

Comparative Genomics Suggests That the Human Pathogenic Fungus *Pneumocystis jirovecii* Acquired Obligate Biotrophy through Gene Loss

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Abstract

Pneumocystis jirovecii is a fungal parasite that colonizes specifically humans and turns into an opportunistic pathogen in immunodeficient individuals. The fungus is able to reproduce extracellularly in host lungs without eliciting massive cellular death. The molecular mechanisms that govern this process are poorly understood, in part because of the lack of an in vitro culture system for *Pneumocystis* spp. In this study, we explored the origin and evolution of the putative biotrophy of *P. jirovecii* through comparative genomics and reconstruction of ancestral gene repertoires. We used the maximum parsimony method and genomes of related fungi of the Taphrinomycotina subphylum. Our results suggest that the last common ancestor of *Pneumocystis* spp. lost 2,324 genes in relation to the acquisition of obligate biotrophy. These losses may result from neutral drift and affect the biosyntheses of amino acids and thiamine, the assimilation of inorganic nitrogen and sulfur, and the catabolism of purines. In addition, *P. jirovecii* shows a reduced panel of lytic proteases and has lost the RNA interference machinery, which might contribute to its genome plasticity. Together with other characteristics, that is, a sex life cycle within the host, the absence of massive destruction of host cells, difficult culturing, and the lack of virulence factors, these gene losses constitute a unique combination of characteristics which are hallmarks of both obligate biotrophs and animal parasites. These findings suggest that *Pneumocystis* spp. should be considered as the first described obligate biotrophs of animals, whose evolution has been marked by gene losses.

Key words: obligate parasite, lifestyle, gene families, neutral drift, convergent evolution.

Introduction

Pneumocystis jirovecii is a fungal parasite causing *Pneumocystis* pneumonia in human beings with deficient immune system, a disease which is often fatal if untreated. The *Pneumocystis* genus includes several species, each displaying strict host specificity for a given mammalian species, for example, *P. jirovecii* infects humans, *P. carinii* rats, and *P. murina* mice. These fungi are members of the Taphrinomycotina, a subphylum of Ascomycota (Redhead et al. 2006), which encompasses organisms with remarkably diverse lifestyles ranging from free-living saprophytes (e.g., *Schizosaccharomyces* spp.) to biotrophic plant pathogens (e.g., *Taphrina* spp.).

Two categories of fungal parasitism are recognized: Necrotrophy where food is obtained from killed host cells, and biotrophy where food is obtained from living host cells (Kemen and Jones 2012). In general, necrotrophs secrete many cell wall degrading enzymes as well as toxins to kill host cells. On the other hand, biotrophs secrete low amounts of lytic enzymes and cause little damage to their host. Biotrophic lifestyle includes a continuum from facultative to obligate states (Kemen and Jones 2012; Spanu 2012). Facultative biotrophs require a living host only for a specific stage of their life cycle and can usually be easily grown axenically in the laboratory. Obligate biotrophs present an absolute requirement for their host to survive and usually cannot

be grown axenically in the laboratory. Evolution toward obligate biotrophy is generally associated with losses of pathways, whose end products can be scavenged from the host (Spanu et al. 2010; Duplessis et al. 2011). In the case of human parasites, true pathogens refer to those able to cause disease in healthy individuals, whereas opportunistic pathogens are those causing disease only in the case of immunosuppression. These definitions are used in this work.

Pneumocystis spp. dwell almost exclusively in mammalian lungs where they apparently do not trigger massive host cell death. They carry out their sex life cycle within their host, which is a hallmark of obligate parasitism and which has been observed among fungi only in plant biotrophs (Spanu 2012). Moreover, attempts to obtain axenic in vitro culture have failed so far. Based on these characteristics, a parallel between *Pneumocystis* spp. and plant obligate biotrophs has been suggested (Cushion and Stringer 2010). Subsequent findings reinforced this resemblance. Indeed, *Pneumocystis* spp. have been suggested to be obligate parasites because of their relatively small genome with a high Adenine and Thymine (AT) content and the loss of the capacity of synthesizing most amino acids (Hauser et al. 2010; Cisse et al. 2012). Moreover, *Pneumocystis* spp. lack virulence factors commonly found in pathogenic fungi, such as glyoxylate cycle, toxins, and secondary metabolism (Cushion et al. 2007; Cisse et al. 2012).

The origin and evolution of biotrophy in *Pneumocystis* spp. can be explored through the hypothetical reconstruction of ancestral gene repertoires. This reconstruction is based on the reliable identification of homologous gene families across related organisms. There are currently two methods for that purpose: The maximum parsimony (MP) and the maximum likelihood (ML) (Wolf and Koonin 2013). MP is designed to find the ancestral state that corresponds to the minimum number of character changes necessary to match the species phylogeny with the observed presence–absence patterns. In MP, different models of evolution can be applied, for example, the irreversible Dollo model (Farris 1977), which assumes that once an ancestral character is lost during the evolution of a particular organism, it cannot be regained. The MP method is based on a stochastic birth-and-death model of evolution. This model assumes that a new gene can appear only once in a phylogeny and that it can further evolve through duplication and loss in the descendant lineages. ML is based on the likelihood that ancestral states would evolve under a stochastic model of evolution to reproduce the observed presence–absence patterns (Cohen et al. 2008; Csuros 2010). Although ML has been suggested to be more robust than MP (Wolf and Koonin 2013), this method requires large amounts of data and is generally not applicable to undersampled clades.

In this study, we have determined ubiquitous and species-specific gene families in the Taphrinomycotina subphylum. We then applied a parsimonious scenario to reconstruct

ancestry of several lineages, and inferred gene families' losses and gains. Our results suggest that the acquisition of biotrophy in *Pneumocystis* spp. was marked by gene losses in biosynthetic pathways, whose products are likely to be scavenged from the lung environment.

