Phylogenetic identification of *Pneumocystis murina* sp. nov., a new species in laboratory mice

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*Pneumocystis* is a fungal genus that contains multiple species. One member of the genus has not been formally analysed for its phylogenetic relationships and possible species status is the *Pneumocystis* found in laboratory mice, *Pneumocystis murina* sp. nov. (type strain ATCC PRA-111 = CBS 114898), formerly known as *Pneumocystis carinii* f. sp. musis. To advance research in this area, approximately 3000 bp of additional DNA sequence were obtained from the locus encoding rRNAs. This sequence and others were used to determine genetic distances between *P. murina* and other members of the genus. These distances indicated that *P. murina* DNA is most similar to that of the species of *Pneumocystis* found in laboratory rats. Nevertheless, *P. murina* is at least as diverged from these other *Pneumocystis* species as species in other fungal genera are from each other. The 18S rRNA gene sequence divergence exhibited by *P. murina* could not be ascribed to accelerated evolution of this gene as similar levels of divergence were observed at seven other loci. When five genes were used to construct phylogenetic trees for five *Pneumocystis* taxa, including *P. murina*, all the trees had the same topology, indicating that genes do not flow among these taxa. The gene trees were all strongly supported by statistical tests. When sequences from the rRNA-encoding locus were used to estimate the time of divergence of *P. murina*, the results indicated that *P. murina* is as old as the mouse. Taken together, these data support previous recognition of multiple species in the genus and indicate that *P. murina* is a phylogenetic species as well.

INTRODUCTION

The genus *Pneumocystis* contains fungi named for a cyst-like multinucleate morphological form that stains strongly with silver and is found together with a uninucleate pleomorphic ‘trophic’ form in alveolar spaces in the lungs of numerous mammalian species. *Pneumocystis* cannot be grown in culture. Nevertheless, molecular studies during the last decade have revealed that this genus contains many distinct organisms (Demanche et al., 2001; Denis et al., 2000; Durand-Joly et al., 2000; Guillot et al., 2001; Hunter & Wakefield, 1996; Keely et al., 1994; Ma et al., 2001; Walker et al., 1998; Norris et al., 2003). The genetic and functional diversity exhibited by these organisms suggests that the genus contains more than one species, and three such species have been formally proposed: *Pneumocystis jirovecii*, the species found in patients suffering from *Pneumocystis* pneumonia, and *Pneumocystis carinii* and *Pneumocystis wakefieldiae*, two species found in laboratory rats (Frenkel, 1976, 1999; Cushion et al., 2004). Laboratory mice contain a distinct type of *Pneumocystis*, *Pneumocystis murina* sp. nov., formerly known as *P. carinii* f. sp. musis (Anonymous, 1994). The name *P. murina* was proposed informally (i.e. without a Latin diagnosis) in 1994 to specify the host species, *Mus musculus* (Eriksson, 1994).

*P. murina* infections in mice serve as the principal model for the study of the immune response. Given its importance as a model of human disease, it is important to clarify the relationship between *P. murina* and other members of the genus. The goal of the work described herein was to better assess the genetic distinctiveness of *P. murina* by systematic comparison of its DNA to that of other *Pneumocystis* species. To enhance the power and accuracy of this comparison, the sequences of the genes encoding the 18S and 5-8S rRNAs were determined along with the internal transcribed spacer (ITS) regions adjacent to these two genes.
These and other sequences were used to assess phylogenetic relatedness, concordance of divergence at different loci and time of genealogical divergence. Analyses of the sequence comparisons employed tools that were not available at the time that the other three *Pneumocystis* species were described. Hence, these studies provide both a foundation for understanding the status of *P. murina* as a possible species and a more-sophisticated view of the relationships between the previously described species in the genus.

Naming new species can be controversial because there are many ways to conceive of a species (Hey, 2001; Keely et al., 2003b; Mayden, 1997). The ‘phylogenetic species concept’ is well suited to the task of erecting an evolutionarily based taxonomic structure for the genus *Pneumocystis*. A phylogenetic species is an evolutionary lineage having a unique combination of DNA sequences (Taylor et al., 2000). Such species can be elucidated by inferences from a bifurcating gene tree, which is usually constructed from a pairwise distance matrix derived from an alignment of orthologous sequences. The distance matrix is produced by determining the best substitution model to fit the data (Posada & Crandall, 1998). The topology of the tree can be evaluated by a test of reliability, such as the common bootstrap statistic (Felsenstein, 1985). Additional tests of statistical significance of tree topology have been developed (Strimmer & Rambaut, 2002). The probability of obtaining a correct species tree can be increased by considering multiple genes at the same time. Analysis of multiple genes also allows application of the genealogical concordance phylogenetic species recognition method, which can detect the occurrence of genetic exchange. If the variation under analysis is occurring within a sexual species, then different genes will tend to produce different trees due to independent assortment of alleles during sexual reproduction (Taylor et al., 2000). By contrast, the absence of gene flow, which predominates when organisms are different species, will tend to give concordant tree topologies due to the fixation of alleles after genetic isolation.

**METHODS**

**Source of *Pneumocystis* organisms.** Organisms were obtained from lung homogenates of 6- to 8-week-old severe combined immunodeficiency (SCID) mice (C3Sn68m.CB17-Prkd<sup>scid</sup>/J), The Jackson Laboratory, Bar Harbor, Maine, USA). SCID mice were immunosuppressed with methylprednisolone (Depo Medrol; Upjohn) as described previously (Cushion et al., 1993a). Organisms were also obtained from infected lungs of wild mice captured in Clermont County, OH, USA. Infected lungs were prepared as described previously (Cushion et al., 1993a). Briefly, lung tissues were homogenized in a Stomacher Lab Blender 80 (Fisher Scientific). Erythrocytes in the homogenate were lysed by treatment with aqueous ammonium chloride (0.85%). *Pneumocystis* organisms were separated from remaining intact host cells by low- and high-speed centrifugations, followed by filtration through 10 μm filters (Mite; Millipore).

