Phialosimplex, a new anamorphic genus associated with infections in dogs and having phylogenetic affinity to the Trichocomaceae

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Anamorphic members of the ascomycete family Trichocomaceae including Aspergillus, Penicillium, Paecilomyces, Geosmithia and Sagenomella have been reported from infections in canines. Six clinical isolates (five associated with infections in canines and one from a human source) demonstrated simple phialides producing conidia in long chains and were investigated for their potential relationship to Sagenomella chlamydospora, a known agent of canine disseminated mycosis. Phylogenetic analyses of internal transcribed spacer (ITS) and small subunit (SSU) region sequences revealed that all of the canine-associated isolates were distinct from Sagenomella species. The new anamorphic genus and species Phialosimplex caninus is described to accommodate the clinical isolates. Sagenomella chlamydospora and Sagenomella sclerotialis are transferred to the new genus as Phialosimplex chlamydosporus comb. nov. and Phialosimplex sclerotialis comb. nov.

Keywords canine fungal infection, Monocillium indicum, Phialosimplex caninus, Sagenomella chlamydospora, Trichocomaceae

Introduction

Aspergillus, Paecilomyces and Penicillium species are among the most frequently reported etiologic agents of opportunistic disseminated mycoses in dogs [1–4]. These fungi are anamorphic members of the ascomycete family Trichocomaceae (Eurotiales). Geosmithia argillacea (also known as Penicillium argillaceum), another member of the family, was reported recently as the cause of systemic mycosis in a German shepherd dog [5].

In 2003, Gené et al. described the new species Sagenomella chlamydospora for a fungus that was reported initially as a Paecilomyces species and that caused fatal disseminated disease with cervical involvement in a Ger-
obtained from these isolates showed a 91–94% sequence similarity to *S. chlamydospora* (AJ519984 based on the ex-type culture) following a GenBank BLAST search. This lack of sequence similarity prompted a further evaluation of the phylogenetic relationship of our isolates with *S. chlamydospora* and other *Sagenomella* species.

Evidence based on phenotypic and molecular analyses of both the ITS and (SSU) rDNA sequences confirm that our six isolates are distinct. We therefore place them within a new anamorphic genus and species in the family Trichocomaceae for which the name *Phialosimplex caninus* gen. et sp. nov. is proposed. The results also show that *S. chlamydospora* and *S. sclerotialis* belong to this genus and thus the new combinations of *Phialosimplex chlamydosporus* (Gené & Guarro) Sigler & Gibas comb. nov. and *P. sclerotialis* (W. Gams & Breton) Sigler & Gibas comb. nov. are proposed. A second report will provide more details about the infections associated with this newly described pathogen.

**Materials and methods**

**Isolates and morphology**

Six isolates were sent for characterization to the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center (UTHSC) at San Antonio, TX. Due to their unusual phenotypic characteristics, the isolates were subsequently forwarded to the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, AB, for additional studies. Table 1 provides details on the provenance and phenotypic features for the isolates. Colonial features and growth rates were examined on potato dextrose agar plates (PDA; BD Diagnostic Systems, Sparks, MD) incubated at 30°C and 35°C for 21 days. For assessing tolerance to cycloheximide, isolates were grown on mycosel agar containing 400 μg-l cycloheximide. Media were dispensed into one half of a two-section Petri plate and incubated at 30°C. Color terms and codes are derived from the color standards of Kornerup and Wanscher [12]. Microscopic observations were made from slide culture preparations using cereal agar prepared in-house as the sporulation medium and incubation for 14 or 21 days at 30°C [13].