These findings provide insights into the intimate relation of these microorganisms with their mammalian hosts.

Materials and Methods

Data Sources and Proteome Completion

Incomplete versions of the proteomes of *P. jirovecii* and *T. deformans* are present in UniProt (www.uniprot.org, last accessed November 11, 2013). Therefore, we completed the protein data sets with manually curated gene predictions (Cisse et al. 2012, 2013). These data sets as well as *P. carinii* predicted proteins and expressed sequence tags are available at http://www.chuv.ch/imul/imu_home/imu_recherche/imu_recherche_hauser/imu-phauser-suppldata.htm (last accessed July 27, 2014). *Pneumocystis carinii* genomic data sets and predicted proteins correspond to data published by others (Cushion et al. 2007; Hauser et al. 2010; Cisse et al. 2012; Slaven et al. 2006). *Pneumocystis jirovecii* genome raw reads and transcriptome assembly, as well as *T. deformans* expressed sequence tags (ESTs) were downloaded from EBI (<http://www.ebi.ac.uk>, last accessed November 11, 2013). The transcriptome assemblies and ESTs were translated into the six open reading frames. The predicted proteomes of *Saccharomyces cerevisiae*, *Ustilago maydis*, *Neurospora crassa*, *Yarrowia lipolytica*, *Ashbya gossypii*, *Rhizopus oryzae*, *Aspergillus fumigatus*, and *Debaryomyces hansenii* were obtained from UniProt (www.uniprot.org, last accessed August 26, 2013). The proteomes of *S. pombe*, *S. octosporus*, *S. japonicus*, and *S. cryptosporus* were from the Broad Institute (<http://www.broadinstitute.org>, last accessed November 5, 2013), and correspond to data published by Rhind et al. (2011). The level of completion of each protein data set was estimated by scanning with 458 core conserved eukaryotic proteins from the CEGMA pipeline (Parra et al. 2007) using hmmer3 (Eddy 2011) with an e value of 10^{-5} as threshold.

Gene Families and Annotation

Gene families were built using OrthoMCL (Li et al. 2003) with a Markov inflation of 1.5 and a maximum e value of 10^{-5} . Repetitive elements were identified using TransposonPSI (<http://transposonpsi.sourceforge.net/>, last accessed July 27, 2014) and TBLASTn searches with an e value of 10^{-10} (Altschul et al. 1997) in Repbase (Jurka et al. 2005). Enrichment in Gene Ontology (GO) terms between different groups was identified using Fisher's exact test with a P value of 0.05 and corrected for multiple testing using BLAST2GO (Conesa et al. 2005). Enzyme prediction and mapping to KEGG biochemical pathways were performed using

BLAST2GO and PRIAM (Claudel-Renard et al. 2003). Proteases were classified according to MEROPS database (Rawlings et al. 2012).

Ancestral Genome Reconstruction and Gene Gains and Losses

The species phylogeny was obtained from our previous study (Cisse et al. 2012) and edited using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>, last accessed July 27, 2014). The true phylogenetic tree along with bootstrap values and branch lengths is shown in [supplementary figure S3, Supplementary Material](#) online. Gene presence and absence patterns were extracted from OrthoMCL proteomes clustering using custom Perl scripts. This matrix was used to infer ancestral genomes using Count (Csuros 2010). Count uses phylogenetic birth-and-death models in a stochastic probability inference. The rate models were computed and optimized under the gain–loss–duplication model with the Poisson family size distribution. The rate of variation across families was set to 4:1:1:4 gamma categories for the edge length, the loss rate, gain rate, and the duplication rate, respectively. The convergence criteria applied were set to 100 rounds for the optimization rounds with a likelihood threshold of 0.1. The family history was determined under the Dollo parsimony assumption.

Specific Gene Searches

All searches were first performed in the proteomes. In case of absence, searches were systematically carried out on the translated transcriptome assemblies, genomes, and finally on raw sequences reads if necessary, to confirm the absence.

RNA Interference Machinery

The *S. pombe* Dicer and Argonaute proteins were used for screening using BLASTp (e value < 0.01) and TBLASTn (e value < 10^{-3}), respectively. The hidden Markov models-based searches were conducted using hmmer3 (0.1) using following Pfam models (Punta et al. 2012): PAZ (PFAM no. accession PF02170), dicer dimerization (PF03368), helicase conserved c-terminal (PF00271), and ResIII (PF04851). The results were manually examined to remove false positives. The *S. cerevisiae* killer virus was used as template for mapping using *P. jirovecii*-unassembled genome reads and Newbler's gsMapper. Homologs to ScV-A (Gag, major capsid protein), ScV-M1 (K1 preprotoxin), ScV-M2 (K2 preprotoxin), ScV-M28 (K28 preprotoxin), and ZbV-M (Zygocin preprotoxin) were searched through the *P. jirovecii* genome and raw reads using TBLASTn (e value < 0.1). Sequence accession numbers and details are given in Schmitt and Breinig (2006).

Thiamine Biosynthesis Pathway

To search the hallmark genes of the thiamine biosynthesis, the *S. pombe* thiamine thiazole synthase (*thi2*), the thiamine pyrophosphokinase (*tnr3*), the phosphomethyl-pyrimidine kinase, and the thiamine biosynthetic bifunctional enzyme (*thi4*) were used for screening using BLASTp (e value < 0.01) and TBLASTn (e value < 10^{-3}). We ran hmmer3 (e value < 0.1) using the different domains composing the *S. pombe* proteins, that is, phosphomethylpyrimidine kinase (PF08543), TENA/THI-4/PQQC family (PF03070), hydroxyethylthiazole kinase family (PF02110), thiamine monophosphate synthase (PF02581), and thiamine pyrophosphokinase (PF04265 and PF04263). For the *thi2* gene, we searched for the InterPro domain thiazole biosynthetic enzyme family (IPR002922). The results were manually inspected to avoid false positives.

