
4 al., 1998; Nahimana et al., 2000a). This is similar to *Taphrina deformans* (Cissé et al., 2013), but  
5 contrasts with most fungi which harbors commonly tens or hundreds of copies of the locus.  
6 Figure 3 shows the genome compositions of *Pneumocystis* species compared to related fungi.  
7 These data highlight the contraction of the protein coding regions as compared to free-living  
8 yeasts, which reflect massive gene losses. Figure 3 also evidences the expansions of the MSG  
9 superfamily, of introns, as well as of the cumulative length of the intergenic regions (IGR). We  
10 previously reported that IGR in *P. jirovecii* occupy a larger genome fraction as compared to free  
11 living yeasts *Saccharomyces cerevisiae* and *S. pombe* despite a significantly smaller genome  
12 (Cissé et al., 2014). This observation holds when we re-evaluate here IGRs in the newly  
13 published full-length genomes of *P. jirovecii*, *P. carinii*, and *P. murina* (Ma et al. 2016a). This  
14 strongly suggests that genome streamlining in *Pneumocystis* species is driven by gene deletions  
15 rather than reduction of IGRs. This observation seems counterintuitive because genome  
16 reduction is almost always associated to a reduction of introns and IGRs in parasites (Keeling  
17 and Slamovits, 2005). Alternatively, large IGRs might favor chromosomal re-arrangements by  
18 increasing the number of possible breakpoints, as hypothesized in fungal microsporidian  
19 parasites (Slamovits et al., 2004; Keeling and Slamovits, 2005). The other characteristics of these  
20 genomes are discussed in the following sections.

21

## 1 **Chromosomal ends**

2 Subtelomeres in microbial parasites are often enriched with multi-copy surface glycoprotein  
3 gene families (Deitsch et al. 2009). These genomic regions are prone to (i) gene silencing that  
4 can be used for mutually exclusive expression, (ii) enhanced mutagenesis, and (iii) ectopic  
5 recombinations facilitated by the formation of clusters of telomeres at the nuclear periphery  
6 (Barry et al., 2003). These regions correspond to an important proportion of the *Pneumocystis*  
7 genomes (ca. 5%), and harbor a superfamily including five to six families of highly polymorphic  
8 multi-copy proteins called major surface glycoproteins (MSG) that are believed to be crucial for  
9 the fungus' lifestyle (Ma et al., 2016a; Schmid-Siegert et al., 2017). These *msg* genes exist only  
10 in *Pneumocystis* species and all species of *Pneumocystis* have their own repertoire, which  
11 suggest they have been acquired in a common ancestor, although their origin is not known. The  
12 absence of homology of these MSGs outside *Pneumocystis* lineages might indicate a transfer  
13 from an unknown species or a gene co-option. *msg* families have been first described and studied  
14 in *P. carinii* (Kovacs et al., 1993; Sunkin et al., 1994; Sunkin et al., 1996; Keely et al., 2005;  
15 Keely and Stringer, 2009), and subsequently analyzed in *P. jirovecii* and *P. murina* (Haidaris et  
16 al., 1998; Kutty et al., 2008; Ma et al., 2016a; Schmid-Siegert et al., 2017). Important differences  
17 exist among *Pneumocystis* species in terms of *msg* gene copy numbers, 60 to 140 copies per cell,  
18 and protein divergence (Ma et al., 2016a). Moreover, one MSG family is present only in *P.*  
19 *jirovecii* (*msg*-IV or -B), whereas another one is present only in *P. carinii* and *P. murina* (MSR  
20 family, *i.e.* MSG-related). MSGs are believed to be involved in antigenic variation (Stringer,  
21 2007). MSGs would also mask glucans at the asci surface from the immune recognition (Kutty et  
22 al., 2016). The antigenic diversity seems to be created via intra-family recombination of *msg*  
23 genes encoding different isoforms, creating mosaic genes, as well as through increased

1 mutagenesis (Kutty et al., 2008; Keely and Stringer, 2009; Schmid-Siegert et al., 2017). The  
2 expression of the most abundant MSG family (*msg-I* or *-A1*) that is present in all species is  
3 subject to mutually exclusive expression of a single isoform in each cell by using a single copy  
4 transcription promoter (the upstream conserved sequence, UCS) (Edman et al., 1996; Kutty et  
5 al., 2001; Sunkin et al., 1996; Wada et al., 1995). The UCS ends by the conserved recombination  
6 joint element (CRJE) which is also present at the beginning of each *msg-I* gene and may serve as  
7 recombination breakpoint (Stringer, 2007). The CRJE would be larger in *P. wakefieldiae* (ca.  
8 330 bps) than in *P. murina* (132), which in turn is larger than in *P. carinii* and *P. jirovecii* (28  
9 and 33, respectively) (Keely et al., 2007). On the other hand, at least in *P. jirovecii*, members of  
10 the other five families possess each their own promoter (Schmid-Siegert et al., 2017), but their  
11 expression patterns remain to be characterized. Recently, one family has been shown in *P.*  
12 *murina* to be expressed only in ascospores within asci and young trophic forms (Bishop et al.,  
13 2018).

14

## 15 **Introns**

16 Introns are extremely abundant in *Pneumocystis* genes and are as many as several tens per gene  
17 with a mean of five, and more than 40% of genes are interrupted by at least four introns (Stringer  
18 and Cushion, 1998; Ma et al., 2016a). Their presence can be equally explained by massive gains  
19 in *Pneumocystis* most recent common ancestry, or retention of ancestral elements that would  
20 have been lost in some Taphrinomycotina lineages such as *Schizosaccharomyces*. The introns are  
21 short (average length of 48 nucleotides), have a strong adenine and thymine bias, and present  
22 typical donor, acceptor and branch site patterns (Slaven et al., 2006). *Pneumocystis* introns  
23 cannot be processed by *S. pombe* and *S. cerevisiae* spliceosomes because of the divergence in  
24 intron-exon boundaries and branching sites within the introns (Thomas et al., 1999). RNA-seq

1 data indicate that intron retention affects ca. 45% of all introns (Ma et al., 2016a). *Pneumocystis*  
2 species contain self-splicing group I introns that are absent in higher eukaryotes such as humans  
3 (Liu et al., 1994), which renders them a prime target for the development of new drugs. These  
4 latter introns catalyze their own excision from RNA transcripts, a reaction that is inhibited by the  
5 drug pentamidine that is used against *Pneumocystis* (Liu and Leibowitz, 1993).