**Sequencing and analysis**

Sequences were obtained at the Fungal Molecular Laboratory at the University of Nebraska Medical Center, Omaha, NE, and at the UAMH. Sequences of the ITS region were obtained for the six clinical isolates listed in Table 1 and for available isolates of *Sagenomella* species and *Monocillium indicum* as listed in Table 2, using the methods as described by Henry et al. [14] and the sequencing primers ITS1 and ITS4 as described by White et al. [15]. Almost complete SSU rDNA sequences were obtained for two clinical isolates (UAMH 10335 and 10337) and for *S. chlamydospora* (UAMH 10961) following procedures for DNA extraction, amplification and sequencing described previously with minor modifications [13]. DNA was extracted using the E.Z.N.A. SP Fungal DNA kit (United Bioinformatica Inc., Saskatoon, SK). The partial SSU region was amplified using primer pair NS1 and NS8 [15]. Sequencing was done with forward primers NS11mun (5’-GCAAATTACCCAATCCCGAC-3’), NS13mun (5’-TGTTTCTAGGACCGCCG-3’), NS151mun (5’-GAAACTACCA GGTCGACAGC-3’) (developed by K.N. Egger, University of Northern British Columbia, Prince George, BC) and reverse primers NS2, NS4 and NS6 [15] using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an ABI 377 Prism Automated Sequencer (Applied Biosystems). The ITS and SSU sequences were edited using Sequencher ver. 4.8 (Gene Codes Corp., Ann Arbor, MI) and aligned manually with sequences retrieved from GenBank using the sequence alignment program Se-Al v2.0a11 [16]. The SSU dataset included sequences available for *Sagenomella* species and for members of the Trichocomaceae obtained by Endo et al. and Thanh et al. [10,11; Group 1 introns were excluded prior to analysis. Parsimony analyses were performed with the PAUP* v.4.0b10 [17] and the robustness of the resultant trees determined using the full heuristic search option (ITS data) and the fast stepwise-addition bootstrap search option for 1000 resamplings (SSU data) [18]. GenBank accession numbers for isolates newly sequenced are listed in Tables 1 and 2.

**Results**

Parsimony analysis of the ITS and SSU datasets comprising 45 and 43 taxa of the Trichocomaceae, respectively, yielded trees having similar topologies and in which three distinct clades were well supported. Clade A included our clinical isolates, in addition to *S. chlamydospora* and *S. sclerotialis* (SSU only), whereas Clade B included all other *Sagenomella* species, including the type species, *S. diversispora* (Figs. 1 and 2); however the species of *Sagenomella* included within Clade B differed slightly according to the available cultures or sequences. The ITS dataset comprised 627 aligned characters, of which 256 were constant, 305 were parsimony-informative and 66 were parsimony-uninformative; the tree shown was one of 6 equally parsimonious trees (Fig. 1). Within Clade A, the clinical isolates grouped with 100% support, as pertaining to the
Table 1  Provenance and morphological features of Phialosimplex caninus isolates examined

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Specimens</th>
<th>Colony diameter (cm)</th>
<th>Colony color</th>
<th>Conidium size</th>
<th>Conidium shape</th>
<th>Diffusible yellow pigment</th>
<th>Pigment on PDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAMH 9569</td>
<td>Canine (M, 14 y) Lymph node, spleen</td>
<td>2.8 (3.2)</td>
<td>Black</td>
<td>2.2–3.5 × 1.8–2.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAMH 10335</td>
<td>Canine</td>
<td>6.5 (7)</td>
<td>Green</td>
<td>2.5–3.5 × 2.3–3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAMH 10336</td>
<td>Canine Lymph node</td>
<td>2.5 (2.2)</td>
<td>Brown</td>
<td>2.2–3.5 × 1.7–2.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAMH 10738</td>
<td>Canine Bone marrow aspirate</td>
<td>7 (6.2)</td>
<td>White</td>
<td>5.5 (6.8)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UAMH 10936</td>
<td>Canine</td>
<td>6.2 (5.6)</td>
<td>Red</td>
<td>2.2–3.5 × 1.8–2.3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The presence of introns was detected in five SSU sequences and these were removed prior to phylogenetic analysis. The sequences for the two clinical isolates, UAMH 10335 and 10337 (2084 nucleotides [nt]) (Table 1), and G. griseoviridis (GenBank AB024591) had a group 1 intron of 357 nt corresponding to subunit position 1165 in a SSU sequence of Saccharomyces cerevisiae (GenBank J01695) [20] and to position 943 in a sequence of Escherichia coli (GenBank J01353) [19] and to position 943 in a sequence of Saccharomyces cerevisiae (GenBank J01695) [20]. Two S. diversispora sequences (AB024588 and AB024589) had a group 1 intron of 345 nt at position 564 in a SSU sequence of E. coli. Intron sequences were lacking in the sequences for S. chlamydospora (UAMH 10961 ex-type culture) and for S. sclerotialis AB024592 (CBS 366.77 ex-type culture). More data are required to determine whether the presence of introns has any taxonomic significance within these groups.

