Comparative Genomics Suggests Primary Homothallism of Pneumocystis Species

João M. G. C. F. Almeida, Ousmane H. Cissé, Álvaro Fonseca, Marco Pagni, Philippe M. Hauser

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal; Institute of Microbiology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland; Vital-IT Group, SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland

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ABSTRACT Pneumocystis species are fungal parasites of mammal lungs showing host specificity. Pneumocystis jirovecii colonizes humans and causes severe pneumonia in immunosuppressed individuals. In the absence of in vitro cultures, the life cycle of these fungi remains poorly known. Sexual reproduction probably occurs, but the system of this process and the mating type (MAT) genes involved are not characterized. In the present study, we used comparative genomics to investigate the issue in P. jirovecii and Pneumocystis carinii, the species infecting rats, as well as in their relative Taphrina deformans. We searched sex-related genes using 103 sequences from the relative Schizosaccharomyces pombe as queries. Genes homologous to several sex-related role categories were identified in all species investigated, further supporting sexuality in these organisms. Extensive in silico searches identified only three putative MAT genes in each species investigated (matMc, matMi, and matPi). In P. jirovecii, these genes clustered on the same contig, proving their contiguity in the genome. This organization seems compatible neither with heterothallism, because two different MAT loci on separate DNA molecules would have been detected, nor with secondary homothallism, because the latter involves generally more MAT genes. Consistently, we did not detect cis-acting sequences for mating type switching in secondary homothallism, and PCR revealed identical MAT genes in P. jirovecii isolates from six patients. A strong synteny of the genomic region surrounding the putative MAT genes exists between the two Pneumocystis species. Our results suggest the hypothesis that primary homothallism is the system of reproduction of Pneumocystis species and T. deformans.

IMPORTANCE Sexual reproduction among fungi can involve a single partner (homothallism) or two compatible partners (heterothallism). We investigated the issue in three pathogenic fungal relatives: Pneumocystis jirovecii, which causes severe pneumonia in immunocompromised humans; Pneumocystis carinii, which infects rats; and the plant pathogen Taphrina deformans. The nature, the number, and the organization within the genome of the genes involved in sexual reproduction were determined. The three species appeared to harbor a single genomic region gathering only three genes involved in sexual differentiation, an organization which is compatible with sexual reproduction involving a single partner. These findings illuminate the strategy adopted by fungal pathogens to infect their hosts.

Pneumocystis species constitute a group of fungi belonging to the Taphrinomycotina subphylum of the Ascomycota which colonize the lungs of mammals. Genetic and transmission analyses revealed that each Pneumocystis species infects specifically a single mammalian host species. Pneumocystis jirovecii, the species colonizing humans, can turn into an opportunistic pathogen in immunosuppressed individuals and cause severe, sometimes fatal pneumonia (Pneumocystis pneumonia [PCP]). PCP is nowadays the second most frequent life-threatening invasive fungal infection worldwide, with above 400,000 annual cases (1). Comparative genomics suggested the loss of synthesis and assimilation pathways in Pneumocystis species and thus that these fungi are obligate parasites without free-living forms (2–4). The characteristics of these fungi suggest that they are biotrophs of mammals, i.e., parasites retrieving energy and compounds from host cells without killing them (4–7).

The life cycle of Pneumocystis species remains poorly known, mostly because of the absence of a method for long-term culture in vitro. During the infection, two types of cellular structures are observed: the trophic cells and the asci (also called trophs and cysts, respectively). Microscopic and cytological studies suggested that the trophic cells may undergo binary fission but also diploidization upon conjugation, followed by meiosis and mitosis to produce asci containing eight haploid ascospores (8, 9). The observation of synaptonemal structures within Pneumocystis cells (10, 11) and the demonstration of the expression of sex-related
genes (5, 12) further confirmed that the reproduction of these fungi probably includes a sexual phase. Quantitative experiments suggested that the main mode of reproduction of *Pneumocystis* species might be meiotic division, whereas the contribution of mitotic division of trophic cells is still unclear (8, 13, 14). Therefore, the sexual phase might be obligatory for the growth of these fungi. Obligate sexuality is also consistent with the fact that the asc might be the only particles aerially transported and responsible for transmission between hosts (14, 15). Obligate sexuality would imply that trophic cells of compatible mating types must always be present within host lungs in order to allow sexual reproduction.