**Electrophoretic karyotype analysis.** *Pneumocystis* chromosomes were prepared for electrophoretic karyotype analysis as described previously (Cushion et al., 1993a, b). Briefly, *Pneumocystis* organisms were treated with DNase I (Boehringer Mannheim) at 10 μg ml<sup>-1</sup> in a solution of 150 mM NaCl/10 mM MgCl<sub>2</sub>/10 mM Tris at pH 7-2 for 30 min at 37°C to digest extracellular DNA. To inactivate the DNase, magnesium ions were removed by washing once with 250 mM EDTA and twice with 125 mM EDTA. Organisms were embedded in 0.8% low-melting-point agarose (Boehringer Mannheim) and treated with 0.25 mg proteinase K ml<sup>-1</sup> (Boehringer Mannheim) in a solution of 1% N-lauroylsarcosine (Sigma Chemical)/0-45 M EDTA/0-01 M Tris at 55°C for 24-48 h. Gels for contour clamped homogeneous electric field (CHEF) electrophoresis contained 1% FMC SeaKem GTG-agarose (SeaKem) prepared in 0.5 × TBE (45 mM Tris/HCl/45 mM boric acid/1-25 mM EDTA) for a total volume of 200 ml and final dimensions of 14 × 21 cm. Electrophoresis was performed using CHEF DR II (Bio-Rad) or a Bio-Rad CHEF DR III apparatus. Gels were run for 104-144 h, at 14°C, in 0.5 × TBE at 3-8 V cm<sup>-1</sup> with a 50 s initial pulse that was gradually increased to 100 s. Agarose gels were stained with SYBR-Gold (Molecular Probes), illuminated by UV light and photographed. The molecular masses of the chromosomes were determined by linear regression using molecular mass standards.

**PCR amplification.** An aliquot of lung homogenate (1 ml) was treated with proteinase K, and genomic DNA was isolated by 2-propanol extraction, as described previously (Keely et al., 2003a). The DNA was dissolved in 0-01 M Tris/0-001 M EDTA, pH 8, and diluted 1:20 in sterile water. One microlitre of the DNA dilution was subjected to PCR. Primer sequences are shown in Table 1. The positions of the primers are shown in Fig. 1. The gene encoding 18S rRNA was amplified with primers p1 (Shah et al., 1996) and p2 (primer B) (Shah et al., 1996) without the restriction sites. ITS1, 5-8S rRNA and ITS2 were amplified with primers p3 and p4 based on previous reports (Edman et al., 1988; Stringer et al., 1989). PCR was performed under the following conditions: 94°C hot start for 2 min, 35 cycles of incubation at 94°C for 20 s, 55°C for 15 s and 72°C for 120 s, and 1 cycle of 72°C extension for 5 min. A portion of the gene encoding the large-subunit mitochondrial rRNA [mtrRNA(LSU)] was amplified with primers PAZ102-H and PAZ102-E (Wakefield et al., 1990) under the following conditions: 95°C hot start for 5 min, 30 cycles of incubation at 95°C for 60 s, 50°C for 120 s and 72°C for 60 s, and 1 cycle of 72°C extension for 5 min. Reaction volumes were 25 μl containing 50 μM each of dATP, dCTP, dGTP, dTPP and 2-5 mM MgCl<sub>2</sub> and 100 ng of each primer, Triple Master polymerase (1:5 U) (Eppendorf) and High Fidelity Buffer were utilized for the amplification of 18S–ITS1–5-8S–ITS2. Taq polymerase (1 U) (Promega) was utilized for the amplification of the mtrRNA(LSU) locus.

**DNA sequencing.** Previous studies have produced several short segments of DNA sequence from *P. murina*. While these sequences have provided clear evidence of the distinctiveness of this organism, they are insufficient for statistical tests of relationships. To facilitate application of such tests, the nuclear gene encoding the 18S rRNA was sequenced. The 18S rRNA gene was amplified twice and both amplicons were separately cloned into the TOPO 2.1 plasmid (Invitrogen), which was introduced into the chemically competent strain of *Escherichia coli* provided with the vector. The 18S rRNA locus was sequenced twice, once from each of the two independently derived amplicons. DNA sequences were determined by the Sequencing Facility at the University of Cincinnati College of Medicine. The amplicon containing the group I intron, ITS1, 5-8S and ITS2 was sequenced from both strands using vector primers (M13Forward, M13Reverse) and those listed in Table 1. Sequences were assembled using DNAMAN software (Lynnon BioSoft). A 350 bp region of the mtrRNA(LSU) locus was cloned into TOPO 2.1 and three plasmids were sequenced with vector primers.

**Sequences compared in this study.** Portions of the following genes were aligned: manganese-cofactored superoxide dismutase...
Table 1. Nucleotide sequences and positions of PCR and sequencing primers

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</table>

*Nucleotide position of primers according to mouse rRNA sequence along the specified gene (GenBank accession no. AY532651). +, Sense strand; –, antisense strand.
†Region of the primer binding site. 18S, 18S-like rRNA; E1, exon 1; E2, exon 2; 26S, 26S-like rRNA.

