Chlamydosauromyces punctatus gen. & sp. nov. (*Onygenaceae*) from the skin of a lizard

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Abstract: *Chlamydosauromyces punctatus* is described for an ascomycete producing punctate, rimmed ascospores within ascomata composed of narrow, thin-walled, branched hyphae and an anamorph of alternate arthroconidia. It is known from a single collection obtained from shed skin of a frilled lizard. DNA sequences from the small subunit (SSU) region of the nuclear ribosomal gene were obtained from the lizard isolate and two other taxa and compared with homologous sequences of onygenalean fungi obtained from GenBank. Phylogenetic analysis supports the inclusion of the genus *Chlamydosauromyces* within the *Onygenaceae*. Results also supported the separation of *Arachniotus ruber* from *Kraurogymnocarpa trochleospora* (*Pseudoarachniotus trochleosporus*), a species that at one time was considered synonymous.

Keywords: *Chlamydosauromyces punctatus, Arachniotus rubber, Kraurogymnocarpa trochleospora, Pseudoarachniotus trochleosporus,* lizard.

Introduction

According to Currah's concepts for the Onygenales (Currah, 1985), ascomycetes within the family *Onygenaceae* have globose, oblate or allantoid ascospores that are pitted, punctate or reticulate. Ascospores with equatorial thickenings or grooves are uncommon within the *Onygenaceae* but may be found among members of the *Gymnoascaceae.* For example, ascospores of *Arachniotus ruber* (Tiegh.) J. Schröt. have a shallow groove bordered by distinct rims. Ascospore walls in species of the *Gymnoascaceae*, however, are generally smooth or slightly irregular in surface ornamentation. An ascomycete having ascospores with equatorial rims and puncta was recovered from shed skin of a frilled lizard during a survey of microfungi from healthy reptiles (Paré *et al*., in press). The objective of the survey was to evaluate prevalence of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (Apinis) Currah, recently identified as an agent of cutaneous mycosis in reptiles, on skins of animals without signs of infection (Paré *et al.*, 1997; Nichols *et al.*, 1999; Thomas *et al*., 2002). All onygenalean fungi recovered in culture were identified to species. To place the unidentified ascomycete within the appropriate family of the *Onygenales* and to evaluate its relationship to known taxa, DNA sequences derived from the small subunit operon (SSU) of the nuclear ribosomal rRNA gene were obtained and compared to published and newly determined sequences (Sigler, Hambleton, Flis & Paré, this volume).

Methods

Samples of shed skin, one from the head and the other from distal region of the tail, were collected from a frilled lizard, *Chlamydosaurus kingii* (Gray), housed at the San Diego Zoo, San Diego, CA, and submitted for culture (Paré *et al*., in press). The skin was cut aseptically into 6 pieces and the pieces were planted onto Mycosel agar (MYC, Difco, Detroit, MI). All colonies recovered were identified to genus. Isolates suspected of being onygenalean fungi were identified to species. The frilled lizard isolate described here was deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH) as UAMH 9990.

Colony and microscopic descriptions are based on potato dextrose agar (PDA, Difco) at 30° and 35°C and on cereal agar (CER), oatmeal agar (OAT), Takashio agar (TAK) (all recipes in Kane *et al.*, 1997) at 30°C. Cycloheximide tolerance was evaluated on Mycosel agar. In vitro hair digestion was assessed following the methods described in Kane *et al.* (1997). Colony color terms are used according to Kornerup and Wanscher (1978). Ascomata and ascospores were examined by light and scanning electron microscopy (SEM). SEM samples were fixed in 2.5% glutaraldehyde in Millonig's buffer (Millonig, 1961), pH 7.3 and

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postfixed in 2% osmium tetroxide in the same buffer. After drying to the critical point, the samples were sputter coated with gold and examined with a Hitachi S-2500 (Hitachi, Ltd.,Tokyo, Japan).

DNA extraction, amplification and sequencing methods were as described in Sigler, Hambleton, Flis and Paré (this volume). SSU DNA sequences were determined for UAMH 9990 and two taxa for which sequences were unavailable in GenBank. These were the ex-neotype strain of *Arachniotus ruber* (UAMH 3543 = CBS 194.64; soil, UK, isolated by G.F. Orr, 1961) and the ex-type strain of *Kraurogymnocarpa trochleospora* (*Pseudoarachniotus trochleosporus*; UAMH 10101 = U.S. Dept. of Agriculture Northern Regional Research Laboratory [NRRL] strain NRRL 3715, sandy clay, Dugway, Utah, G.F. Orr, 1964). The sequences were manually aligned with 26 sequences obtained from GenBank for species of the *Onygenales* and allied fungi. Species of the *Eurotiales* served as outgroup. GenBank accession numbers are given in Fig.1. The data matrix was subjected to parsimony analysis using the heuristic search option of PAUP* v. 4.0b8 (Swofford, 1999) with 500 replicates of random stepwise addition of taxa, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Bootstrap percentages used to assess support for the branching topologies were determined from 1000 resamplings of the data set using the full heuristic search option and random sequence addition.