6         Given the important genome reduction at the *Pneumocystis* genus level, the presence of a  
7 high intron density per gene suggests a selective constraint to conserve them. Intron loss is  
8 dominant in fungi (Stajich et al., 2007), and this tendency is even more pronounced in some  
9 parasites such as microsporidia (Keeling et al., 2010). The intron history is highly flexible within  
10 the Taphrinomycota, with the plant-associated *Neolecta* having a high intron density similar to  
11 *Pneumocystis* (Nguyen et al., 2017), and the intron-poor free-living yeast *S. pombe* (Wood et al.,  
12 2002). The non-sense-mediated mRNA decay machinery is conserved in *Pneumocystis* species  
13 (Ma et al., 2016a). Under neutral scenario (no advantage) and widespread intron retention, most  
14 of the introns would produce non-functional transcripts tagged for destruction. This would be an  
15 incredible waste of resources in absence of another function. The latter could consist in  
16 alternative splicing increasing transcript diversity and regulating gene transcription or mRNA  
17 stability. Consistently, the *P. carinii* inosine 5'-monophosphate dehydrogenase pre-mRNA is  
18 differentially spliced, which was suggested to reflect changes in environmental stresses (Ye et  
19 al., 2001). These considerations suggest that introns might be neutral elements involved in many  
20 cellular processes via a greater proteome diversity, possibly including acting as a favorable  
21 substrate to facilitate shifts in lifestyle (*i.e.* parasite transition from one host species to another, or  
22 from plant to animal).

23

## 1 Mitogenomes

2 The mitochondrial genomes of *P. carinii* (Sesterhenn et al., 2010; Ma et al., 2013), *P. jirovecii*  
3 (Cissé et al., 2012; Ma et al., 2013), and *P. murina* (Ma et al., 2013) have been sequenced. The  
4 mitogenome sizes range from 24 to 35-kb with a substantial size variability among isolates in all  
5 species. *P. carinii* and *P. murina* mitogenomes end with single-stranded loop sequences that  
6 would allow forming linear concatemers and protecting the ends of the molecule. The presence  
7 of these repeats might account for the variable size of *P. carinii* mitogenomes. *P. jirovecii*  
8 mitochondrial genome is circular since it lacks inverted terminal repeat allowing circulation. The  
9 significance of circularity versus linearity is unknown. Related Taphrinomycota of the genera  
10 *Schizosaccharomyces*, *Taphrina*, and *Neolecta* have circular genomes (Bullerwell et al., 2003;  
11 Cissé et al., 2013; Tsai et al., 2014; Nguyen et al., 2017), which might indicate that the circular  
12 form is ancestral. Interestingly, *P. carinii* and *P. murina* mitogenomes are highly co-linear  
13 whereas *P. jirovecii* mitogenome presents some re-arrangements, similarly to the nuclear  
14 genomes (see below Chromosomal re-arrangement section). The gene content is highly  
15 conserved among the three *Pneumocystis* species, although there is a substantial nucleotide  
16 divergence among species (27 to 31%) (Ma et al., 2013). These mitogenomes encode ca. 17  
17 genes commonly found in mitochondrial fungal genomes such as ATP synthases, cytochrome *c*  
18 oxidases, NADH dehydrogenases, and the full repertoire of at least 20 transfer RNAs.

19 Reports investigating the dynamics of the mitochondrial genes during infection have revealed  
20 that mitogenomes would be very plastic in terms of copy number variations (Valero et al., 2016),  
21 and of genetic diversity including heteroplasmy (Alanio et al., 2016). The subsequent sections of  
22 this review focus on nuclear genomes.

23

## 1 **EVOLUTION**

2 Comparison of the gene families and pathways present in *Pneumocystis* genomes to those in  
3 selected fungi has revealed numerous losses / contractions and relatively few expansions (Table  
4 2). The hypothetical evolutionary history of *Pneumocystis* species derived from these  
5 observations is represented in Figure 4 and discussed in the following sections.

6

### 7 **Losses in the metabolic and cellular machineries**

8 Massive gene losses suggest that *Pneumocystis* species are auxotroph for essential nutrients,  
9 which might explain the recurrent failures of *in vitro* culturing attempts. The lost pathways  
10 include basic components of metabolic machinery such as the synthesis of amino acids or  
11 carbohydrates (Table 2). The loss of purines catabolism seems unique to *Pneumocystis* (Chitty  
12 and Fraser, 2017). *Pneumocystis* species are able to synthesize fecosterol and episterol but lack  
13 enzymes to convert them into ergosterol. Consequently, their membranes contain cholesterol  
14 instead of ergosterol, which probably explains their resilience to azole treatment. *Pneumocystis*  
15 organisms are also able to synthesize a unique class of sterols, the “pneumocysterols” (Kaneshiro  
16 et al. 1994; Kaneshiro et al. 1999; Florin-Christensen et al. 1994; Giner et al., 2002). It  
17 interesting to note the early steps of the sterol biosynthetic pathway leading to the formation of  
18 pneumocysterol and episterol are conserved in *Pneumocystis* species, and only the final steps  
19 toward ergosterol/cholesterol production are missing (Joffrion et al., 2010). This is exemplified  
20 by the fact that key enzymes for the formation of ergosterol (i.e. *erg3*, *erg4* and *erg5*) are not  
21 identifiable within the genomes. Analysis of the sterol biosynthesis machinery suggest that these  
22 species may be able to synthesize ergosterol/cholesterol precursors such as zymosterol, fecosterol

1 and episterol. Thus, the sterol pathway may have been re-routed and branch to form unique  
2 sterols, the pneumocystisterols.

3 Overall, these observations are consistent with the idea that losses of metabolic genes correlate  
4 with an increased dependency of the parasite on its host. Therefore, nutrients need to be  
5 scavenged from the host, which often mechanistically involves large batteries expanded  
6 transporters (*e.g.* as observed in microsporidia [Cuomo et al., 2012]). This is not the case in  
7 *Pneumocystis* since transporters families are also greatly reduced (Cissé et al., 2014; Ma et al.,  
8 2016a). For instance, the amino acid permeases and transporters that can respectively carry  
9 amino acids and oligopeptides are greatly reduced relatively to other Taphrinomycota (one copy  
10 of general amino acid permease versus 21 copies in *S. pombe*). Transmembrane proteins such as  
11 those of the major facilitator superfamily, sugar transporters, or more specific transporters (*e.g.*  
12 efflux pumps) are significantly reduced in *Pneumocystis*. The reduction of the transporters  
13 battery might be compensated by the use of highly selective transporters for critical compounds.  
14 The recent discovery of the import of *myo*-inositol in *Pneumocystis* cells via a low affinity but  
15 highly selective system supports this idea (Cushion et al., 2016). Unfortunately, high affinity  
16 transporters cannot be identified solely by computational means. Alternatively, simple diffusion  
17 across the membrane may occur, as evidenced in *P. carinii* for amino acids uptake using *in vitro*  
18 experiments (Basselin et al, 2001a; Basselin et al, 2001b). Basic cellular machinery is also  
19 affected by the loss of several fungal specific transcription factor families and the RNA  
20 interference machinery (Table 2).