(SODA), accession nos Z79785, AF146751, AF146753, AF146754 and AF146752; dihydroprotease synthase (DHPS), accession nos M86602, U66283, AF139132, AT070270 and AF322064; dihydrofolate reductase (DHFR), accession nos AF322061, AF175561, AF90368, AY101748 and AF186097; mtrRNA(LSU), accession nos U20169, S42926, AF461783 and S42915, and two P. murina sequences, AF257179 and Peters et al. (1994); small-subunit mitochondrial rRNA [mtrRNA(SSU)] (Hunter & Wakefield, 1996); ITS1–5-8S rRNA–ITS2 (Hsu et al., 2001; Ortiz-Rivera et al., 1995; Nimri et al., 2002), accession nos L27658 and AY328078; 18S rRNA, accession nos X12708 and L27658, Pneumocystis f. sp. mustalae (Shah et al., 1996), P. jiroveci (Shah et al., 1996); thymidylate synthase, accession no. S77510, P. jiroveci (Mazars et al., 1995) and P. murina (Mazars et al., 1995); 5-enolpyruvylshikimate-3-phosphate synthase (AROM), P. jiroveci (Banerji et al., 1995), P. murina (Banerji et al., 1995) and P. carinii (Banerji et al., 1995); AB000948 (Taphrina carnea), AB000960 (Taphrina virginica), AB000958 (Taphrina robinsoniana), AB000955 (Taphrina nana), D12531 (Taphrina wisneri), AB000949 (Taphrina communis), AB000959 (Taphrina ulmi), Z735578 [Saccharomyces (Sac.) cerevisiae]; AY406227 (Saccharomyces bayanus); AB040998 (Saccharomyces mikatae); AB040997 (Saccharomyces kudriavzevii); Z75580 (Saccharomyces kluverii); X97806 (Saccharomyces paradoxus); Z75577 (Saccharomyces castelli). Outgroups and other fungi utilized for the rDNA alignment: Oryza sativa, AF069218; Candida albicans, E15168; Schizosaccharomyces (Sch.) pombe, X58056.

Phylogenetic tree construction. Sequences were aligned using DNAMAN software using the default settings. The alignments were optimized by introducing a limited number of gaps, which were not counted in relatedness calculations. In addition, ambiguous regions in the alignment were not scored. The DNA alignments contained 939, 815, 600, 229, 219, 332, 327 and 1800 nt for SODA, DHPS, DHFR, mtrRNA(LSU), mtrRNA(SSU), thymidylate synthase, AROM and 18S rDNA, respectively. For supertree analysis of five Pneumocystis taxa, SODA, DHPS, DHFR, mtrRNA(LSU) and mtrRNA(SSU) sequences were concatenated to form a sequence 2800 nt long.

Relatedness of pairs of aligned sequences for each individual gene and the concatenated sequences were calculated by MEGA 2.1 software utilizing p-distance and pairwise deletion (Kumar et al., 2001). P-Distance is the proportion of nucleotide sites at which the two sequences compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. Nearby-joining (NJ) trees were constructed with MEGA 2.1 (Kumar et al., 2001) and 1000 bootstrap replications were performed for each tree.

The p-distance nucleotide substitution model may not be optimal for phylogenetic analysis of Pneumocystis genes. Thus, optimal models were estimated for SODA, DHPS, DHFR, mtrRNA(LSU), mtrRNA(SSU), 18S rDNA and concatenated alignments by maximum-likelihood analysis utilizing the hierarchical likelihood ratio test implemented in MODELTEST 3.6 (Posada & Crandall, 1998) for PAUP* 4.0b10 (Swofford, 1998) and TREESTRUCTURE version 5.1 (Strimmer & von Haeseler, 1996). One-thousand bootstrap replications were performed and the bootstrap values for internal branches were recorded.

Different models and parameters were proposed by the hierarchical likelihood ratio test in MODELTEST (Posada & Crandall, 1998). The
Hasegawa, Kishino and Yano (HKY) model (Hasegawa et al., 1985) was optimal for mtRNA(LSU), mtRNA(SSU), DHPS and DHFR. This model assumes that transitions and transversions each have different rates of change and assumes that the base composition frequencies are unequal. The estimated base composition frequencies for mtRNA (LSU) were: A, 0.3-3923; C, 0-1111; G, 0-3268; T, 0-3228. In addition, a transition/transversion (Ti/tv) ratio of 1-4392 was estimated. For mtRNA(SSU), the estimated base composition frequencies were: A, 0-3491; C, 0-1016; G, 0-2109; T, 0-3384; and the Ti/tv ratio was 1-6497. A gamma shape parameter of 0-6877 was estimated to allow for a broad distribution of rates among nucleotide sites (this model is referred to as HKY + G). HKY + G was also optimal for DHPS and DHFR. The estimated base composition frequencies for DHPSs were: A, 0-3288; C, 0-1264; G, 0-1914; T, 0-3534; the Ti/tv ratio was 5-5724, and the gamma shape parameter was 0-2445. The estimated base composition frequencies for DHFR were: A, 0-3073; C, 0-1405; G, 0-2146; T, 0-3376; the Ti/tv ratio was 2-0145, and the gamma shape parameter was 0-7177. Interestingly, HKY + G was determined to be optimal for these mitochondrial genes and the DHPS gene from Pneumocystis derived from Old World and New World monkeys (Hugot et al., 2003).

The general time reversible (GTR) model (Rodriguez et al., 1990) was determined to be optimal for the SODA, 18S rDNA and concatenated alignments. This model is more complex than HKY because it estimates six nucleotide substitution rates (rAC, rAG, rAT, rCG, rCT and rGT) and assumes unequal base composition frequencies. Interestingly, it was shown recently to be the best model for a combined alignment of three Pneumocystis genes derived from Old World and New World monkeys (Hugot et al., 2003).

For the SODA locus, the GTR model with a proportion of invariable sites fitted the data the best (referred to as GTR + I). The proportion of invariable sites was 0-4094; the estimated base composition frequencies were: A, 0-3470; C, 0-9957; G, 0-1455; T, 0-4118; and the rate matrix was: A-C, 1-0000; A-G, 7-7290; A-T, 1-8147; C-G, 1-8147; C-T, 7-7290; G-T, 1-0000. GTR, with a gamma shape parameter and a proportion of invariable sites (referred to as GTR + G + I), was optimal for the 18S rDNA sequence alignment. The estimated base composition frequencies were: A, 0-2591; C, 0-2073; G, 0-2622; T, 0-2714; the gamma shape parameter was 0-6220, the proportion of invariable sites was 0-3987, and the substitution rate matrix was: A-C, 1-0000; A-G, 2-6109; A-T, 1-0000; C-G, 1-0000; C-T, 4-5713; G-T, 1-0000. Similarly, the GTR model of substitution with a gamma shape parameter (GTR + G) was optimal for the concatenated sequence alignment. Estimated base composition frequencies were: A, 0-3141; C, 0-1499; G, 0-1942; T, 0-3508. The gamma shape parameter was 0-7925 and the rate matrix was: A-C, 1-0000; A-G, 5-9787; A-T, 1-6157; C-G, 1-6157; C-T, 5-9787; G-T, 1-0000.