There are two main systems of sexual reproduction among fungi: heterothallism, involving two compatible mating types, and homothallism, involving a single self-compatible mating type. In heterothallic ascomycetes, the MAT loci contain divergent genes (idiomorphs) in opposite mating types (16). In so-called primary homothallism, involving a single self-compatible mating type, the MAT loci are divergent genes present in the genomes of these species, genes relative to their localization close to the homeobox domain (PTHR11850 or TPR00900). In this case, the homothallic behavior results from mating type switching and the presence of three MAT loci in the same genome: one active and two silent. In *P. pombe*, the MAT loci are flanked by cis-acting sequences that are involved in a switching mechanism which exchanges the expressed cassette. Primary homothallism is observed in many filamentous ascomycetes such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. As far as homothallism species are concerned, their particular system of reproduction is still unknown. The genomic region surrounding the pheromone receptor ste3 gene has been postulated to constitute a MAT locus in these fungi. It would also include genes encoding the protein kinase Ste20 and the homeodomain transcriptional regulator Ste12 and thus, surprisingly, would resemble the MAT locus of the basidiomycete *Cryptococcus neoformans* (17).

In the present study, we investigated the system of reproduction of *P. jirovecii* and *Pneumocystis carinii*, the species infecting rats, by the analysis of their genome content and comparative genomics. We also investigated the issue in the *Taphrina deformans* relative *Taphrina deformans*, a plant pathogen whose sexual reproduction is also poorly characterized. In order to identify the sex-related genes present in the genomes of these species, genes from the well-annotated *Taphrina deformans* member *S. pombe* were used as homology references.

**RESULTS**

**Identification of sex-related genes.** Due to the low conservation and great diversity of the sex-related genes among fungi, the identification of homologs of sex-related *S. pombe* proteins within the genomes of *P. jirovecii*, *P. carinii*, and *T. deformans* was done using the bioinformatics strategy shown in Fig. S1 in the supplemental material. The procedure relied on tBLASTn search and recognition by manual inspection of specific domain architecture (see details in Materials and Methods). The 103 *S. pombe* genes used in sequence queries were selected on the basis of their annotation in UniProt and Gene Ontology (GO) terms, as well as of their belonging to relevant protein complexes (see Table S1 in the supplemental material). Genes were classified in one or two of seven role categories: cell fusion, signal transmission, signal transduction, signal regulation, meiosis, mating type locus silencing (RNA interference pathway), and mating type locus switching. Using this strategy, we established the presence or absence of sex-related genes within the genomes of the three fungi (see Table S2 in the supplemental material; the locus reference or genomic location of the genes identified is given in Table S3 in the supplemental material).

Genes homologous to all seven role categories of sex-related *S. pombe* proteins were present in the three genomes investigated (see Table S2 in the supplemental material, green areas). In agreement with their notoriously difficult identification, the presence or absence of the pheromone precursor genes could not be assessed (mam2, mam1, mam2, and mam3). The genes encoding the two pheromone receptors (mam2 and ste3) and the elements of the pheromone-induced signaling cascade (e.g., gpa1, byr2, byr1, spk1, and ste11) were present in all three genomes investigated, except that *T. deformans* lacked mam2. There was a remarkable similarity of the presence and absence of the genes in the three species investigated, with 51 genes present (see Table S2 in the supplemental material, green areas), and 25 absent (brown areas). Twelve genes were absent only in the two *Pneumocystis* species, suggesting specific features of this genus (signal transmission, pmnl1; signal transduction, ste4; signal regulation, rst2, sax1, and sax2; mating type silencing: ago1, arb1, arb2, chp1, dac1, lst2, obr1, and rdr1). Thus, more genes were detected in *T. deformans* than in the two *Pneumocystis* species. The Argonaute small interfering RNA chaperone (ARC) complex was absent in the *Pneumocystis* species (mating type silencing: ago1, arb1, and arb2), a feature which we already reported (4). The presence or absence of the gene products of the three species within the reconstructed pathways of *S. pombe* is shown in the supplemental material (see Fig. S2 in the supplemental material).