21

## 1 **Evolutionary basis of gene loss**

2 Gene loss is a common trend in parasitic and symbiotic species, which often harbor a small sized  
3 genome (Keeling and Slamovits, 2005; Wolf and Koonin, 2013). The driving factors are often  
4 unknown or specific to the lifestyle of the species under study. A central question in evolutionary  
5 biology is whether gene loss is neutral or adaptive. In *Pneumocystis* species, there are footprints  
6 of both processes and we discuss here a few examples.

7         The neutral theory is usually sufficient to explain gene loss in parasites (O'Malley et al.,  
8 2016). Organisms with narrow host niche such as *Pneumocystis* are predicted to have small sized  
9 populations with increased **genetic drift** (bold: see glossary) (Papkou et al., 2016). The main  
10 mechanisms for gene loss are pseudogenization and sudden DNA deletions. Pseudogenization  
11 consists in the accumulation of deleterious mutations in non-essential genes ultimately leading to  
12 the loss (Kuo and Ochman, 2009; Wernegreen, 2015). The proportion of pseudogenes in *P.*  
13 *jirovecii* is low and equivalent to that present in free-living yeasts (0.02 pseudogene per protein-  
14 coding gene [Cissé et al., 2014]). This observation might indicate that pseudogenization is not  
15 the main driver of gene loss in this species. The following considerations do not undermine this  
16 observation but suggest that caution must be exercised: (i) this rate of pseudogenization is valid  
17 only for *P. jirovecii* and for the single isolate which genome was sequenced (Cissé et al., 2012),  
18 and (ii) only genes including stop codons were considered, that is, other types of gene  
19 inactivation were not considered (*e.g.* untranslated RNA genes or unfixed mutations). Gene loss  
20 can also be result of deletions independent of selection such as the movement of transposable or  
21 integrated viruses (reviewed by Albalat and Canestro, 2016).

22         The adaptive theory of gene loss implies a selective advantage and has been  
23 demonstrated to have occurred in many pathogenic lineages, for example for the Allergen 1 in

1 *Cryptococcus neoformans* (Jain et al., 2009), and for the *de novo* biosynthesis of nicotinic acid  
2 genes in *Candida glabrata* (Domergue et al., 2005). In *Pneumocystis*, the loss of chitin might  
3 have been lost to allow avoiding recognition from the host immune system (Ma et al., 2016a).  
4 The gene families and pathways cited in Table 2 are missing in the three *Pneumocystis* genomes  
5 available (Ma et al 2016a), which suggests that these losses occurred before the radiation of the  
6 genus. An unexpected consequence is that the observed gene losses might not reflect the current  
7 selective forces, and therefore might not be relevant for the host specificity.

8

### 9 **Chromosomal re-arrangement**

10 The chromosome level assemblies revealed that an important chromosomal re-arrangement  
11 occurred among *Pneumocystis* species (Ma et al., 2016a). The re-arrangement, however,  
12 followed the species tree, that is, the macrosynteny is broken between rodents infecting  
13 *Pneumocystis* (*P. carinii* and *P. murina*) and the humans infecting species (*P. jirovecii*), whereas  
14 *P. carinii* and *P. murina* genomes are highly collinear. Nevertheless, the gene order is conserved  
15 in syntenic regions among the three species (>92% of the genes), and ca. 83% of gene families  
16 are orthologous, with 4 to 30% of divergence at the nucleotide sequence level. The high gene  
17 conservation among the three species suggest that re-arrangements occurred mostly in the  
18 intergenic regions (IGR). In fungi, IGRs are often enriched in regulatory functions such as signal  
19 transduction or binding sites of transcription factors (Noble and Andrianopoulos, 2013).  
20 Chromosomal translocations impact gene expression as well as long-distance gene-to-gene  
21 contact via chromatin interactions, and thus might be involved in speciation (Rieseberg, 2001;  
22 Bakloushinskaya, 2016). Protein evolution is also faster in re-arranged chromosomes than  
23 collinear chromosomes because re-arrangements reduce homologous recombination and

1 facilitate positive selection (Rieseberg, 2001). A key question here is whether chromosomal re-  
2 arrangements are involved in the adaptation of each *Pneumocystis* species to its host. Future  
3 studies are required to probe an eventual role of these re-arrangements in *Pneumocystis*  
4 evolution.

## 6 **Loss of complex multicellularity**

7 The recent sequencing of the *Neolecta irregularis* genome revealed that the Taphrinomycotina  
8 last common ancestor was probably multicellular (Nguyen et al., 2017). These findings suggest  
9 that *Pneumocystis* organisms evolved from a plant-associated or soil-adapted multicellular  
10 organism. The shift in cell morphology to single celled organisms is associated with the deletion  
11 of an ancestral morphogenic kit that included many cell differentiation and cell-to-cell signaling  
12 genes. These losses are not specific to *Pneumocystis* and were observed in a wide range of  
13 unrelated yeasts (Nguyen et al., 2017; Nagy et al., 2014; Nagy, 2017), which suggests a  
14 convergent evolution. The transition from a hyphal to yeast form takes place in many fungal  
15 lineages and is often triggered by a thermal stimulus (Köhler et al., 2017), CO<sub>2</sub> levels (Hall et al.,  
16 2010), or pH (Davis, 2009), and is directly linked to the ability to invade hosts. Notable  
17 examples include the dimorphic human pathogenic fungi *Histoplasma*, *Blastomyces*,  
18 *Coccidioides*, and *Paracoccidioides* (Beaman et al., 1981; Medoff et al., 1987; Inglis et al.,  
19 2013).

20 The ancestral morphogenic kit for complex multicellularity (fruiting bodies) is lost in  
21 *Pneumocystis*. However, *Pneumocystis* species are able to produce biofilms (Cushion et al.,  
22 2009), which is an undifferentiated form of aggregative multicellularity often seen in bacteria  
23 (Claessen et al., 2014). Inversely, the yeast *Saitoella complicata* grows primarily by budding

1 (Goto et al., 1987), despite having the cellular machinery for the production of fruiting bodies  
2 (Nguyen et al., 2017). Comparative genomics and epigenomics would be extremely valuable to  
3 explore the molecular process underlying the loss of the multicellular phenotype. These  
4 considerations highlight the fact that phenotypes cannot be explained solely by gene loss and  
5 gain balance, and that other subtle mechanisms need to be considered.  
6