Nitrogen Metabolism

We used the *T. deformans* nitrite and nitrate reductases as query in order to screen using BLASTp (e value < 0.01) and TBLASTn (e value < 10^{-3}). Domain-based searches were conducted with hmmer3 (0.1) with the following characteristic domains: FAD-binding (PF00970), oxidoreductase NAD-binding (PF00175), nitrite/sulfite reductase ferredoxin-like half (PF03460), and nitrite and sulphite reductase 4Fe–4S (PF01077). We retrieved all proteins containing at least one domain and aligned them using MAFFT (Kato and Standley 2013) with E-INS-i option to take into account of the presence of multiple domains. As these proteins belong to large protein families, the results were manually examined to remove false positives (e.g., flavoproteins, sulfite reductases, or cytochrome reductases).

Sulfite Metabolism and Molybdopterin Biosynthesis

The *S. pombe* proteins were used for the screening using BLASTp (e value < 0.1) and TBLASTn (e value < 10^{-3}). The molybdopterin biosynthesis genes were identified in *T. deformans* based on protein similarity with the genes of the obligate biotroph *Albugo laibachii* (Kemen et al. 2011). Domain-based searches were conducted using hmmer3 (e value < 0.1) with the following characteristic domains: FAD binding (PF000667), oxidoreductase NAD-binding (PF00175), Mo-co oxidoreductase dimerisation (PF03404), oxidoreductase molybdopterin-binding domain (PF00174), Molybdenum cofactor synthesis C (PF06463), Radical SAM superfamily (PF04055), MoeA (PF02391), molybdopterin binding (PF00994), MoeA C-terminal region (PF03454), and MoeA N-terminal region (PF03453).

Transporters

The transporters were identified using two complementary approaches. The proteomes were compared with the tcdb

proteins collection (Saier et al. 2009) using ssearch (Pearson 1991). In parallel, putative amino acid transporters were identified using BLAST2GO with BLASTp (10^{-10}) and InterProscan options. The sequences were then classified according to the tcdb families' classification system to assess putative substrate. Statistical tests were performed using (R Development Core Team 2008).

Data Accessibility

Proteome clustering results and matrix, scripts, and sequences alignments are available from GitHub (https://github.com/ocisse/Pneumocystis_comparative, last accessed July 27, 2014).

Results

Identification of Orthologous Gene Families

To investigate the evolutionary dynamics of gene families in the Taphrinomycotina subphylum, we established the orthologous relationships among the proteomes of seven Taphrinomycotina, four Saccharomycotina, two Pezizomycotina, one Ustilaginomycotina, and one Mucormycotina. This corresponded to a set of 101,030 predicted proteins. Based on the presence of 458 core eukaryotic proteins (Parra et al. 2007), *P. jirovecii* predicted proteins and its de novo assembled transcripts showed a completeness of 89% and 70%, respectively. There was an overlap of 98% between these two latter sets, suggesting that most of the *P. jirovecii* genes were captured (supplementary table S1, Supplementary Material online). Except that of *P. carinii* with 79%, the fungal proteomes had a completeness of at least 97%. The proteomes clustering yielded 8,368 families and 2,143 single taxa clusters (i.e., containing at least two proteins from the same species), which included 81% of *P. jirovecii* predicted proteins.

P. jirovecii Proteome Content

Sixty-eight percent of the 3,898 *P. jirovecii* predicted proteins had at least one ortholog in the proteome of another fungus, including in a distantly related one, suggesting an ancient origin (supplementary fig. S1, green and blue slices, Supplementary Material online). These conserved genes were primarily devoted to the basal cellular and metabolic activities (supplementary table S1, Supplementary Material online). Four percent of the *P. jirovecii* proteins were genus specific and enriched for glycoprotein biosynthetic activity (supplementary fig. S1, red slices, Supplementary Material online). As a comparison, the *Schizosaccharomyces* genus-specific genes represented 10% (pink slices), suggesting a greater genetic innovation than in the *Pneumocystis* genus. Two percent of *P. jirovecii* proteins, which encode mostly hypothetical proteins, were included in gene families specific to this organism (light gray slices).

Gene Families' Gain and Loss Patterns in Reconstructed Ancestors

Biotrophy in *Pneumocystis* and *Taphrina* lineages would have appeared in ancestors as a consequence of a shift of host or environment. Thus, the gene families' losses and gains in these ancestors should provide insights regarding the possible acquisition of biotrophy. To obtain a dynamic view of these events, we derived a gene presence-absence matrix from the gene clustering and projected these patterns onto a species phylogeny obtained from 458 shared single copy orthologs (Cisse et al. 2012). We then applied a parsimonious evolutionary scenario using the MP method based on the Dollo model to infer gene gain and loss events. The reconstructed ancestor of *Pneumocystis* spp. "a4" appeared to have had at least 3,113 gene families, which indicated a reduction of approximately 30% as compared with the ancestors of *T. deformans* "a5" and of *Schizosaccharomyces* spp. "a3" (fig. 1). There was a significant difference in the number of gene families' gains between Taphrinomycotina members and their reconstructed ancestors (Wilcoxon rank sum test [one-side test], $P=0.02$), with the highest number in the ancestor of *Schizosaccharomyces* spp. "a3" ($n=529$). The difference in the number of gene losses among the Taphrinomycotina members was also significant (Wilcoxon rank sum test, $P=0.01$), with the highest number in the last common ancestor of the *Pneumocystis* spp. "a4" ($n=1,819$). These observations held true when the numbers of gene gains and losses were normalized by proteome size (data not shown).