Tree concordance analysis. COMPONENT software (version 2.0, R. D. M. Page; http://taxonomy.zoology.gla.ac.uk/rod/cpw.html) was utilized to determine the total possible topologies for the set of taxa. The tree topology and branch lengths that have the greatest likelihood of generating the data were determined (Strimmer & von Haeseler, 1996). Gene tree topologies were evaluated by the one-sided and two-sided Kishino–Hasegawa methods implemented in TREEPUZZLE version 5.1 (Strimmer & von Haeseler, 1996). Log-likelihood, likelihood ratios and standard errors (SE) were calculated for each gene alignment and set of trees. Confidence sets were defined by the upper and lower bounds of an approximate 95% confidence interval (−1-96 × SE and +1-96 × SE).

Phylogenetic speciation. 18S rDNA gene NJ trees were constructed from distance matrices produced by GTR + G + I (see above for parameter values) and Kimura’s two-parameter (K2P) (Kimura, 1980) nucleotide substitution models. K2P is a special case of GTR: it assumes transitions have one rate and transversions have another; it also assumes nucleotide base frequencies are equal (i.e. πA = πC = πG = πT) (Kimura, 1980). NJ trees were calibrated with an evolutionary rate of 1-26 × 10−16 substitutions per site per lineage per year, which is based on the fungal fossil record (Berbee & Taylor, 2001). Times of divergence were determined for each node, as described previously (Nei et al., 2001). ITS1–5-8S rRNA–ITS2 divergence times were estimated as described previously (Kasuga et al., 2002). Poisson correction distances were determined for DHFR as described previously (Nei et al., 2001). Poisson correction corrects for multiple hits and assumes (1) equality of substitution rates among sites and (2) equal amino acid frequencies. These distances were utilized to construct a NJ tree, which was linearized and calibrated using 140 million years as the time of divergence of C. albicans and Sac. cerevisiae (Berbee & Taylor, 2001).

RESULTS AND DISCUSSION

P. murina has a distinct genome

Soon after the discovery of P. murina outbreaks in SCID mice (Roths et al., 1990; Shultz et al., 1989; Walzer et al., 1989), its genome was observed to be distinct from other Pneumocystis species (Stringer & Cushion, 1998; Weinberg & Durant, 1994). To illustrate the extent of these differences, chromosome-sized DNA bands of P. murina, P. carinii and P. wakefieldiae were separated by CHEF electrophoresis, a pulsed-field gel technique that produces distinct electrophoretic profiles (karyotypes). P. murina organisms produce a karyotype of 17 bands ranging in size from 634 to 309 kb (Fig. 2). By contrast, the karyotype of P. wakefieldiae separates into 14 bands ranging in size from 660 to 308 kb, while P. carinii produces 15 bands ranging in size from 679 to 308 kb. Summation of the bands (including those that are likely to represent co-migrating chromosomes; shown as black bands in Fig. 2) reveals an estimated genome size of 8-2 Mbp for both P. murina and P. carinii, with a slightly smaller estimate for P. wakefieldiae, 7-7 Mbp.

Besides the difference in karyotype profiles, hybridization of gene probes to the Southern-blotted karyotypes of these organisms demonstrates a lack of concordance. For example, a probe to the subtilisin-like protease gene sequence localizes to a single chromosome band of 580 kb in the P. murina genome (Lee et al., 2000) but is distributed on multiple chromosomes in P. carinii (Lugi et al., 1997). A similar lack of concordance has been reported for the single-copy locus that encodes a sequence (upstream conserved sequence) involved in the control of major surface glycoprotein expression in P. carinii (Lee et al., 2000; S. P. Keely, M. J. Linke, M. T. Cushion & J. R. Stringer, unpublished data).

P. murina isolated from SCID and wild mice are genetically similar

It was shown previously that multilocus enzyme electrophoresis (MLEE) is useful for the investigation of species-level genetic diversity of Pneumocystis organisms (Mazars et al., 1997). In this study, MLEE analysis was performed for Pneumocystis organisms isolated from 22 weaning...
rabbits, 30 rats and 17 mice [13 BALB/c/U42 (BU) hybrid white mice and four outbred U42 white mice]. It was shown that three distinct populations occurred in rats, but only one was found in mice and one in rabbits (Mazars et al., 1997). This suggested that organisms isolated from mice and rabbits have very little or no genetic diversity.

To further investigate the genetic diversity in mice, we amplified and sequenced a portion of the mtrRNA(LSU) locus from a wild mouse putatively infected with P. murina. A comparison of 210 nt of this sequence (‘wild-type’) and the homologous region from a SCID mouse (Peters et al., 1994) indicated that there was one G→A substitution and four one-nucleotide indels between them. A second comparison between the wild-type sequence and another P. murina sequence (GenBank accession no. AF257179) showed that there were one T→C substitution, two G→A substitutions and three one-nucleotide indels. Thus, the wild-type sequence was >98 % identical to those from two laboratory mice. This high level of similarity is indicative of strain-level but not species-level variation (Stringer, 1996). MLEE and sequence analysis taken together suggest that one species propagates in mice. However, these data do not exclude the possibility of discovering additional Pneumocystis species in mice.