## 1 **POPULATION GENETICS**

### 2 **Strain typing**

3 Given the high homogeneity of genomic sequences at the nucleotide sequence level among *P.*  
4 *carinii* isolates, strain typing for this species relied on chromosomes' size analyses which  
5 allowed identifying numerous different karyotypic forms (Lundgren et al., 1990; Cushion, 1998;  
6 Wakefield, 1998a; Nahimana et al., 2001). On the other hand, the low but significant  
7 heterogeneity in many genomic loci among *P. jirovecii* isolates allowed using multilocus  
8 sequence typing (Wakefield, 1998b). The latter method represents nowadays the most used  
9 technique for *P. jirovecii* strains identification. The discrimination power of eight distinct loci  
10 has been validated and extensively used for epidemiological studies of *P. jirovecii* pneumonia  
11 (Maitte et al., 2013). Genotypes identification is performed by PCR of multiple loci followed by  
12 direct DNA sequencing (Sanger), restriction fragment length polymorphism, single-strand  
13 conformation polymorphism, type-specific oligonucleotide hybridization, tandem repeats  
14 number analysis, or high-throughput amplicon sequencing (Hauser et al., 1997; Hauser et al.,  
15 1998; Lee et al., 1993; Lu et al., 1995; Ma et al., 2002; Alanio et al., 2016; Esteves et al., 2016).  
16

### 17 **Genetic diversity**

18 The conclusions drawn from the studies concerning *Pneumocystis* genetic diversity were often  
19 contradictory. Low levels of genetic diversity as defined by Shannon diversity and Simpson  
20 indexes (Shannon, 1948; Simpson 1949) have been reported at the *P. jirovecii* and *P. carinii*  
21 internal transcribed spacers of the nuclear rDNA operon using PCR-based Sanger sequencing  
22 (Palmer et al., 2000; Beser et al., 2011). On the other hand, moderate to important levels of  
23 diversity measured in term of DNA polymorphisms in *P. jirovecii* using multilocus sequence

1 typing have been reported (Matos and Esteves, 2010; Jarboui et al., 2013; Sun et al., 2015;  
2 Alanio et al., 2017). The lack of whole genome sequence data, differences in sampling strategies,  
3 differences in interpretation, as well as the likely frequent *in vitro* formation of PCR chimeras  
4 (Beser et al., 2007), make difficult the reconciliation of these conclusions.

5         Moreover, sexual recombination could explain partly these conflicting conclusions.  
6 Indeed, sexual reproduction is one of the main mechanisms to generate genetic diversity in fungi.  
7 It is believed to favor adaptation in fluctuating conditions while purging deleterious alleles  
8 (Heitman, 2010). *Pneumocystis* are probably homothallic species (Almeida et al., 2015; see life  
9 cycle section), and self-fertilization favors mating by avoiding the search of a compatible  
10 partner, a strategy thought to be favorable to and adopted by several human pathogens such as  
11 *Cryptococcus* and *Candida* species (Heitman, 2010). Sexual reproduction is based on classical  
12 Mendelian segregation, which supports both cross- and self-fertilization (Buscaglia et al., 2015).  
13 *Pneumocystis* would be able to perform both clonal and sexual propagation with various degrees  
14 of inbreeding or outcrossing. These variations in the multiplication process could explain the  
15 conflicting patterns of genetic diversity reported.

16         Polymorphism rates change substantially across loci and chromosomes in various  
17 species, including fungi, plants, and animals (Ellegren and Galtier, 2016). Genetic diversity is  
18 influenced by three main forces: mutation, demography (migration and **bottlenecks**), and  
19 selection (**selective sweeps** or **clonal interference**). Demography and selection create  
20 differences in the effective population size, whilst variations in mutation rate may create  
21 differences in the level of genetic diversity according to the geographical location. Neutral  
22 mutation rates in eurotiomycetes are typically between  $1 \times 10^{-8}$  and  $1 \times 10^{-9}$  substitutions per site  
23 per year (Kasuga et al., 2002), and a rate of  $1.2 \times 10^{-10}$  for the 18S rDNA has been used to

1 estimate *Pneumocystis* species divergence (Keely et al., 2003a). However, the genome-wide  
2 mutation rates for these species are unknown and expected to fluctuate greatly among genomic  
3 regions. For example, subtelomeric regions harboring MSGs have high substitution rates (Keely  
4 and Stringer, 2009; Schmid-Siegert et al., 2017), whereas ribosomal regions display a normal  
5 rate (Fischer et al., 2006). Moreover, given their likely variations according to the host, the  
6 mutation rates for each species must be determined independently. Care must be taken inferring  
7 these rates because recombination can be mutagenic and its impact as well as other confounder  
8 effects need to be addressed.

9         The size of the populations of *Pneumocystis* species are not known, but they are expected  
10 to be small because of their narrow host ranges. *P. jirovecii* would have a small population size  
11 relative to the species infecting micro-mammals, thus reflecting the small size of human  
12 populations relative to those of rodents. Variations in population size over time affect the genetic  
13 diversity, *e.g.* a strong population **bottleneck** creates a loss of allele diversity due to increased  
14 **genetic drift**. Using non-recombining neutral loci, realistic mutation rates, and appropriate  
15 molecular clock models, past population history can be traced back using coalescent theory  
16 based applications such as skyline plot methods (Drummond et al., 2005; Heled and Drummond,  
17 2008). These demographic reconstructions would provide key metrics such as ancestral  
18 population sizes and evolutionary rates.

19         Interestingly, the strongest prediction of genetic diversity in many species is the life  
20 history, not the population history (Ellegren and Galtier, 2016). This means that there is a strong  
21 correlation between phenotypic traits (*e.g.* mating system, generation times) and the genetic  
22 diversity. For example, homothallism is expected to have long term evolutionary cost fitness  
23 because selfing populations experience reduced recombination rates and size, which ultimately

1 reduce the strength of purifying selection and increase genetic drift (Charlesworth and Wright,  
2 2001; Hill and Robertson, 1966; Otto and Lenormand, 2002; Pollak, 1987). The homothallism  
3 used by *Pneumocystis* species is also often associated to higher probability to experience  
4 population **bottlenecks** via founder effects and linked selection (Jarne, 1995; Charlesworth and  
5 Wright, 2001). There is a complex interplay between demographic, selective factors, and genetic  
6 diversity. Alternative scenarios, such as purifying selection purging deleterious alleles, which is  
7 known as “background selection” (Charlesworth, 1994), need also to be considered. In  
8 conclusion, many factors may have influenced genetic diversity of *Pneumocystis* species, which  
9 remains unclear.