**MAT genes.** Genes homologous to the four *S. pombe* MAT genes were sought in the three species investigated (see Table S2 in the supplemental material, boxed genes). Extensive in silico searches using our bioinformatics strategy (see Fig. S1 in the supplemental material) revealed the presence of only three genes in each *Pneumocystis* species and in *T. deformans*, namely, the homologs of *S. pombe* matMc, matMi, and matPi. The gene matMc encodes a transcription factor with the high-mobility-group domain (PTHR10270:SF176 or PTHR10270:SF159), matMi encodes a mating type M-specific polypeptide, and matPi encodes a transcription factor with a homeobox domain (PTHR11850 or PTHR11850:SF31). The matMi-encoded proteins have no known domains and are poorly conserved between species, so that their identification did not rely on domain architecture as for the other genes but on their localization close to matMc and opposite orientation relative to matMc.

Multiple sequence alignment revealed a fair degree of similarity of the matMc-encoded proteins identified, in particular at the high-mobility-group domain, with 27% overall identity with the *S. pombe* protein for all three *P. jirovecii*, *P. carinii*, and *T. deformans* proteins (Fig. 1A). The degree of similarity was lower between the matPi proteins and concentrated at the homeobox domain present at the end of the proteins, with 19% overall identity with the *S. pombe* protein for *P. jirovecii* and 18% for both *P. carinii* and *T. deformans* (Fig. 1B). Reasonable alignment of the matMi proteins could not be generated because of their low similarity, the identity with the *S. pombe* protein being only 12, 15, and 20% for *P. jirovecii*, *P. carinii*, and *T. deformans* proteins, respectively. Despite this low similarity, the phylogeny of the three putative MAT
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The genomic region surrounding the MAT genes appeared to be fully syntenic between the two *Pneumocystis* species with six genes conserved (Fig. 3). The three putative MAT genes were located on a single contig in *P. jirovecii* and thus on a single DNA molecule in the genome. These genes are on two contigs in *P. carinii* and *T. deformans*, but this may result from DNA fragmentation during genome sequencing and assembly, especially in *P. carinii* because of its overall high synteny with *P. jirovecii*. Limited synteny existed between the other species, with only one gene conserved in addition to the MAT genes (*yox1* between *Pneumocystis* species and *T. deformans*; *rga7* between *P. jirovecii* and *T. deformans*). The finding of *rga7* next to the *matMc/matMi* gene pair in *T. deformans* is particularly relevant since this arrangement is conserved in all Schizosaccharomyces species (18).

**Analysis of the putative MAT genes of several *P. jirovecii* isolates.** The putative MAT genes *matMc*, *matMi*, and *matPi* were amplified from the *P. jirovecii* isolates present in six different patients with PCP and sequenced. The sequences of all isolates were exactly the same as that present in the *P. jirovecii* genome sequence, except in the isolates of three patients where a deletion of 5 bp was located between the putative *matMc* and *matMi* genes (CCTTG at positions 57275 to 57279 in sequence CAKM01000262.1 of the *P. jirovecii* genome [contig 262]). One of the three patients harbored at least two coinfecting genotypes, one in the majority which presented the deletion and one in the minority which did not.

**Genomic region surrounding the gene encoding the pheromone receptor Ste3.** We analyzed the genomic region surrounding the *ste3* gene because it has been postulated to constitute...
a MAT locus in *P. carinii* (17). This genomic region was reported to include the protein kinase Ste20 and the homeodomain transcriptional regulator Ste12. In both *P. carinii* and *P. jirovecii*, BLAST analyses using *S. pombe* sequences as queries revealed that the so-called *ste20* gene was in fact an *shk2* homolog with a pleckstrin homology-like domain (IPR011993) and the so-called *ste12* gene was a truncated version of *ste11*, a signal transduction kinase gene. We identified *ste3* and *shk2* on the same contig in *P. carinii*, whereas *shk2* was on the same contig as the three putative MAT genes in *P. jirovecii* but fairly distant (ca. 80 kb). The complete

a MAT locus in *P. carinii* (17). This genomic region was reported to include the protein kinase Ste20 and the homeodomain transcriptional regulator Ste12. In both *P. carinii* and *P. jirovecii*, BLAST analyses using *S. pombe* sequences as queries revealed that the so-called *ste20* gene was in fact an *shk2* homolog with a pleckstrin homology-like domain (IPR011993) and the so-called *ste12* gene was a truncated version of *ste11*, a signal transduction kinase gene. We identified *ste3* and *shk2* on the same contig in *P. carinii*, whereas *shk2* was on the same contig as the three putative MAT genes in *P. jirovecii* but fairly distant (ca. 80 kb). The complete
ste11 gene was identified in distinct locations in the two Pneumocystis genomes (see Table S3 in the supplemental material).