Based on molecular analysis, we describe the new genus and species, Phialosimplex caninus gen. et sp. nov., for the clinical isolates and propose the transfer of the species S. chlamydospora and S. sclerotialis to Phialosimplex (see Taxonomy). The morphological characteristics differentiating the three accepted species are summarized in Table 3.
Taxonomy

*Phialosimplex* Sigler, D.A. Sutton, Gibas, Summerbell & Iwen gen. nov.

Mycobank MB513392


**Species typica:** *Phialosimplex caninus* Sigler, D.A. Sutton, Gibas, Summerbell & Iwen sp. nov.

Colonies are pale, white, cream to yellowish white. Hyphae are narrow, branched, septate. Conidiogenous cells are simple phialides borne laterally on the vegetative hyphae or occasionally on short, unbranched conidiophores. Conidiogenous cells are simple phialides (mostly monophialidic) that sometimes proliferate to form a second opening (polyphialidic). Phialides are narrow, cylindrical to slightly swollen at the base or below the midpoint and taper to a narrow neck with indistinct collarette. Conidia are borne in long chains or aggregate in heads and are hyaline, smooth, subglobose, pyriform, obovoid or ovoid with a truncate base. Chlamydospores and sclerotia are absent or present. A teleomorph is unknown. *Phialosimplex* is an anamorphic genus morphologically resembling *Sagenomella* and placed within the order Eurotiales, family Trichocomaceae.

**Phialosimplex caninus** Sigler, D.A. Sutton, Gibas, Summerbell & Iwen sp. nov.

Table 1. Fig. 3 A–C and Fig. 4 A–E.

<table>
<thead>
<tr>
<th>UAMH No.</th>
<th>Species name (Type status)</th>
<th>Source</th>
<th>Location</th>
<th>Other collection numbers</th>
<th>GenBank no. ITS (SSU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1499</td>
<td><em>Monocillium indicum</em> (T)</td>
<td>Soil</td>
<td>India</td>
<td>IMI 62202</td>
<td>GQ169328</td>
</tr>
<tr>
<td>929</td>
<td><em>Sagenomella diversispora</em></td>
<td>Soil</td>
<td>Netherlands</td>
<td>CBS 354.36 MUCL 9029</td>
<td>GQ169318</td>
</tr>
<tr>
<td>1419</td>
<td><em>Sagenomella diversispora</em> (T, <em>Paeonulomyces diversispora</em>)</td>
<td>Soil</td>
<td>Canada</td>
<td>DAOM 87662 CBS 430.67</td>
<td>GQ169319</td>
</tr>
<tr>
<td>1655</td>
<td><em>Sagenomella sp.</em></td>
<td>Air</td>
<td>USA</td>
<td></td>
<td>GQ169320</td>
</tr>
<tr>
<td>2873</td>
<td><em>Sagenomella diversispora</em></td>
<td>Soil</td>
<td>Canada</td>
<td></td>
<td>GQ169321</td>
</tr>
<tr>
<td>2888</td>
<td><em>Sagenomella striatispora</em></td>
<td>Soil</td>
<td>Canada</td>
<td>CBS 394.69 IMI 148005</td>
<td>GQ169327</td>
</tr>
<tr>
<td>2890</td>
<td><em>Sagenomella humicola</em> (T, <em>Paeonulomyces humicola</em>)</td>
<td>Soil</td>
<td>Canada</td>
<td>IMI 113166 ATCC 18506</td>
<td>GQ169323</td>
</tr>
<tr>
<td>4873</td>
<td><em>Sagenomella sp.</em></td>
<td>Wood</td>
<td>Canada</td>
<td></td>
<td>GQ169324</td>
</tr>
<tr>
<td>9571</td>
<td><em>Sagenomella sp.</em></td>
<td>Plant root</td>
<td>USA</td>
<td>CBS 113280</td>
<td>GQ169325</td>
</tr>
<tr>
<td>10961</td>
<td><em>Sagenomella chlamydospora</em> (T)</td>
<td>Canine</td>
<td>Spain</td>
<td>CBS 109945 IMI 387422</td>
<td>(GQ169327)</td>
</tr>
</tbody>
</table>