10

### 11 **Population structure**

12 The population structure of *Pneumocystis* species is also controversial. Indeed, data support an  
13 absence of strong subdivision in *P. jirovecii* (Parobek et al., 2014) and *P. carinii* (Palmer et al.,  
14 2000), whereas other data support possible geographical clusters in *P. jirovecii* (Esteves et al.,  
15 2016; Alanio et al., 2017). Importantly, Matos and Esteves (2010) noted that the infections are  
16 not necessarily clonal and recombination between multi-locus genotypes is possible. All these  
17 inferences are based on a relatively small number of markers (*e.g.* ITS, mitochondrial large  
18 subunit rDNA), and need to be validated at the genome scale using appropriate Bayesian  
19 methods based on unlinked multi-allelic genotypes, such as STRUCTURE (Pritchard et al.,  
20 2000). In the meantime, interesting clues can be extracted from the biological cycle. The  
21 question is whether the fluctuation of the population structure is caused by variations in spore  
22 dispersal or in sexual recombination. The asci are 4-6  $\mu\text{m}$  in size, which is small enough to be  
23 airborne dispersed efficiently over long distances. The asci cell wall is enriched with

1 glycoproteins, melanin,  $\beta$ -glucans, and mannans without outer chain (Kottom and Limper, 2000;  
2 Icenhour et al., 2003; Icenhour et al., 2006b; Ma et al., 2016a), which might allow them to resist  
3 desiccation and UV irradiation usually fatal to many fungal spores (Golan and Pringle, 2017;  
4 Latgé, 2007). The viability of spores for extended periods of time is supported by the detection  
5 of *P. jirovecii* mRNA in hospital air samples (Latouche et al., 2001; Maher et al., 2001). Their  
6 resistance to physical assaults is suggested by their detection in air spora trapped in rural  
7 locations (Wakefield, 1996).

8         Dispersal of fungi can occur in two modes: (i) multiple sequential short-distance  
9 dispersal, and (ii) a single successful long-distance move of spores ultimately coinciding with  
10 optimal conditions for the growth of the fungus (Golan and Pringle, 2017). The former option  
11 produces a strong population subdivision, while the latter ends up with no or weak population  
12 structure because the same genotype(s) will be spread over large geographical distance. If long  
13 distance dispersal occurs on a global scale, it will result in a global population structure (Pringle  
14 et al., 2005). Rare long-distance dispersal would involve stochastic founding events, which can  
15 be revealed by population structures with an excess of rare alleles. Future studies combining  
16 genetic and geography are needed to fully access the population structures of *Pneumocystis*  
17 species.

18

### 19 **Clonal evolution or predominant sex/recombination?**

20 *P. jirovecii* infections are most often caused by multiple populations co-infecting the same  
21 individual (Hauser et al., 1997; Nahimana et al., 2000b; Palmer et al., 2000; Ma et al., 2002;  
22 Alanio et al., 2016). Multilocus genotypes (MLGs), which refer to a unique combination of  
23 alleles, can persist over long periods of time (4 to 9 years), and be observed across different

1 countries (Wakefield et al., 1994; Esteves et al., 2010). Recombination was also detected among  
2 MLGs (Esteves et al., 2010), which would explain the reported lack of strong population  
3 subdivision, at least in *P. jirovecii* (Parobek et al., 2014). Under **panmictic** population  
4 conditions, MLGs should not persist in the population because they will be disturbed consistently  
5 by recombination.

6 Buscaglia et al. (2015) proposed that “a highly structured (*i.e.* clonal) population  
7 indicates that the main mode of reproduction for such a species lacks genetic exchange (*i.e.* is  
8 primarily asexual) or sex occurs only rarely”. In *Pneumocystis*, MLGs do recombine which  
9 would indicate a limited global population structure (Esteves et al., 2010; Parobek et al., 2014).  
10 Thus, the definition proposed by Buscaglia et al. would suggest a widespread sexual  
11 reproduction in *Pneumocystis* species. However, some MLGs persist over time, which suggests  
12 that these species might be mostly clonal and only rarely engage to sexual events. This latter  
13 scenario would be consistent with the theory of predominant clonal evolution (Tibayrenc and  
14 Ayala, 2012; Tibayrenc and Ayala, 2014), which proposes that restrained recombination is not  
15 strong enough to disturb the pattern of clonal structure. The frequency of recombination events at  
16 the genome level is unknown in *Pneumocystis*, which currently prevents reaching definitive  
17 conclusions.

18

### 19 **Intra individual short-term evolution**

20 Infections are usually caused by multiple *P. jirovecii* strains acquired from different origins  
21 (infections *de novo* but also possibly re-activation of organisms). The balance between different  
22 strains will likely change over the course of the disease because of either drug treatment,  
23 pressures from the host immune system, and/or varying metabolism and fitness among the strains

1 present. Other pathogens such as *Candida* and *Cryptococcus* species evolve rapidly within their  
2 hosts by acquiring new mutations or changes in genomic heterozygosity associated with drug  
3 resistance (Ford et al., 2015; Chen et al., 2017). It is unclear if these mutations result from  
4 positive selection or DNA repair errors in *Cryptococcus* (Rhodes et al., 2017), although it is also  
5 possible that DNA repair errors are selected by positive selection. Competition among  
6 multiclonal parasite populations within the same host can, in theory, promote parasite diversity  
7 (Bashey, 2015). The full extent of *Pneumocystis* short-term evolution within their host is  
8 unknown. Interestingly, Alanio and colleagues used a set of markers to evidence changes in  
9 population composition during *P. jirovecii* infections (Alanio et al. 2016). Multiple strains  
10 infections are frequently found in pathogens and may have clinically relevant consequences  
11 (Balmer and Tanner, 2011). Different strains might have different susceptibility to treatment or  
12 evolve differently so that they may escape detection by the immune system or diagnostics tools.  
13 We anticipate that the characterization of multiclonal infections will have serious implications  
14 for the treatment and the management of *P. jirovecii* pneumonia. Experimental setups will  
15 become realistic when long-term *in vitro* culture method will become widely reproducible.  
16

## 1 **PERSPECTIVES AND CONCLUSIONS**

2 The research on *Pneumocystis* is still in its infancy mainly because of the lack of culture *in vitro*,  
3 but the availability of genomic data will help exploring the mysteries of their evolution. The next  
4 cornerstone will be the establishment of a long-term culture system and genetic manipulation.  
5 The upcoming expectation goes far beyond the *Pneumocystis* research community and will allow  
6 exploring key questions in evolutionary cell biology such as the evolution of parasitism and  
7 multicellularity. The study of *Pneumocystis* organisms has the unique interest that they are the  
8 only strictly mammalian-adapted fungal pathogens. Thus, determining the molecular basis of  
9 their adaptation and speciation are of uttermost importance. The key questions are: what are the  
10 determinants of the genome reduction? What are the molecular determinants of the host  
11 specificity and speciation? Why introns are so abundant and what are their function(s)? What are  
12 the impact of multiclonal infections and short-term evolution within host in the context of drug  
13 resistance and development of vaccines? How do natural populations of *Pneumocystis* evolve in  
14 different hosts?  
15

1

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7

1 **GLOSSARY**

2

3 **Background selection**

4 Reduction of genetic diversity at linked loci owing to selection against deleterious mutations.

5

6 **Bottleneck**

7 A sharp and rapid reduction in the size of a population.

8

9 **Clonal interference**

10 Phenomenon in population genetics of organisms with significant linkage disequilibrium (*i.e.*  
11 absence of recombination), especially in asexual organisms. It occurs when two (or more)  
12 different beneficial mutations arise independently in different individuals.