Gene Losses Associated with the Acquisition of Obligate Biotrophy in the *Pneumocystis* Genus

We focused on two time points in relation to the possible acquisition of obligate biotrophy in *Pneumocystis* ancestry: The common ancestor of the *Taphrina* and *Pneumocystis* genera "a5," and the ancestor of the *Pneumocystis* genus "a4." Notably, *T. deformans* and *Pneumocystis* spp. display distinct behaviors, that is, the former can be grown axenically in vitro (Fonseca and Rodrigues 2011), whereas the latter ones could not so far. Thus, we hypothesized that gene losses during the transition from the "a5" to "a4" occurred. We optimized rates for gene birth-and-models and applied Dollo parsimony to infer gene families' gain and loss events. The investigation of the matrix of these events highlighted 2,324 ancestral genes that are present in "a5," and subsequently lost in "a4." In this analysis, we required that the inferred ancestral genes were also conserved in both *Taphrina* and *Schizosaccharomyces* lineages. These genes were significantly enriched for the following GO terms: Oxidation-reduction (Fisher's exact test, $P=4.5 \times 10^{-11}$), carbohydrate metabolism ($P=2.3 \times 10^{-4}$), amino acid transport ($P=8.7 \times 10^{-4}$), and sulfur compound metabolic process ($P=0.009$) (supplementary table S3, Supplementary Material online).

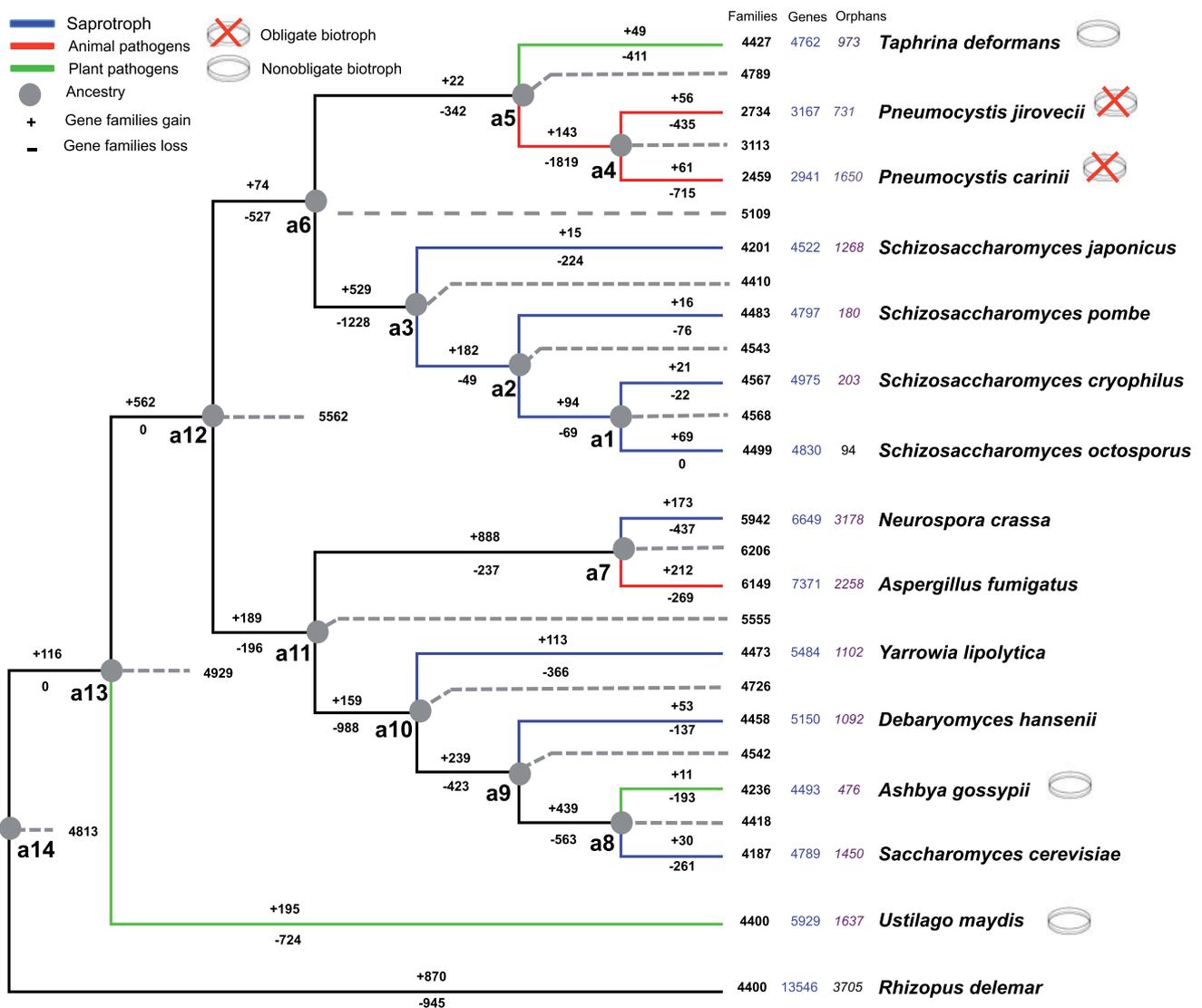


FIG. 1.—Lifestyle and projected gene families' gain and loss patterns in representative fungal genomes. The gene families' loss and gain patterns are projected onto each branch of the species phylogeny. The fungal species phylogeny corresponds to a cladogram derived from the ML tree of our previous study (Cisse et al. 2012; supplementary fig. S3, Supplementary Material online). The numbers on the branch of the phylogenetic tree corresponds the gene families' gains (+) and losses (–). The lifestyle of the different species is indicated by the color of the branch (blue for saprotrophs, red for animal pathogens, and green for plant pathogens). Biotrophic organisms are indicated by Petri dishes, red crosses indicating the absence of a culture system. Ancestral gene families' content was reconstructed using the Dollo parsimony assumption (represented by the gray dotted lines and the letter "a" which stands for ancestor). For each species, the number of gene families, genes, and orphan genes is displayed. The number of gene families is also displayed for inferred ancestral taxa.