Analysis of the 18S rRNA locus of P. murina

The 18S rRNA gene of P. murina contained 2179 nt and a composition of 27·1 % A, 28 % T, 19·3 % C and 25·6 % G. These percentages are the same as those in the three other Pneumocystis species. The region spanning nucleotides 1774–2165 is occupied by a 391 bp group I intron. The composition of the intron is slightly different from the coding region: 29·9 % A, 27·0 % T, 17·6 % C and 25·4 % G. The intron is located at the same site as it is in P. carinii (Liu & Leibowitz, 1993). It is 89·4 and 78·8 % identical to the introns in P. carinii (Liu & Leibowitz, 1993) and ferret Pneumocystis, respectively. Interestingly, the intron is not present in the 18S rRNA gene of P. wakefieldiae and P. jirovecii (Cushion et al., 1993b; Liu et al., 1992; Ortiz-Rivera et al., 1995). The variability with respect to the presence of this intron shows that P. murina is different from P. wakefieldiae and P. jirovecii. However, the intron information is difficult to use as an index of degree of divergence because the frequencies of intron insertion and deletion are not known.

Table 2 shows that the coding region of the P. murina 18S rRNA gene was most similar to 18S rRNA genes in P. carinii (16 nucleotide substitutions between the sequences) and P. wakefieldiae (22 substitutions). By contrast, the mean number of nucleotide substitutions for all Pneumocystis was 38 ± 4·33 SE (range, 16–51). Thus, the P. murina 18S rRNA gene is most closely related to its orthologues in the two Pneumocystis species found in rats. At 38, the average number of nucleotide substitutions for all Pneumocystis is substantially greater than the mean for Taphrina (23·6 ± 2·98 nucleotide substitutions per gene, range 10–38) and

Fig. 2. Schematic of karyotypic profiles of P. carinii, P. wakefieldiae and P. murina. Organisms were purified from the lungs of immunosuppressed rodents and embedded in low-melting-point agarose, and their DNA was separated using CHEF electrophoresis at 3·8 V cm⁻¹ with a 50 s initial pulse and 100 s final pulse as reported previously (Cushion et al., 2001). Sizes of the DNA bands in each karyotype profile were determined by linear regression based on the migration distance of each of the bands of the lambda size markers and their known size (48·5 kb concatamers). The mean migration distances from three different gels were used to determine the sizes of the Pneumocystis bands and their lambda size markers. P. murina organisms were obtained from C3H mice from Jackson Laboratories and Surfactant Protein A knockout mice in a Black Swiss background (gift from Dr Michael Linke, University of Cincinnati) (Linke et al., 2001).
several times greater than Saccharomyces \((7.2 \pm 1.55\) nucleotide substitutions per gene, range 0–18). Similarly, the number of substitutions between the three Pneumocystis types found in rodents is comparable or greater to that exhibited by half of the Taphrina species and most of the Saccharomyces species.

**Divergence of the P. murina 18S rRNA gene cannot be attributed to accelerated evolution and is indicative of overall genomic divergence in the genus**

To gauge the significance of these 18S rRNA substitutions, two avenues were explored. First, the possibility that the P. murina 18S rRNA gene might have evolved faster was tested. Tajima’s relative rate test (Tajima, 1993) was devised to detect such a situation, should it occur. The test operates on the proposition that when divergence rates are constant within a genus, two species in that genus will be approximately equally diverged from a distant outgroup species, such as one from a different genus. To conduct the test, pairs of Pneumocystis 18S rRNA coding sequences were compared to an outgroup sequence provided by Taphrina and Sch. pombe. Table 3 shows that each pair of Pneumocystis sequences exhibited approximately equal divergence from the outgroup sequence. Hence, the test provided no indication that the rate of change in the 18S coding sequences is different among species in the genus Pneumocystis.

The second avenue to gauge the significance of the 18S rRNA data was to examine other areas of the genome. Seven other loci were subjected to distance analysis. The distance values obtained mirrored those seen at the 18S rRNA locus (Table 4). Thus, the divergence of P. murina from other members of the genus occurs throughout the genome to an extent commensurate with what would be expected for a separate species.

**P. murina formed a monophyletic clade with P. carinii and P. wakefieldiae**

An NJ tree was constructed from an alignment of the 18S rDNA sequences of five Pneumocystis taxa, seven Taphrina taxa, Sch. pombe, C. albicans and Sac. cerevisiae (Fig. 3A). As expected, the archiascomycetes (Taphrina taxa) and

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<td>0·0046</td>
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<td>9</td>
<td>8</td>
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<tr>
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<td>0</td>
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<td>18</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table 2. Matrix for Pneumocystis, Taphrina and Saccharomyces species**

For each matrix, upper right are pairwise p-distances and lower left are the number of nucleotide differences for each pair of sequences. Pneumocystis species: PM, P. murina; PC, P. carinii; PW, P. wakefieldiae; PF, ferret Pneumocystis; PJ, P. jirovecii. Taphrina species: TN, T. nana; TC, T. carnea; TR, T. robinsoniana; TO, T. communis; TU, T. ulmi; TW, T. wiesneri; TV, T. virginica. Saccharomyces species: Sklu, Sac. kluveri; SM, Sac. mikatae; SB, Sac. bayanus; SP, Sac. paradoxus; Sca, Sac. castellii; SC, Sac. cerevisiae; Skud, Sac. kudriavzevii.
Table 3. Tajima’s relative rate test for the Pneumocystis 18S rRNA gene

<table>
<thead>
<tr>
<th>Outgroup</th>
<th>P. murina versus*</th>
<th>Nucleotide substitutions†</th>
<th>Probability‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. wiesner</td>
<td>P. carinii</td>
<td>5/9</td>
<td>0.285</td>
</tr>
<tr>
<td>P. wakefieldiae</td>
<td></td>
<td>8/9</td>
<td>0.808</td>
</tr>
<tr>
<td>P. jirovecii</td>
<td></td>
<td>15/19</td>
<td>0.493</td>
</tr>
<tr>
<td>Ferret Pneumocystis</td>
<td></td>
<td>15/20</td>
<td>0.398</td>
</tr>
<tr>
<td>Sch. pombe</td>
<td>P. carinii</td>
<td>5/8</td>
<td>0.405</td>
</tr>
<tr>
<td>P. wakefieldiae</td>
<td></td>
<td>7/10</td>
<td>0.467</td>
</tr>
<tr>
<td>P. jirovecii</td>
<td></td>
<td>12/18</td>
<td>0.273</td>
</tr>
<tr>
<td>Ferret Pneumocystis</td>
<td></td>
<td>17/15</td>
<td>0.724</td>
</tr>
</tbody>
</table>