13

14 **Effective population size**

15 The size that a theoretical population evolving under a Wright-Fisher model would need to be in  
16 order to match aspects of the observed genetic data.

17

18 **Genetic drift**

19 Fluctuation of allele frequency among generations in a population owing to the randomness of  
20 survival and reproduction of individuals, irrespective of selective pressures.

21

22 **Haploid selfing**

23 Refers to true homothallic species. A species able to accomplish their entire sexual reproduction  
24 without the need of a partner.

25

26 **Heterozygosity**

27 Measure of the genetic diversity, which represents the presence of different alleles at one or more  
28 loci on homologous chromosomes. Often presented as a probability that two randomly sampled  
29 gene copies in a population carry distinct alleles.

30

31

1 **Linkage disequilibrium**

2 Nonrandom association of alleles at two loci often but not always due physical linkage. Such  
3 association is broken over time by recombination.

4 **Panmictic population**

5 Random mating among individuals in an idealized population.

6

7 **Selective sweep**

8 Elimination or reduction of genetic diversity in the neighborhood of a beneficial allele that  
9 increases in frequency in the population, typically after an environmental change.

10

11 **Selective sweeps**

12 Elimination or reduction of genetic diversity in the neighborhood of a beneficial allele that  
13 increases in frequency in the population, typically after an environmental change.

14

15

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## 1 LEGENDS FIGURES

### 2 **Figure 1** | Cell cycle

3 The whole cell cycle of *Pneumocystis* species would take place within the host's lungs, airborne  
4 asci ensuring transmission to new hosts. The cycle is thought to include two phases: sexual and  
5 asexual. The trophic forms tightly adhere to the host's alveolar epithelial pneumocytes type I,  
6 whereas asci are generally localized within the alveolar lumen. The ring shown in green might  
7 allow the formation of a rent upon contact with humidity and so the release of the ascospores.  
8 This ring may correspond to the parentheses-like structure visible on Figure 2. This Figure does  
9 not include new features relatively to models previously proposed.

10

### 11 **Figure 2** | Cluster of *P. jirovecii* asci

12 Cluster of *P. jirovecii* asci stained with Grocott's Methenamine silver (Churukian and Schenk,  
13 1977) within a patient's bronchoalveolar lavage. The structures darker than the rest of the wall  
14 on each ascus are the parentheses-like structure (picture from the Institute of Microbiology,  
15 Lausanne University Hospital).

16

### 17 **Figure 3** | Genome composition of *Pneumocystis* and related fungi

18 Protein coding genes, intergenic spaces, and intron positions were obtained from NCBI  
19 (<https://www.ncbi.nlm.nih.gov/>, last accessed 2018-03-20). Curated *Schizosaccharomyces*  
20 *pombe* and *Saccharomyces cerevisiae* intron data were extracted respectively from Pombase  
21 database (Wood et al., 2002; <https://www.pombase.org/downloads/intron-data>, last accessed  
22 2018-03-20) and *Saccharomyces* Genome database (Cherry et al., 1998;  
23 <https://www.yeastgenome.org>, last accessed 2018-03-20). Repeats include DNA transposons,  
24 retrotransposons, and simple low complexity repeats proportions as roughly estimated using

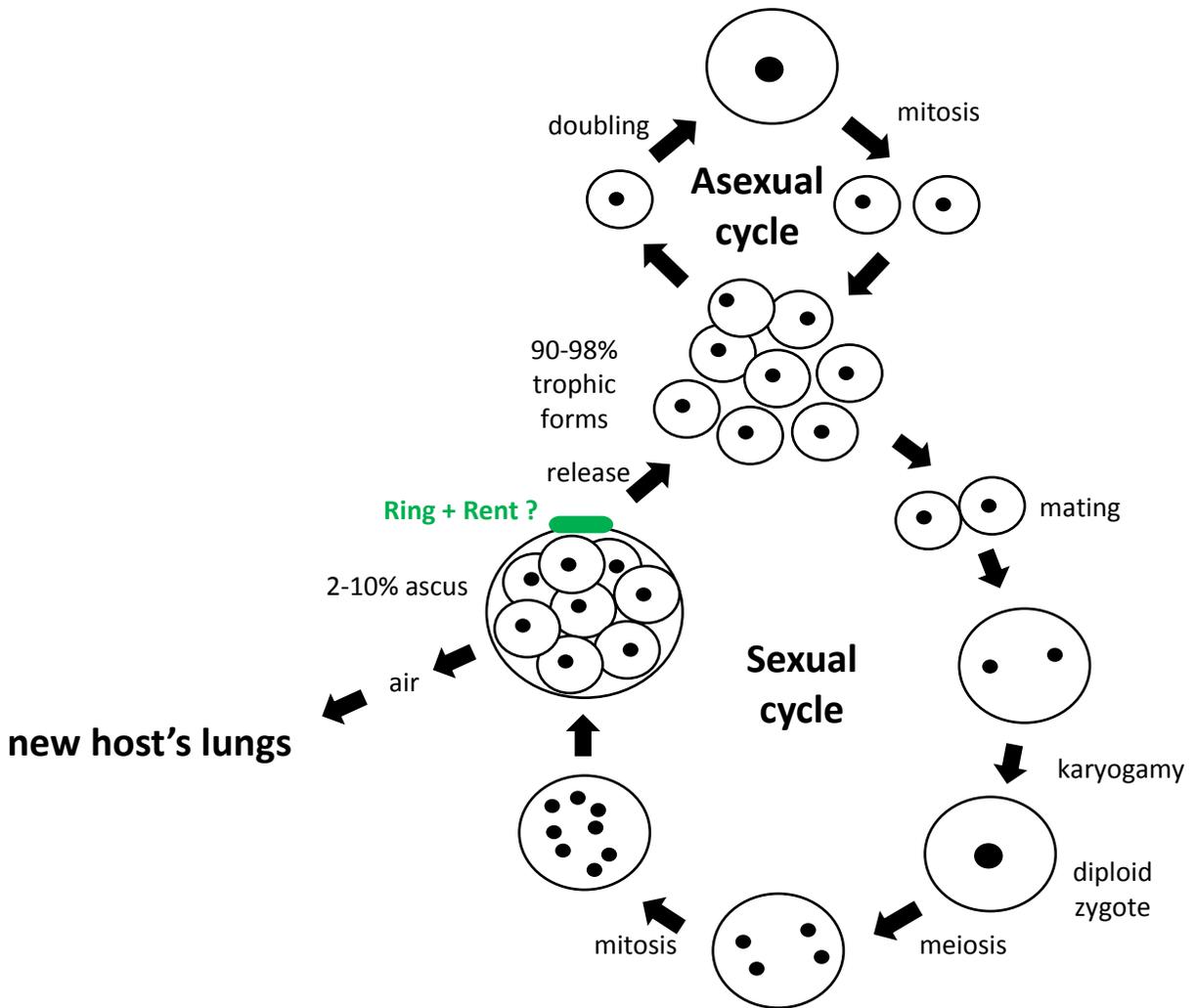
1 RepeatMasker (Smit et al., 2013) and RepBase database (Bao et al., 2015). The proportions of  
2 MSGs were calculated based on data from Ma et al. (2016a). Ribosomal DNA cassettes include  
3 each three genes (rDNA) and two internal transcribed spacers (ITS): 18S rDNA-ITS1-5.8 rDNA-  
4 ITS2-26S rDNA. *S. pombe* genome encodes roughly 140 copies of a cassette of a size of 5.8 kb.  
5 In *S. cerevisiae*, ca. 150 tandem copies of a 9.1 kb cassette are present (Venema and Tollervey,  
6 1999). In contrast, *Pneumocystis* species harbor each a single rDNA cassette of 11 kb.