Abbreviations: T = Ex-type culture; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; DAOM, National Mycological Herbarium, Ottawa, ON; IMI, CABI Genetic Resource Collection, Egham, UK; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB.
Phialosimplex caninus associated with infections in dogs

Fig. 1 One of six equally parsimonious trees (CI 0.458, RI 0.542, HI 0.771) inferred from maximum parsimony analysis of internal transcribed spacer (ITS) rDNA sequences showing the placement of Phialosimplex and Sagenomella species within the Trichocomaceae. Spiromastix grisea and Eremascus albus are outgroup taxa. Bootstrap values above 50% are shown. The GenBank accession number together with the culture collection number for each isolate is listed if available. GenBank accession numbers for newly sequenced isolates are listed in Tables 1 and 2. T v ex-type culture.

of 2.5 to 2.8 cm (Table 1). Growth rates on PDA at 35°C are nearly equivalent (Fig. 3 middle column). Most isolates (except UAMH 10936) produce a diffusible yellow pigment on PDA or PYE. Colonies on PYE after 21 days at 30°C are similar macroscopically to those on PDA, but the diffusible pigment is darker golden yellow (5B7) to brown

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(6D/E8) (Fig. 3 right column). All isolates were inhibited in the presence of cycloheximide. Conidiogenous cells are simple phialides borne laterally on the vegetative hyphae or occasionally on short, unbranched conidiophores. Phialides are typically monophialidic, sometimes proliferating to form a second opening (polyphialidic), and are narrow, cylindrical to slightly broader at the base or swollen below the midpoint, tapering at the neck and bearing an indistinct collarette (Fig. 4 A–E). They measure 4.5–16 μm (average 11.9 μm) long, 2–4 μm (average...
2.1 μm) wide at the base and 0.7–1.5 μm (average 1.1 μm) at the tip. Conidia are smooth, hyaline, single-celled and produced in long chains or in heads (Fig. 4 B,C). Conidia in chains are subglobose, 2.2–4 μm long by 1.8–3.7 μm wide. Conidia in heads are obovoid, 2–4.5 μm long by 1.5–3.2 μm wide. Ascomata, sclerotia and chlamydospores are absent.

**Phialosimplex chlamydosporus** (Gené & Guarro) Sigler comb. nov. Table 3, Figs. 3D and 4F–G

Mycobank MB513394

*P. chlamydosporus* differs from *P. caninus* by developing abundant solitary chlamydospores borne laterally on short unbranched or branched stalks (Table 3). Chlamydospores are subglobose to globose or obovate, somewhat thick-walled, hyaline and measure 2.8–5.5 μm long by 2.5–4.8 μm wide (Fig. 4F). Phialides are simple, narrow, cylindrical to slightly swollen centrally, and measure 3–14 μm long, 1.5–2.3 μm wide at the base, 1–1.5 μm wide at the tip (Fig. 4G). Conidia are smooth, hyaline and single-celled and produced in chains or in heads. Conidia in chains are oval to pyriform, 2.3–5.8 μm long and 1.7–3 μm wide, while those in heads are 1.8–3.5 μm long by 1.3–2 μm wide. Colonies on PDA after 21 days at 30ºC and 35ºC are white, thin, glabrous with patches of felty aerial mycelium (Fig. 3D). Growth is similar on PYE. No diffusible pigment is produced.