*Mouse Pneumocystis was compared to four different Pneumocystis populations.
†The number of 18S rRNA nucleotide substitutions for each pair of species compared, relative to the outgroup. To the left of the solidus is the number for P. murina; to the right is the number for the other species.
‡The chi-square statistic probability of rejecting the molecular clock (i.e. null hypothesis). Probability values of less than 0.05 are significant.

hemiascomycetes (C. albicans and Sac. cerevisiae) formed separate clusters. Pneumocystis taxa formed a cluster between these two. Hence, monophyly was strongly supported for three groups: Pneumocystis, Taphrina and the hemiascomycetes.

Within the Pneumocystis group, P. murina formed a monophyletic clade with the two other Murinae (i.e. Pneumocystis species found in rats and mice) Pneumocystis, P. carinii and P. wakefieldiae (bootstrap value, 99 %). Within the Murinae clade, P. murina had an affinity for P. carinii (bootstrap value, 71 %). Branch lengths in the Murinae clade were similar to those of Taphrina, as would be expected from the distance values presented above.

To explore the reproducibility of these relationships, additional phylogenetic trees were constructed. An NJ tree was derived from the entire 18S rRNA locus sequence and consisted of the same five Pneumocystis taxa shown in Fig. 3(A) and many additional Ascomycota representatives, such as 15 euascomycete species (e.g. Neurospora crassa) and four hemiascomycete species (e.g. C. albicans and Sac. cerevisiae), as described in Berbee & Taylor (2001). Another NJ tree was derived from a portion of the thymidylate synthase gene and included the five Pneumocystis taxa shown in Fig. 3(A), as well as C. albicans, Sac. cerevisiae and three protozoa (Keely et al., 1994; Cushion et al., 2004). As in Fig. 3(A), similar branch patterns and statistical support were seen for Pneumocystis in these two trees (trees not shown). These relationships were confirmed by maximum-likelihood analysis of 56 nucleotide substitution models (see Methods). Thus, all trees suggested that the Murinae clade is monophyletic.

Concordance of gene genealogies

A phylogenetic species is an evolutionary lineage that has a unique combination of DNA orthologue sequences (Taylor et al., 2000). The evolutionary history of phylogenetic species can be depicted by a bifurcating gene tree. The branches can be evaluated by a test of reliability, such as the common bootstrap statistic, and robustness can be assessed by comparing the concordance of topologies of multiple gene trees.

NJ trees were constructed from SODA, DHPS, DHFR, mtrRNA(LSU) and mtrRNA(SSU) gene sequences and compared to 18S rDNA. As expected, P. murina and P. carinii clustered together and 100 % bootstrap support was observed in all trees. This level of significance was also observed for the cluster of P. jirovecii and non-human primate-derived Pneumocystis (Demanche et al., 2001; Denis et al., 2000; Guilhot et al., 2001; Hugot et al., 2003). To further increase the signal-to-noise ratio, a supertree was constructed from a concatenated gene alignment. All of the branches had 100 % bootstrap support (Fig. 4).

The trees were subjected to a second statistical test using the Kishino–Hasegawa (KH) method implemented in TREEPUZZLE version 5.1 software (Strimmer & von Haeseler, 1996). This test compares the log-likelihood differences between all possible trees and the best tree (i.e. the tree with

Table 4. Pairwise distances (%) of seven Pneumocystis genes

<table>
<thead>
<tr>
<th>Species compared</th>
<th>SODA</th>
<th>DHPs</th>
<th>DHFR</th>
<th>mtrRNA</th>
<th>AROM</th>
<th>TS*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nt</td>
<td>aa</td>
<td>nt</td>
<td>aa</td>
<td>nt</td>
<td>aa</td>
</tr>
<tr>
<td>P. murina and P. carinii</td>
<td>16</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>P. murina and P. jirovecii</td>
<td>27</td>
<td>23</td>
<td>15</td>
<td>19</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>P. carinii and P. jirovecii</td>
<td>33</td>
<td>25</td>
<td>16</td>
<td>18</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>P. jirovecii and primate†</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>19</td>
<td>21</td>
</tr>
</tbody>
</table>

NA, No sequence available.

*TS, Thymidylate synthase.
†P. jirovecii was compared to non-human primate-derived Pneumocystis from owl-monkey and macaque.
Phylogenetic tree depicting the relationships between *Pneumocystis* species and among higher fungi. (a) The tree was constructed using the NJ method, pairwise deletion and Kimura two-parameter (K2P) in MEGA version 2.1 software (Kumar et al., 2001). One-thousand bootstrap replications were performed and the values (expressed as percentages) are shown at the nodes. Bar indicates K2P distance. (b) The tree was linearized and divergence times were calculated as described previously (Nei et al., 2001) using an evolutionary rate of 1.26% substitution per site per 100 million years (Berbee & Taylor, 2001).

These data showed that all of the loci analysed are diverged from each other to the same extent. Such concordance of gene trees is indicative of genetic isolation, which leads to the formation of species.

**Phylodating *P. murina***

The rRNA locus has been used to estimate the time that various fungi diverged from each other. This locus is particularly useful for this purpose because it contains regions that evolve at different rates, thereby allowing comparisons above and below the genus level. For example, there is a 10- to 20-fold difference in the rate of nucleotide substitution between ITSs and the 18S rRNA locus (Kasuga et al., 2002). In order to determine the divergence times for *P. murina*, the branch lengths in Fig. 3(A) were re-estimated under the assumption of a molecular clock (Takezaki et al., 1995). This re-estimate produced a ‘linearized tree’, shown in Fig. 3(B). The molecular clock appeared to have no significant effect on the topology of the tree. The linearized tree was calibrated to time by using a rate of 1.26 × 10−6 substitutions per site per lineage per year, which has been proposed to be the rate for fungi (Berbee & Taylor, 2001). Times of divergence were determined for each node utilizing the MEGA program, as described previously (Nei et al., 2001). As an internal control, the divergence time was calculated for *C. albicans/Sac. cerevisiae* to be 142 million years, which agrees with a previous estimate (Berbee & Taylor, 2001).