7  
8 **Figure 4** | Graphical overview of the hypothetical evolutionary history of *Pneumocystis* species  
9 *Pneumocystis* species divergence timing has been determined elsewhere (Keely et al., 2003a;  
10 2004a; Beimforde et al., 2014). Losses of multiple metabolic pathways, as well as contraction  
11 and expansion of specific gene families are presented (Table 2). Note that the timing and order of  
12 losses is unknown. The gain and loss of specific functions for *Pneumocystis* is inferred here to  
13 have occurred in the last most recent ancestor common of *Pneumocystis* species (MRCA)  
14 because the underlying genes are absent in the genomes of all *Pneumocystis* sequenced to date.  
15 The MSG superfamily emerged in *Pneumocystis* ancestry and displays a substantial level of  
16 lineage specific divergence (represented by blue triangles). Intron loads are similar among  
17 *Pneumocystis* species, which might suggest a common origin. The fission yeast clade diverged  
18 ~250 MYA ago (Rhind et al. 2011) and has lost most of the introns acquired from an intron rich  
19 ancestor (Roy et al., 2005; Stajich et al. 2007; Rhind et al., 2011). Although there is no dating  
20 estimates for the intron loss in fission clade, the absence of recent intron gains and the low rates  
21 of intron loss (Zhu and Niu, 2013) suggest that the majority of introns were lost before the  
22 diversification of the fission yeast clade. The colors of the lines representing the evolving species  
23 signify different nutritional modes (dark green, saprophytism; light green, gradual shift from  
24 saprophytism to the parasitism; yellow, animal parasitism). We assume that the MRCA of

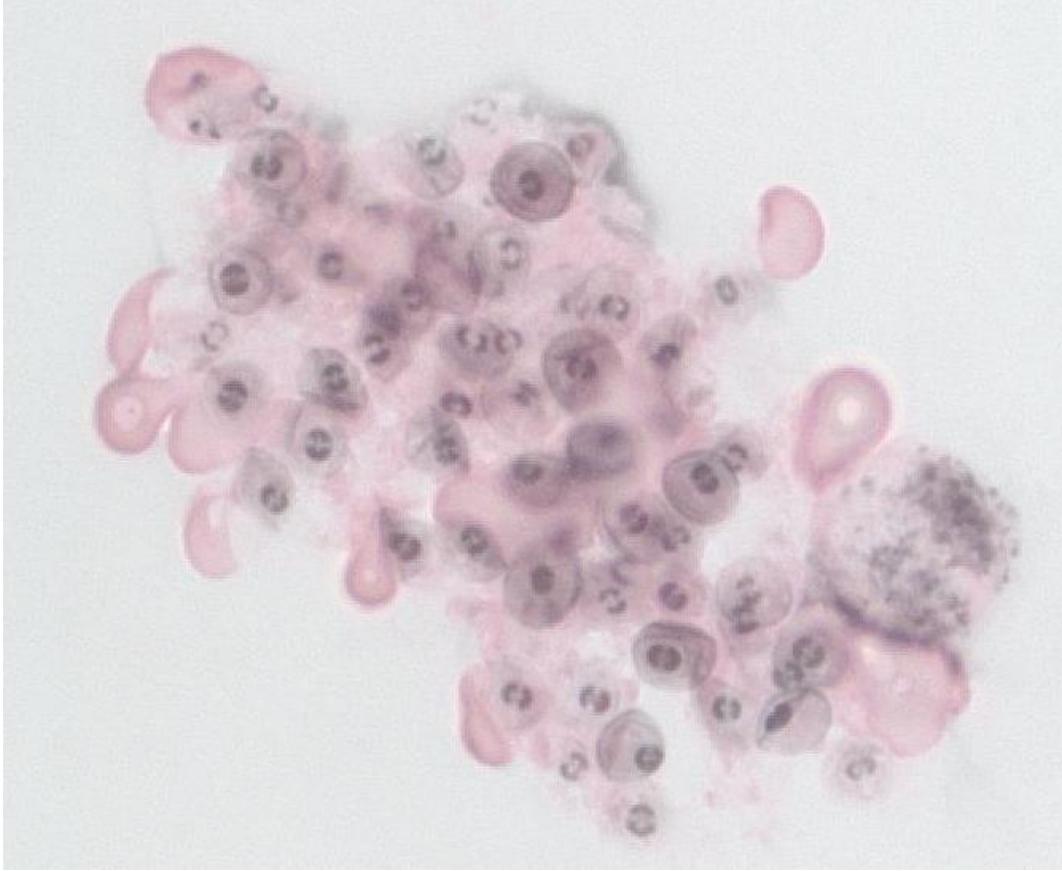
1 Taphrinomycota subphylum was a multicellular or dimorphic saprotroph based on ancestral traits  
2 reconstruction (Schoch et al., 2009; Nguyen et al., 2017). The phylogenetic relationship  
3 presented here is consistent with published phylogenies (Liu et al., 2009; Sugiyama et al., 2006).  
4 RRM correspond to RNA binding proteins harboring an RNA recognition motif.