To investigate whether gene losses could have occurred through massive pseudogenization, we conducted a comprehensive search of pseudogenes in *P. jirovecii* (supplementary material, Supplementary Material online; *P. carinii* could not be analyzed because of its high fragmentation). We found evidence of 75 pseudogenes (supplementary table S7, Supplementary Material online), which correspond to a ratio of 0.02 pseudogene per annotated protein-coding gene. This value is close to those of 0.03 and 0.01 reported, respectively, for *S. cerevisiae* and *S. pombe* (Echols et al. 2002; Wood et al. 2002).

Loss of Amino Acid and Purine Metabolisms

Among the 2,324 genes present in ancestor "a5" but lost in the *Pneumocystis* genus, 183 enzymes were mapped, and 42% of them turned out to be involved in the amino acid and purine metabolisms (fig. 2). The overrepresentation of the amino acid metabolism category is consistent with the previously reported loss of most enzymes dedicated to the biosynthesis of amino acids (Hauser et al. 2010; Cisse et al. 2012). These acquired auxotrophies imply scavenging of these compounds from the host or lung environment. Many fungi use

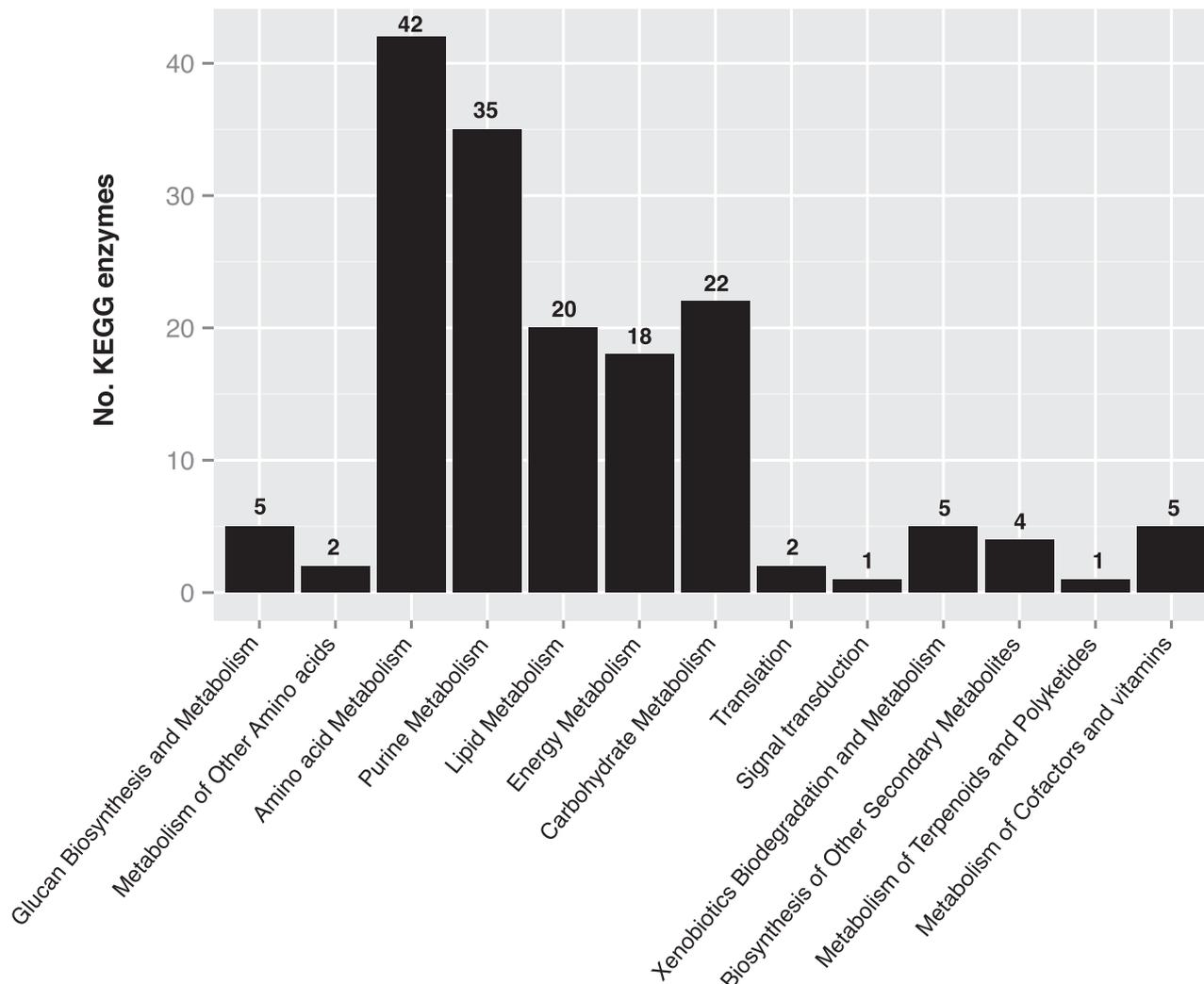


Fig. 2.—Functional categories of ancestral genes lost in the *Pneumocystis* genus. The 2,324 ancestral genes that appeared to be present in the last common ancestor “a5,” and subsequently lost in the *Pneumocystis* genus, were reconstructed using MP and mapped onto KEGG biochemical pathways atlas.

various amino acid permeases and oligopeptide transporters to scavenge amino acids from their environment. For example, the biotroph rut fungus *Uromyces fabae* uses an AAT1p amino acid transporter for histidine uptake (Struck et al. 2002), and the necrotroph fungus *Fusarium oxysporum* uses a general permease for amino acids uptake (Divon et al. 2005). We screened the proteomes of all species used in this study to identify transporters. *Pneumocystis jirovecii* and *P. carinii* displayed a significant reduction in the amino acid/polyamine/organocation family as compared with other members of the Taphrinomycotina subphylum (Fisher’s exact unilateral test, P value = 1.3×10^{-7} ; [supplementary table S3, Supplementary Material online](#)). The major facilitator superfamily, which are versatile transporters that can carry amino

acids, also showed a significant reduction in *Pneumocystis* spp. (Fisher’s exact unilateral test, P value = 4.08×10^{-15} ; [supplementary table S3, Supplementary Material online](#)).