**DISCUSSION**

In order to better characterize the life cycle of Pneumocystis species and *T. deformans*, we searched for sex-related genes in their genomes. We detected many genes involved in various sex-related processes, such as mating, pheromone signaling, and meiosis. Some of these genes have been previously identified in *P. carinii* (17, 19–23) and *T. deformans* (24), but we report them in *P. jiroveci* for the first time. The expression of several putative mating or meiosis genes in *P. carinii* has been documented elsewhere (5, 12). Together, these observations strongly support the hypothesis that a sexual phase occurs in the life cycle of the *Pneumocystis* species and *T. deformans*.

Our analyses reveal a conserved syntenic genomic conformation of the MAT locus between the two *Pneumocystis* species, despite a high level of divergence between the MAT genes. This suggests that the conservation of the locus configuration might be more critical for sexual reproduction than that of the mating genes. The most striking feature of our findings was that only three genes homologous to the four MAT genes were identified in each *Pneumocystis* species, as well as in *T. deformans* (matM, matMi, and matPi). It must be stressed that the matMi genes identified remain hypothetical because of their low similarity with that of *S. pombe*. It is likely that no other MAT genes were present because (i) the results were similar in the two *Pneumocystis* species and (ii) the genomic and transcriptomic data analyzed are expected to cover most of the genomes. Moreover, we did observe the same synteny and three MAT genes also in the *Pneumocystis murina* genome, but our data are unpublishable because this genome sequence was released prior to publication under specific terms (see *Pneumocystis murina* Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org/). One limitation of our study is that MAT genes too divergent from those of *S. pombe* would not have been detected. However, *S. pombe* harbors two different types of DNA binding domains (high-mobility group and homeobox), which increased the probability of detecting such genes. The proximity of three putative MAT genes identified in the *Pneumocystis* species and *T. deformans* is atypical and suggests a fusion of two MAT loci, one of type minus (M) with matMc and matMi and one incomplete of type plus (P) with only matPi. Consequently, the transcription factors encoded by matMc (high-mobility group) and matPi (homeobox) would be sufficient to trigger sexual development. Such fusion of two MAT loci has been previously observed in other fungi, for example, in the *Stemphylium* genus (25), and the loss of MAT transcription factors occurred in *Candida* species other than *C. albicans* (26). A similar fusion of the two MAT loci M and P is also present in three *Schizosaccharomyces* species other than *S. pombe*, with only matMc in their type M loci (18).

The number and the organization of the putative MAT genes in the *Pneumocystis* species and *T. deformans* allow formulation of a hypothesis concerning the system of sexual reproduction of these fungi. First, the presence of only three MAT genes seems incompatible with secondary homothallism. Indeed, this system requires generally more genes, i.e., six corresponding to three MAT loci in *S. pombe* and *S. cerevisiae* and four corresponding to two MAT loci in some methylotrophic yeasts, as recently described (27). This is consistent with the fact that we did not detect any cis-acting sequences for mating type switching flanking the putative MAT genes. Second, the three MAT genes in *P. jiroveci* are present on a single DNA molecule (Fig. 3), and this is also the case in *P. murina* (our unpublished findings). The close proximity of these genes on a single DNA molecule is particularly important because it is not compatible with heterothallism. Indeed, if the genome sequences had been derived from a mixture of two compatible heterothallic mating types, two MAT loci would have been observed located on two distinct DNA molecules, not on a single one. The alternative possibility, i.e., that the genome sequences correspond to a single heterothallic mating type, is also unlikely. First, the MAT locus identified appears to include both types, M and P. Second, ascI are present in most *Pneumocystis* infections, if not all, i.e., sexuality appears obligatory. In particular, this was the case in the clinical specimen used for *P. jiroveci* genome sequencing because the diagnosis was made by silver staining of the walls of ascI (3).