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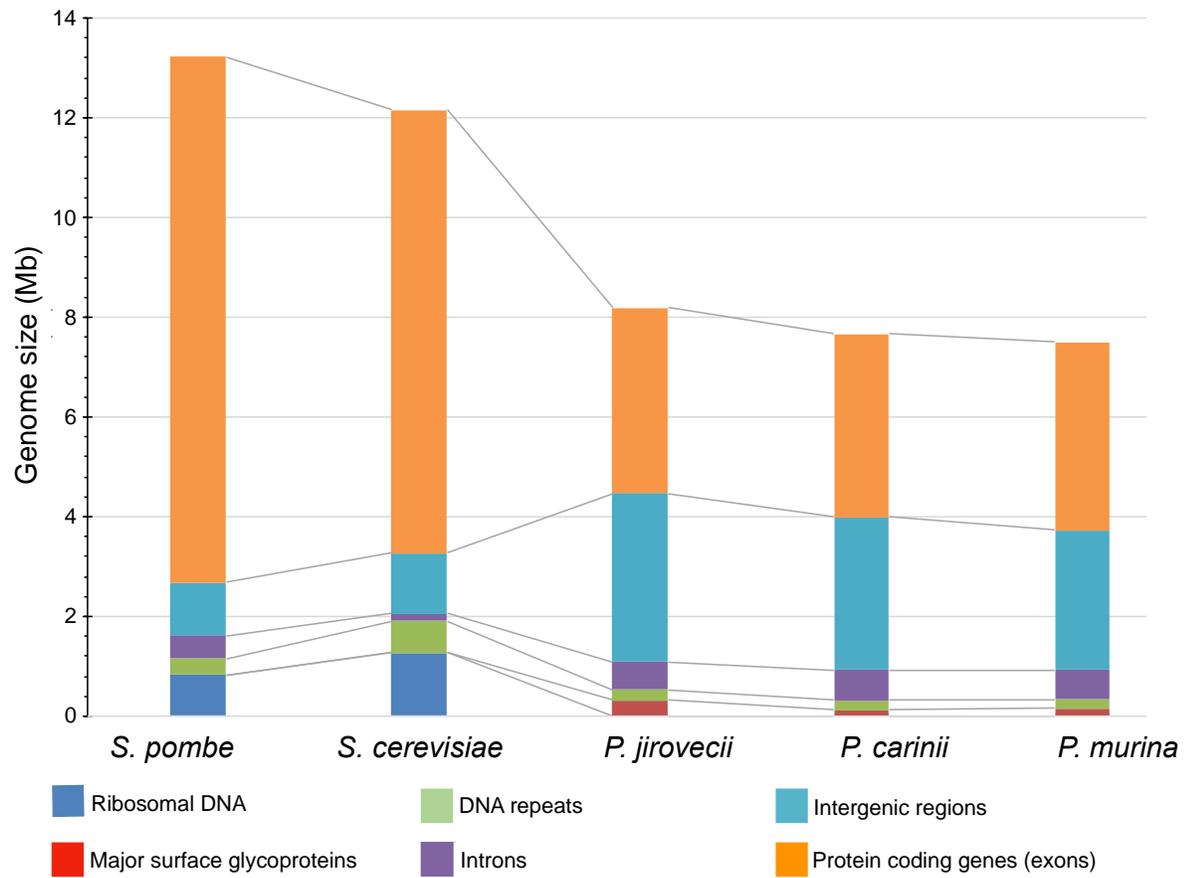
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7 **Figure 1.**



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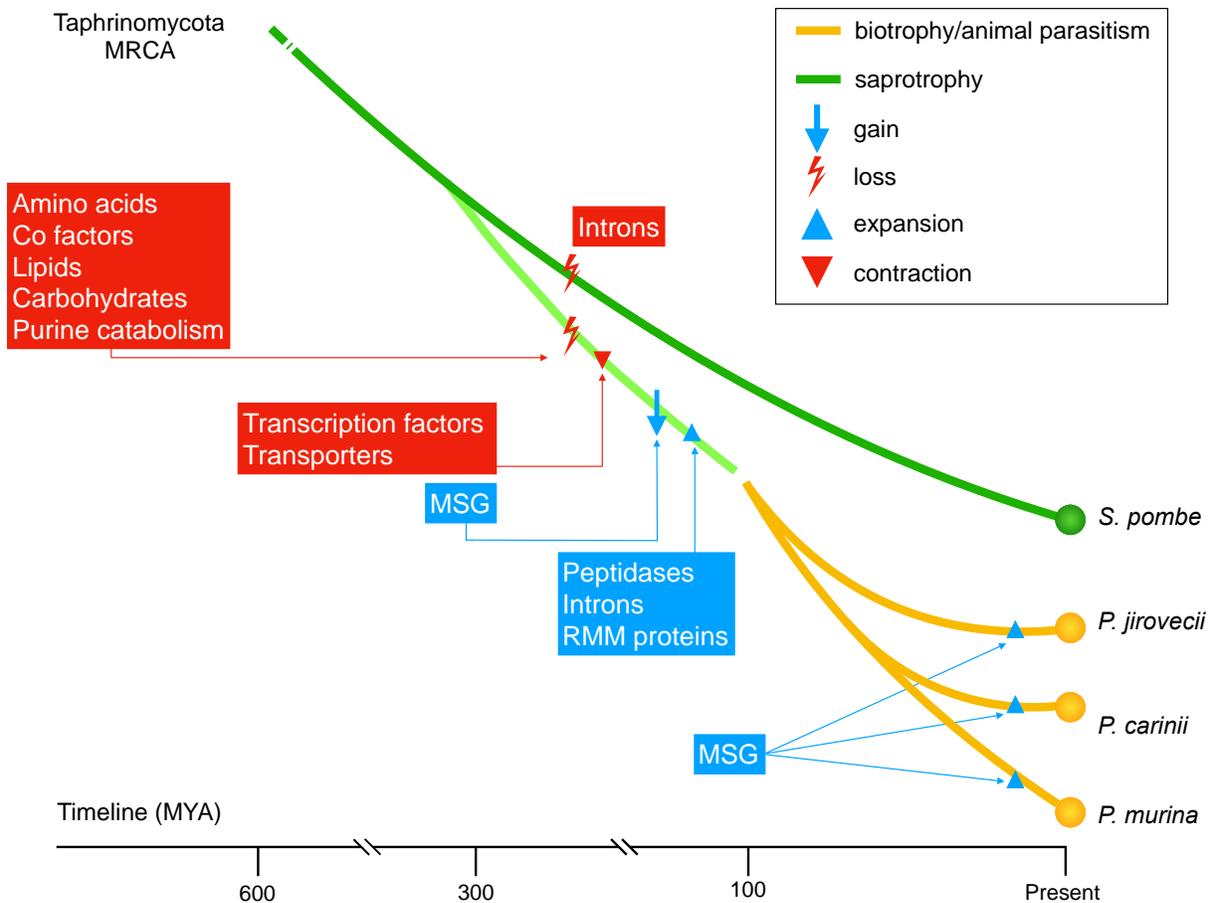
**Figure 2.**

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**Figure 3.**



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**Figure 4.**