Loss of Purine Degradation Pathway

Nineteen percent of the 183 ancestral enzymes conserved in *T. deformans* but lost in the *Pneumocystis* genus are related to purine metabolism (fig. 2). This metabolism includes the synthesis and degradation of adenine and guanine, which are fundamental and well-conserved pathways across all life forms (Caetano-Anolles et al. 2007). The purine degradation allows the recycling of purines and their use as secondary source of nitrogen in case of limiting conditions. We detected the presence of the hallmark genes of the inosine

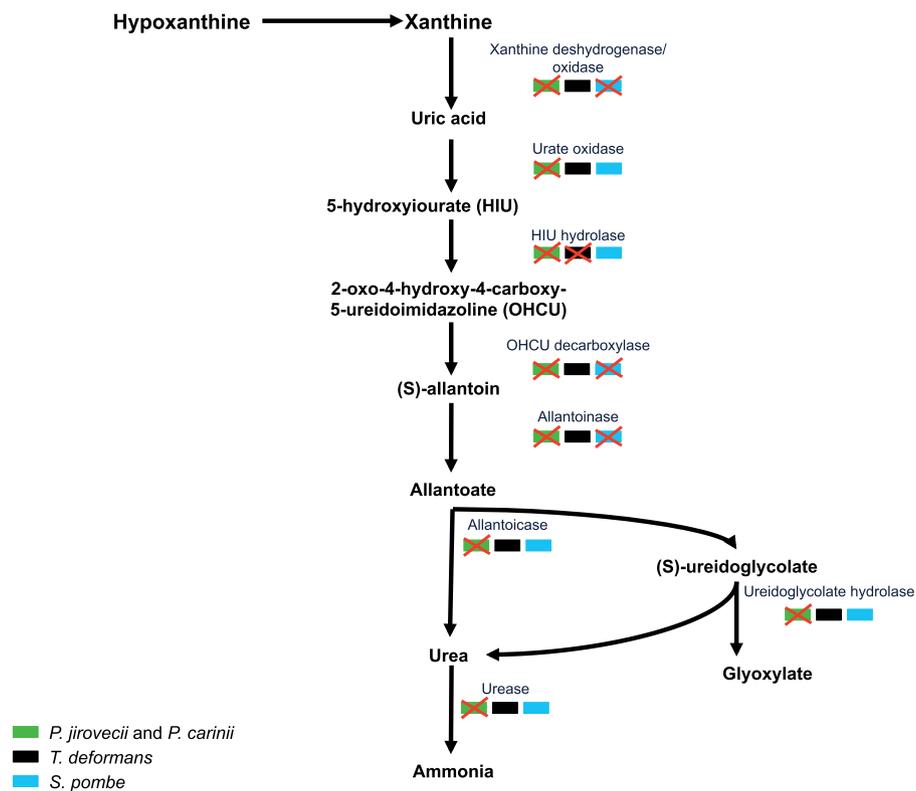


FIG. 3.—Purine degradation pathway in members of the Taphrinomycotina subphylum. The scheme indicates the presence or absence of purine metabolism genes in *Pneumocystis jirovecii*, *P. carinii*, *Taphrina deformans*, and *Schizosaccharomyces pombe*. The map diagram is based on the purine degradation pathway described by Lee et al. (2013). The colored rectangles represent the genes in the organisms. *Schizosaccharomyces pombe* is able to use different purines as starting point for purine degradation as described elsewhere (Kinghorn and Fluri 1984) (e.g., hypoxanthine, xanthine, uric acid, allantoin, and allantoic acid). The pathway has not been experimentally characterized in *T. deformans*.

5'-phosphate and uridine 5'-phosphate biosynthesis pathways in *P. jirovecii* and *P. carinii* (supplementary table S5, Supplementary Material online). These compounds are the precursors of the synthesis of purines and pyrimidines, suggesting that the de novo purine biosynthesis takes place in *Pneumocystis* spp. However, no matches for all the catabolic enzymes involved in the degradation of purines were detected in *P. jirovecii* and *P. carinii*, whereas most of them were present in *T. deformans* and *Schizosaccharomyces* spp. (fig. 3 and supplementary table S4, Supplementary Material online).

Loss of Nitrogen and Sulfur Assimilation Pathways

Nitrogen and sulfur are essential for life. The key enzymes of the inorganic nitrogen and sulfur metabolisms, that is, the nitrate and nitrite reductases, as well as the sulfite reductase and oxidase, were found to be absent in the *Pneumocystis* and *Schizosaccharomyces* genera, while being preserved in *T. deformans* (supplementary table S4, Supplementary Material online). The nitrate reductase is a molybdenum-dependant enzyme, which is involved in the nitrate reduction during the nitrogen acquisition (Campbell 1999). Consistently,

the complete molybdopterin biosynthesis pathway was lost in the *Pneumocystis* and *Schizosaccharomyces* genera (supplementary table S4, Supplementary Material online).