As shown in Fig. 3(B), *Pneumocystis* appears to have diversified approximately 100 million years ago. *P. murina* appears to have split from *P. carinii* about 39 million years ago. The deeper position of the *P. wakefieldiae* branch suggests that it may have evolved millions of years prior to this time. To test these estimates, an NJ tree of DHFR was constructed and linearized utilizing a calibration point of 140 million years for *C. albicans/Sac. cerevisiae* (tree not shown). As observed for the 18S rRNA gene, *P. murina* split from *P. carinii* approximately 33 million years ago.

The evolution of *P. murina* may be beyond the resolution of individual genes such as the 18S rRNA gene because they are slow-evolving (Philippe et al., 1994). To circumvent this possibility, additional approaches were utilized to calculate divergence times. One way is to compare loci that are known to undergo rapid evolution. Fungal ITSs evolve faster than adjacent rRNA genes and are useful for determining divergence dates below the genus level (Kasuga et al., 2002). To this end, the ITS1–5.8S rRNA–ITS2 (ITS–5.8S) region of *P. murina* was analysed by aligning it individually to ITS–5.8S of *P. wakefieldiae*, *P. carinii*, and macaque *Pneumocystis*. The Kimura two-parameter (K2P) corrected pairwise distance between *P. murina* and *P. carinii* was 0.20. This value was also seen for *P. wakefieldiae*, but greater K2P values were observed for *P. jirovecii* (0.33) and macaque...
Pneumocystis (0–36). These K2P values are larger than those of several cross genera fungal pairs: Histoplasma capsulatum and Blastomyces dermatitidis, Arthroderma in-curatum and Trichophyton rubrum, Erephasus albus and Ascophphaera apis (Kasuga et al., 2002). We next estimated the divergence time for P. murina utilizing the ITS–5′8S as described by Kasuga et al. (2002). Let $T = K/2r$, where $T$ is the divergence time, $K$ is the K2P corrected distance since divergence and $r$ is the evolutionary rate of ITS–5′8S. The mean evolutionary rate for several sister fungi is $1.4 \pm 1 \times 10^{-9}$ substitutions per site per lineage per year (Kasuga et al., 2002). Therefore, using this rate, $P. murina$ split from $P. carinii$ approximately 71 million years ago. The reason this estimate is twice that of the 18S rDNA locus is unclear, but it is known that fungal ITS evolutionary rates can vary several fold (Kasuga et al., 2002). Rate variability is also seen in animals ($3 \times 10^{-9}$ to $8 \times 10^{-9}$) and plants ($1.7 \times 10^{-9}$ to $8 \times 10^{-9}$) (Depes et al., 1992; Richardson et al., 2001). Since the rate ($r$) of Pneumocystis ITS–5′8S is unknown, it was estimated with the equation $r = K/2T$, where $T$ is the divergence time inferred from DHFR and rRNA NJ trees. The mean rate is $2.8 \times 10^{-9}$, which shows that the ITS–5′8S locus evolved 20 times faster than the 18S rRNA gene. Using this rate, $P. murina$ evolved about 36 million years ago, which is consistent with the evolution of its host (Nei et al., 2001).

Another approach to estimate the divergence dates of Pneumocystis species is to examine the evolution of their host mammals. This entails the hypothesis that Pneumocystis species co-evolved with their hosts. Two recent evolutionary studies of Pneumocystis derived from different mammalian orders support this view (Demanche et al., 2001; Hugot et al., 2003). According to fossil records, rats and mice diverged from each other about 14 million years ago. However, since fossil dates provide only a minimum estimate of divergence dates between species, they may not be useful for confirming molecular dates. Molecular data suggest an older split of 30–40 million years ago (Nei et al., 2001). This estimate is remarkably consistent with the rRNA and DHFR values, suggesting that $P. murina$ has been living in its host as a separate species for 30–40 million years. These data suggest that either $P. murina$ is a species or the rate of evolution between 18S rRNA in these fungal genera is different. It is important to note that Pneumocystis has only one gene encoding rRNA (Giuntoli et al., 1994). By contrast, nearly all known fungi have hundreds of rRNA-encoding genes (Bollon, 1982). The relationship of gene number and rate of evolution is not clear, but it is possible that one gene will evolve more rapidly than 100 of them.

**Conclusion**

To summarize, the divergence of the 18S rDNA gene of $P. murina$ is greater than it is among species in other fungal genera that evolved tens of millions of years ago. Divergence is not limited to the 18S rDNA gene, but occurs throughout the genome of $P. murina$. Molecular dating studies confirmed that $P. murina$ is very old, at least as old as its mammalian host, the mouse. Gene trees are concordant, which is consistent with a long period of genetic isolation of $P. murina$. Finally, there is every indication that few Pneumocystis species share the same habitat, since they are host-restricted (Durand-Joly et al., 2002; Giglotti et al., 1993). Thus, even if they could exchange genes, multiple factors prevent this from happening, thereby allowing speciation to occur. Thus, the picture is clear; the genetic divergence and age of $P. murina$ show that it is a phylogenetic species. Therefore, a new name, Pneumocystis murina, is proposed to reflect this new knowledge.