**Phialosimplex sclerotialis** (W. Gams & Breton) Sigler comb. nov.

Mycobank MB513395

Based on the original description [8], *P. sclerotialis* is distinguished from other *Phialosimplex* species by the presence of white globose sclerotia in older cultures and by the absence of chlamydospores (Table 3). *P. sclerotialis* is similar to the other species in having pale colonies, in being thermotolerant, growing faster at 33–36ºC than at 24ºC, and in producing conidia in chains from simple phialides measuring 5–15 μm long, 1.2–1.8 μm wide at the base and 1.0 μm wide at the tip.

**Discussion**

Based on combined morphological and molecular data, this report describes the new anamorphic genus *Phialosimplex* encompassing one new species and two species formerly accommodated in *Sagenomella* (Figs. 1 and 2). *Phialosimplex caninus* associated with infections in dogs...
Fig. 3 Colonial morphologies of *Phialosimplex caninus* and *P. chlamydosporus* are shown after 21 days incubation on PDA at 30°C (left column), PDA at 35°C (middle column) and on PYE and Mycosel agar in biplates at 30°C (right column). Figs. A to C show faster and slower growing isolates. Yellow diffusible pigments are present on PDA for *P. caninus*, but are stronger on PYE.
Phialosimplex caninus associated with infections in dogs

Fig. 4 Microscopic morphologies of *Phialosimplex caninus* (A-E) and *P. chlamydosporus* (F-G) are shown from slide culture preparations using phase contrast microscopy. Figures A-E show conidia in chains and in heads produced from phialides that are narrow, cylindrical to slightly broader at the base or swollen near the midpoint (arrows) and that taper to an indistinct collarette (open arrow, Part E). Phialides are typically monophialidic but sometimes polyphialidic (arrowheads). Isolates shown are UAMH 1037, 10738 and 10936. Figures F-G show chlamydospores, phialides and conidia of *P. chlamydosporus* (UAMH 10961). All scale bars = 2 μm. D and E were taken with an oil immersion objective.
and Sagenomella, phialides are simple and produced laterally on hyphae; are cylindrical to centrally swollen, and may proliferate to form a second opening (polyphialides). In the other genera, phialides are typically borne on complex branched conidiophores (except Torulomyces); are often flask-shaped, and do not proliferate. Although Torulomyces has been distinguished from Penicillium by its solitary phialides, the type species (T. lagena) has been reclassified in Penicillium with a Eupenicillium limonium teleomorph [21]. Similarly, several species of Geosmithia are now classified within Penicillium because Geosmithia sensu stricto comprises anamorphs of Bioneuriacae.

Phialosimplex species differ from Sagenomella species by having lightly pigmented conidia, by producing conidia also in heads and by demonstrating good growth at 35°C. The species accepted here, including P. caninus, P. chlamydosporus and P. sclerotialis, are differentiated by conidial shape, presence of diffusible yellow pigments on PDA and the presence of chlamydospores or sclerotia (Table 3).

In addition to P. chlamydosporus, a species known thus far only from disseminated mycosis in a dog [6,7], P. caninus is recognized here as another potential agent of canine infection. In the case of infection involving P. chlamydosporus, and in four of the five cases of disseminated canine disease reported here for P. caninus, the fungus was isolated from lymph nodes or vertebrae (Table 1). As noted by Zanatta et al. [4], swelling of the lymph nodes is considered an early sign of mycotic discospondylitis in dogs, and the disease usually progresses to involve numerous sites. Based on the relatively small number of cases examined thus far, it is not clear whether P. caninus is associated with a particular type of clinical disease.