Loss of Thiamine Biosynthesis Pathway

Thiamine is an essential cofactor required by almost all living organisms. No matches for the key enzymes thiamine thiazole synthase and accessory enzyme kinases, except the thiamine pyrophosphokinase, were detected in *P. jirovecii* and *P. carinii* (supplementary table S4, Supplementary Material online). These enzymes appeared to be highly conserved in other members of the Taphrinomycotina subphylum, except the thiamine thiazole synthase in *T. deformans*.

Loss of RNAi Machinery

The RNAi machinery is involved in the genome defense by controlling the movement of repetitive mobile elements and the regulation of chromatin modification. No matches for the Argonaute and Dicer proteins were detected in *P. jirovecii* and *P. carinii*, whereas they were present in *T. deformans*

and *Schizosaccharomyces* spp. (supplementary table S4, Supplementary Material online).

Virulence Associated Proteases

We analyzed several secreted peptidases of the MEROPS sub-families, that is, A01, C13, G01, M35, S08, S09, S10, and S14. These are important fungal virulence factors (Monod et al. 2002), which might be involved in the degradation of the extracellular matrix in human lungs. The number of these proteases in *Pneumocystis* spp. is slightly lower (0.3–0.4% of their proteomes) than in most of the other Taphrinomycotina members (0.4–0.7%) (supplementary table S5, Supplementary Material online). Interestingly, *P. jirovecii* and *P. carinii* along with *T. deformans* harbor a specific ATP-dependent clp protease of the S14 family involved in the hydrolysis of proteins (UniProt accession: LOPC06), which is lacking in the *Schizosaccharomyces* spp. On the other hand, *Pneumocystis* spp. showed an underrepresentation of the A01 and S09 families which have been suggested to be crucial virulence factors in many fungi such as the human pathogen *Candida albicans* (Schaller et al. 2005).

Pneumocystis spp. Genomic Features

In order to investigate their evolution, several features of *Pneumocystis* spp. genomes were analyzed (supplementary material, Supplementary Material online). We estimated the genome-wide Ka/Ks ratio by the analysis of 898 single copy orthologous genes shared by *Pneumocystis* and *Schizosaccharomyces* spp. The mean Ka/Ks values of these genes were, respectively, 0.06 and 0.05 in the two genera (supplementary fig. S2A, Supplementary Material online). These values are smaller than one which suggests that these genes evolve under purifying selection.

The proportion of repetitive DNA in *P. jirovecii* genome, including transposons, retrotransposons, and low complexity DNA, but excluding the subtelomeric gene families, was estimated to 9.8% (supplementary table S6, Supplementary Material online; *P. carinii* could not be analyzed because of its high fragmentation). This value is higher than those of the plant pathogen *T. deformans* (1.5), of the free-living yeasts *S. cerevisiae* (4.8) and *S. pombe* (5.2), and of the extreme obligate parasite *Encephalitozoon cuniculi* (0.7).

Further analyses revealed intergenic region lengths in *P. jirovecii* which are similar to those observed in the free-living yeasts and *T. deformans*, but larger than those of *E. cuniculi* (supplementary fig. S2B, Supplementary Material online). The gene density observed in *P. jirovecii* (481 genes per Mb) was similar to those of *S. cerevisiae* (487), *S. pombe* (554), and *T. deformans* (431), but clearly lower than that observed in *E. cuniculi* (816; supplementary table S6, Supplementary Material online).

Discussion

Biotrophy is a character that has emerged independently in various fungal clades as well as in oomycetes (Kemen and Jones 2012; Spanu 2012), and which has been described exclusively in plant parasites so far. This study was undertaken to explore the signature of biotrophy in the human fungal parasite *P. jirovecii*. By reconstructing the ancestral genomes of the Taphrinomycotina subphylum, we discovered several gene losses in the *Pneumocystis* genus compatible with its obligate biotrophy. It is unlikely that we missed these genes because of the incompleteness of the genomes as 1) the losses were observed in both *P. jirovecii* and *P. carinii*, and 2) the searches were conducted on independent and partially overlapping genomic and transcriptomic data sets, which are expected to cover 100% of the genomes.

Because a large proportion of the genome was found to be under purifying selection, there is a high probability that the losses of genes we detected in *Pneumocystis* spp. have resulted from neutral genetic drift rather than from positive selection. Genetic drift might have been favored by the relative small effective population size of *Pneumocystis* spp., which is expected from the strict host specificity and absence of free-living forms of these fungi. The relatively large proportion of repetitive DNA, large intergenic region lengths, and low gene density in *P. jirovecii* are also more compatible with the genetic drift hypothesis than with positive selection. Furthermore, the ratio of pseudogenes in *P. jirovecii* was found to be small and similar to those of nonbiotrophic free-living organisms, suggesting that a massive appearance of such nonfunctional genes has not been a major mechanism of gene loss.

Most amino acids biosyntheses were lost in *Pneumocystis* spp. (Hauser et al. 2010; Cisse et al. 2012), a hallmark of obligate parasitism. Such loss was not observed in plant biotrophs so far (Spanu 2012), but occurs systematically in organisms feeding on other animals, such as protozoan parasites of humans, as well as, for example, in *Homo sapiens* (Payne and Loomis 2006). In fungal biotrophs, losses of biosynthetic pathways are generally compensated by expanded transporters families to scavenge the products of these pathways (Spanu et al. 2010; Duplessis et al. 2011). However, we observed a reduced panel of transporters susceptible to carry amino acids in *Pneumocystis* spp. This reduction may be linked to the loss of the amino acids biosynthesis pathways and suggests the use of an alternative strategy. Biochemical experiments showed that amino acids uptake in *P. carinii* may occur by a facilitated diffusion across the membrane (Basselin, Lipscomb, et al. 2001; Basselin, Qiu, 2001). *Pneumocystis* spp. have been previously suggested to scavenge other crucial compounds from their hosts, that is, sterols (Joffrion and Cushion 2010).