Recognizing species is not a simple matter. However, once sufficient sequence data are available, there are many tools that provide investigators with the means to determine the statistical significance of observed sequence divergence among members of the genus Pneumocystis. The work described above showed that the amount of sequence information needed to obtain statistically significant results exceeds that recommended previously in published guidelines. However, the following steps will provide ample support of proposed new species names. In addition to the International Code of Botanical Nomenclature requirements described elsewhere (Stringer et al., 2001), researchers should submit at least four sequences, one mitochondrial and three nuclear (and all other gene sequences), to GenBank. They should also analyse genes that have been analysed in other species in the genus. This approach will allow further gene tree concordance analysis. The length of the sequence alignments and extent of genetic variation in them should be sufficient to perform these tests. DNA and any other genetic reagents such as DNA clone libraries should be submitted to public culture collections or similar repositories.

**Description of Pneumocystis murina** sp. nov.

Keely, Fischer, Cushion & Stringer

Pneumocystis murina (mu.ri’na. L. adj. murina murine, of the mouse, after the host in which the organism is found, Mus musculus).

Formerly known as Pneumocystis carinii f. sp. muris (Anonymous, 1994).

Non-filamentous yeast-like organisms (trophic forms) resident in the pulmonary alveoli of Mus musculus. Extracellular and adhere to Type I pneumocytes of the alveolar lumen with clusters of admixed presumptive developmental stages extending into the alveolar lumen. The vegetative cells (trophic forms), measuring 1–5 μm, are uninucleate, of irregular shape, thin-walled and composed of two plasma membranes. Asc (cysts), measuring 5–8 μm, are thick-walled, globose, with two plasma membranes and contain eight round to ovoid ascospores, each 1–2 μm; when empty, they appear faliform or irregular. P. murina is morphologically indistinguishable at the light microscopic level.
level from *Pneumocystis* species that reside in other mammalian lungs, but ultrastructural studies show the filopodia of *P. murina* to be thinner and more abundant than those of *Pneumocystis* from rabbits (Dei-Cas et al., 1994; Nielsen et al., 1998).

*P. murina* is very different at the DNA sequence level from other *Pneumocystis* species. *P. murina* 18S rRNA gene sequences are most similar to those from *P. carinii* and *P. wakefieldiae*, with a 0-9 and 1-1% divergence versus a 2-5 and 1-8% divergence with the same sequences from *P. jirovecii* and *Pneumocystis* from ferret. DNA sequences from regions in the genes of the *P. murina* SODA diverged from those of *P. carinii* by 16% and *P. jirovecii* by 27%; DHPS by 6 and 15%; DHFR by 17 and 31%; mtrRNA(LSU) by 8 and 20%; mtrRNA(SSU) by 10 and 18%; thymidylate synthase by 6 and 21%; ITS regions within the nuclear rRNA locus by 28 and 45%; and in the pentavalent AROM synthase by 6 and 21%; *mtrRNA* regiones intra nuclearem ribosomalem DNA mutandae sunt ut exempla

The type strain is ATCC PRA-111T (＝CBS 114898T). Extracted from lungs of 6- to 8-week-old SCID mice (C3SnSmn.CB17-PrkdcSCID/J, The Jackson Laboratory, Bar Harbor, Maine). Cryopreserved samples are stored at the Cincinnati Veterans Affairs Medical Center, Cincinnati, OH.

**Latin diagnosis of Pneumocystis murina sp. nov. Keely, Fischer, Cushion & Stringer**

Non filiosae similes fermento formae (formae trophicae) quae habitant in alveolis pulmonis Mus musculus. Extra-cellulares et haestae in Typi Primi pneumocystis alveolaris luminis cum corymbis admixtis progrединium formarum extendentium in alveolae lumen. Cellae holitariae (formae trophicae), 1–5 μm, sunt unincusculae, irregulares, tenuitunicatae, consistae de duabus membranis plasmaticis. Ascii (cysti), 5–8 μm, sunt crassitunicati, globosi, cum duabus membranis plasmaticis, et continent octo rotundos ad ovatiles ascospores, quibque 1–2 μm; vacui, apparent falcoformi aut irregulares. *P. murina* est morphologiciter inspectandus, facil microscopio visus, a Pneumocystis qui in pulmonibus mammalium habitat, sed studia ultrastructuralia demonstrant filopodia *P. murina* esse tenuioria et abundanter quam filopodia Pneumocystis e leporibus (Dei-Cas et al., 1994; Nielsen et al., 1998).

*P. murina* est dissimilior ordine DNA quam aliae Pneumocystis speciae. *P. murina* 18S rRNA ordines genium erant similimiae illis a *P. carinii* et *P. wakefieldiae*, cum 0-9% et 1-1% differentia versus 2-5% et 1-8% differentia cum codem ordine a *P. jirovecii* et Pneumocystis ferretiae. DNA ordines a regionibus in *P. murina* dismutatae manganisicofactorati superoxidi erant dissimiles ab illis *P. carinii* a 16% et *P. jirovecii* a 27%; dihydropteroti synthasi a 6 et 15%; dihydrofolati reductasi a 17 et 31%; mitochonldrals magni subunitatis RNA a 8 et 20%; mitochondrialis parvi subunitatis ribosomalis RNA a 10 et 18%; thymidylati synthasi a 6 et 21%; internae transcriptae sepaerantes regiones intra nuclearem ribosomalem DNA locum a 28 et 45%; et in pentaventali AROM geni, a 7 et 11%, propriae. *P. murina* et *P. carinii* habere intronum in 3′ regione 18S rRNA genis, sed *P. wakefieldiae* caret hunc intronum (Edman et al., 1988; Liu & Leibowitz, 1993).

**TYPUS:** Consociatae Civitates Americae, Cincinnatensis, OH. Extractae e pulmonibus sex–octo hebdomadis SCID muris (C3SnSmn.CB17-PrkdcSCID/J, Officina lacsoni, Vectis Portus, Maine). Curae compagium tradidae sunt ut exempla cryoservat Collectione Americano Culturarum Typarum (numerus accessionis ATCC PRA-111T). Exempla cryoservata servata sunt ad Medium Medicum Reurn Veteranorum Cincinnatensis, Cincinnatensis, OH.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