We are aware of one published report of probable P. caninus infection. Mackie et al. described a case of granulomatous lymphadenitis and splenitis in a dog in which the fungus involved was identified tentatively as Monocillium indicum [22]. Although the isolate from that case was not available for study, it was described as producing conidia in fragile chains from solitary, centrally swollen phialides. An unpublished photograph available to the senior author (LS) revealed phialides strongly resembling those described here for P. caninus. A potential relationship between P. caninus and M. indicum was evaluated by morphological examination and ITS sequence comparison of the ex-type culture (UAMH 1499, Table 2). M. indicum differs from P. caninus by producing sclerotia and by having phialides that are conspicuously thick-walled and refractile in the lower region, swollen near the middle and tapered to a thin-walled, sinuous apex. Some phialides terminate in a swollen vesicle that collapses resembling a flared cup shaped collarette, but these collapsed structures do not produce conidia. The sequence of M. indicum could not be aligned with members of the Trichocomaceae; therefore it was excluded from the analysis shown here. A Blast search with the ITS sequence revealed closest relatives among the Hypocreaceae but there were no sequences showing high similarity. According to Gams [23], Monocillium species are anamorphs of Niesslia species, which are now placed in the family Niessliaceae of the Hypocreales [24].

The third species in Phialosimplex, P. sclerotialis, may also have potential to cause infection. In the SSU analysis (Fig. 2), the subgroup comprising P. sclerotialis includes five isolates. One is reported in the GenBank record (EU140822) as being from a case of human keratitis in Taiwan. Although the record states that the isolate represents a new species, we were unable to find a published description. The other isolates are from environmental sources, including the ex-type culture (GenBank EU140822, CBS 366.77), obtained from fodder grasses in France, and three isolates from deep sea basin in India. No ITS sequences were available for members of this group, but pairwise comparison of SSU sequences revealed that type isolate differed by only 2 nt from the others.

Currently, the Mycobank [available at: http://www.mycobank.org] and Index Fungorum [available at: http://www.indexfungorum.org] list 13 and 12 Sagenomella species respectively. One species listed on Mycobank appears to be unpublished. With the transfer of S. chlamydospora and S. sclerotialis to Phialosimplex (Clade A, Fig 2) and the placement of Sagenoma viride (anamorph Sagenomella sagenomatis) outside Sagenomella (Clade C), six species are substantiated in our analysis as members of the genus Sagenomella (Clade B). These are S. diversispora, the type species (including its synonym Paecilomyces variabilis), S. griseoviridis, S. humicola, S. striatispora, S. verticillata and the Sagenomella anamorph of T. ocol. Pigmented conidia are found in S. diversispora (roughened and smooth conidia of varying shapes) and S. striatispora (limoniform striated conidia) [8,25]. S. humicola produces brown chlamydospores and hyaline limoniform conidia [8,25]. Faintly pigmented conidia occur in S. griseoviridis and in S. verticillata [8] as well as in T. ocol [9] but the latter two species often demonstrate phialides borne on branched conidiophores. S. griseoviridis has been considered close to S. verticillata and this is confirmed by our SSU data. S. oligospora, having strongly warty conidia [8], was excluded from the Trichocomaceae by Endo et al. [10]. No sequences are available for the three remaining Sagenomella species so their disposition has not been resolved by the present or prior molecular analyses [10,11]; however, none of the species matches Phialosimplex morphologically. Sagenomella bohemica and Sagenomella ryukyuensis produce phialides on branched conidiophores and the latter also expresses a Talaromyces teleomorph [26,27]. Sagenomella alba fails to grow above 27°C [8]. No species of Sagenomella, as
redefined here, has been associated with animal or human infection. This apparent absence of pathogenicity is correlated with an inability to grow at 35–36°C among the isolates examined by us (Table 2) and others [8,9].

In conclusion, *Phialosimplex caninus* and *P. chlamydosporus* are here reported as additional members of the Trichocomaceae having potential to cause opportunistic disseminated mycoses in dogs. While comparison of ITS (or other) sequences is now often very helpful in determining the identity of an unknown isolate, the sequence databases still have many gaps, as we found in this study. Although the closest match to our clinical isolates was a species of *Sagenomella*, the paucity of *Sagenomella* species available for comparison led to the detailed comparison reported here and to the discovery of a novel genus.

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