The loss of the transcription factor gene matPc (high-mobility group) in the *Pneumocystis* species and *T. deformans* suggests that the P-specific genes may not be expressed and that P cells may be lacking. Thus, these species may use a single mating type for sexual reproduction, a system previously observed in *C. neoformans* (28) and *Candida albicans* (29). An alternative hypothesis is that expression of the P-specific genes, including that encoding the P-factor, is ensured thanks to a rewiring of the MAT pathways, a phenomenon frequently observed in fungi (30, 31). The latter hypothesis would be consistent with the presence of the receptor mam2 to the P-factor in the *Pneumocystis* species. The presence of matPi in all three genomes investigated might reflect that both this gene and the matMi gene seem necessary for the expression of mei3 and thus for entry into meiosis (32). Further experiments are required in order to decipher the system of sexual reproduction in the *Pneumocystis* species and *T. deformans*.

Our results do not allow us to ascertain that the genes identified are *bona fide* MAT genes of these species, because we cannot rule out the possibility that there are other transcription factors involved in mating type determination elsewhere in the genomes. Also, one cannot formally exclude secondary homothallism. However, the close vicinity and arrangement of the putative MAT genes are consistent with primary homothallism in the *Pneumocystis* species as well as in *T. deformans*. The latter system of reproduction implies the presence of the same MAT locus in all isolates of each species that we investigated. This is what we found in the *P. jiroveci* isolates of six patients, further supporting the hypothesis of primary homothallism. This hypothesis is also compatible with the *T. deformans* cell cycle. Indeed, single haploid yeast cells inoculated on peach leaves are able to produce, without cell-cell conjugation, dikaryotic hyphae that give rise to ascI containing eight ascospores (33, 34).

Primary homothallism has been hypothesized to be advantageous for pathogens (35), including *Pneumocystis* species (28, 29). Our results are compatible with this hypothesis. Although it involves a single strain, primary homothallism has been shown in *C. neoformans* to avoid accumulation of deleterious mutations, as well as to increase genetic diversity and virulence (36). In the case of *P. jiroveci*, the fact that the majority of infections involve two or more distinct genotypes (37) suggests that the genetic diversity may be further increased by mating among these genetic variants. The presence of many genes that we classified in the silencing and switching categories in the *Pneumocystis* species and *T. defor-
<p><em>mans</em> appears contradictory to the putative absence of secondary homothallism. Nevertheless, their presence could be simply due to their involvement in other cellular or metabolic processes. For example, some swi-encoded proteins are known to be necessary for DNA metabolism. On the other hand, the absence of two silencing (clr1 and swit6) and two switching (sap1 and swit2) genes in all three species investigated might be significant because these genes are apparently dedicated to silencing or switching of MAT genes (see Table S1 in the supplemental material).

As far as <em>P. carinii</em> is concerned, we confirmed the location of <em>ste3</em>, the so-called <em>ste20</em> gene, and the so-called <em>ste12</em> gene within the genomic region which was postulated to be a MAT locus resembling those of the basidiomycete <em>C. neoformans</em> (17). However, the so-called <em>ste20</em> and <em>ste12</em> genes turned out to be shk2 and a truncated version of <em>ste11</em>, respectively. These different findings may result from the constant improvement of the databases, the increase in knowledge about these genes since 2001, and the fact that in this study we dealt with more than one genome. The confusion between <em>ste12</em> and <em>ste11</em> probably results from the fact that <em>S. cerevisiae</em> <em>ste12</em> is the ortholog of <em>S. pombe</em> <em>ste11</em>. Consequently, the genomic region surrounding <em>ste3</em> in the <em>Pneumocystis</em> species may constitute a cluster of sex-related genes, rather than a MAT locus resembling that of <em>C. neoformans</em>. The computational observation of putative MAT genes does not prove conclusively that a species is sexual, because these genes may have been conserved from a sexual ancestor. In conclusion, our analyses suggest the working hypothesis that primary homothallism is the system of reproduction of the <em>Pneumocystis</em> species as well as of <em>T. deformans</em>.</p>
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