Nitrogen and sulfur assimilation pathways as well as the purine catabolism were lost in *Pneumocystis* spp. In the absence of assimilation from inorganic sources, nitrogen and sulfur are essential compounds which can be obtained from

the degradation of purines, or from proteolysis. Purine catabolism is unlikely to be a source in *P. jirovecii* and *P. carinii* because we did not detect the key genes of this pathway. In the absence of the sulfite oxidase, which is required to metabolize cysteine and methionine, proteolysis does not seem to be the source of sulfur, source which thus remains to be characterized. On the other hand, proteolysis could be the source of nitrogen which would be consistent with the fact that animal tissues are rich in proteins. This would contrast from the saprotroph *S. pombe*, which uses the purine degradation for this purpose (Kinghorn and Fluri 1984). The loss of the purine degradation has not been observed in fungal biotrophs so far and thus represents a specificity of *Pneumocystis* spp., which might also be related to their adaptation to animal hosts. On the other hand, the loss of inorganic nitrogen and sulfur assimilation is frequently observed in fungal plant biotrophs (Spanu 2012), but this is not a hallmark of biotrophy as it is also observed in *Schizosaccharomyces* spp. that are saprotrophs. An interesting alternative to the possibility of acquiring essential compounds from the host is the Black Queen hypothesis (Morris et al. 2012). This hypothesis proposes that the loss of capability at the individual level can be compensated by the community. In the case of *Pneumocystis* spp., this community would be the other microorganisms sharing the same environment. This could be investigated through studies of the metabolic capacities of the lung microbiota associated with *Pneumocystis* pneumonia.

The key enzymes for thiamine biosynthesis were not detected in *Pneumocystis* spp. This loss is also a hallmark of biotrophy (Spanu 2012) and suggests that this vitamin is available from the mammalian host lungs. The missing thiamine thiazole synthase is a turnover suicide enzyme that needs to be replaced after each reaction (Chatterjee et al. 2011).

Our searches revealed that the RNAi machinery is lost in *Pneumocystis* spp. This loss would have occurred after the divergence of the *Taphrina* and *Pneumocystis* genera but before diversification in the *Pneumocystis* genus. The removal of RNAi has been suggested to confer a selective advantage to pathogens because it would enhance genome evolution by allowing the free movement of transposons and retrotransposons (Oliver and Greene 2009). The RNAi machinery is also absent in the fungi *S. cerevisiae*, *U. maydis*, and *Cryptococcus gattii*, as well as in the protozoan parasites *Trypanosoma cruzi* and *Plasmodium falciparum* (Nicolas et al. 2013). The significance of this loss remains to be elucidated.

Our findings suggest that obligate biotrophy arose in *Pneumocystis* spp. in conjunction with gene losses of multiple pathways. The vital compounds that can be scavenged from the host lungs probably allowed these losses. Together with other characteristics, that is, a sex life cycle within the host, the absence of massive destruction of host cells, the absence of an axenic in vitro culture system so far, and the lack of virulence factors, these gene losses strongly suggest that *Pneumocystis* spp. should be considered as obligate biotrophs. The

reduction of the genome size of *Pneumocystis* spp. contrasts with the increase of this feature often observed in fungal plant obligate biotrophs (Spanu 2012). This increase corresponds to a proliferation of retrotransposons, which would create genetic variability and diversity including panels of effectors. As in oomycetes, the genome size reduction in *Pneumocystis* spp. might be compensated by the genetic diversity generated by sexuality, suggesting that they might be "obligate sexual organisms" (Spanu 2012). Another hypothesis is that the niches in animal hosts do not require a variety of effectors to sustain infection but rather an increased fitness. This increased fitness might be obtained by the reduction of genome size, a feature which is observed in most human parasites, eukaryotic as well as prokaryotic (Sakharkar 2004; Hauser et al. 2010).

In contrast to necrotrophy, biotrophy implies a long-lasting and tight relationship with the living host and its immune system. Such relationship requires mechanisms which are expected to be specific to each host. One mechanism might be the antigenic variation encoded by the contingency gene families present at the telomeres of *Pneumocystis* spp., which may help maintaining the infection despite the host immune system. Thus, one can speculate that the strict host specificity and the biotrophy of *Pneumocystis* spp. were acquired simultaneously. The obligate nature of biotrophy may have emerged later and implied coevolution with the host.

A limitation of our study was the use of the Dollo parsimony model, which assumes that lost ancestral traits cannot be reacquired. Indeed, the model is not appropriate to detect genes brought by horizontal gene transfer events, which are thought to be relatively frequent in lower eukaryotes such as fungi (Fitzpatrick 2012). The possible occurrence of such transfers, which may have brought important new traits within the *Pneumocystis* genus, deserves further studies.

In conclusion, *Pneumocystis* spp. harbor a unique combination of characteristics which are hallmarks of both obligate biotrophs and animal parasites. The evolutionary events underlying biotrophy in *Pneumocystis* spp and the closely related hemibiotroph *T. deformans* appear drastically different, which suggests a parallel rather than a convergent evolution. *Pneumocystis* spp. should be considered as the first described obligate biotrophs of animals, which can turn into opportunistic biotrophic pathogens upon immune deficiency of the host. The lifestyle of *P. jirovecii* differs from that of other human fungal parasites. Indeed, *Malassezia* and *Candida* spp. can be considered as obligate commensals and opportunistic necrotrophs, whereas Dermatophytes would include both facultative and obligate true necrotrophs, which, possibly, might be transiently commensals.

Supplementary Material

Supplementary material, tables S1–S7, and figures S1–S3 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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