Results

For the three strains newly sequenced, nearly complete SSU sequences were obtained and deposited in GenBank. These were 1738 nucleotides (nt) in length for the lizard isolate, UAMH 9990 (AY177297); 1710 nt for the ex-type strain of *Kraurogymnocarpa trochleospora* UAMH 10101 (AY177295); and 1749 nt for the ex-type strain of *Arachniotus ruber* UAMH 3543 (AY177296). The sequences are complete at the 3' end, finishing with CATTA box that precedes the first internal transcribed spacer region. The SSU data matrix comprised 29 taxa and 1697 aligned characters. Of these, 1482 were constant and 120 were parsimony informative; 34 ambiguously aligned characters were excluded. Parsimony analysis resulted in a single most parsimonious tree (Fig. 1) of 376 steps, with a consistency index of 0.537 and a retention index of 0.699. Results of the bootstrap analysis are shown on Fig. 1.

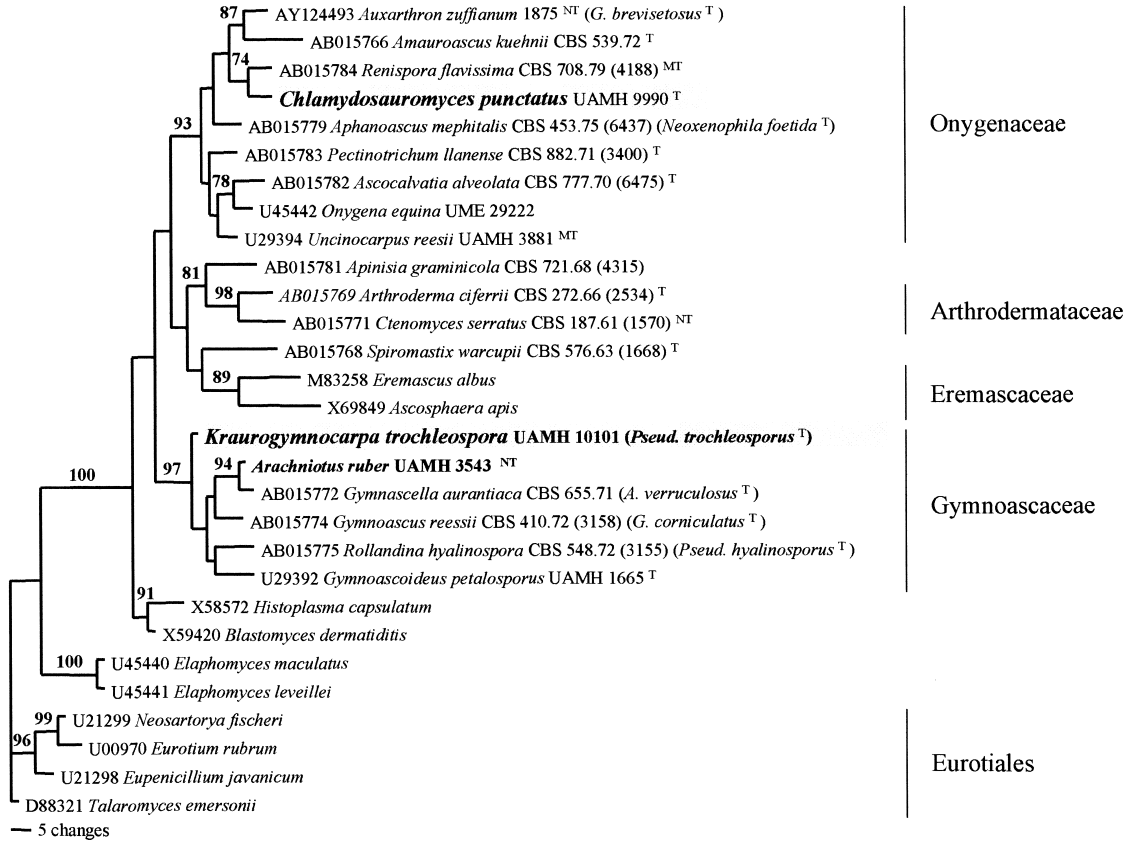
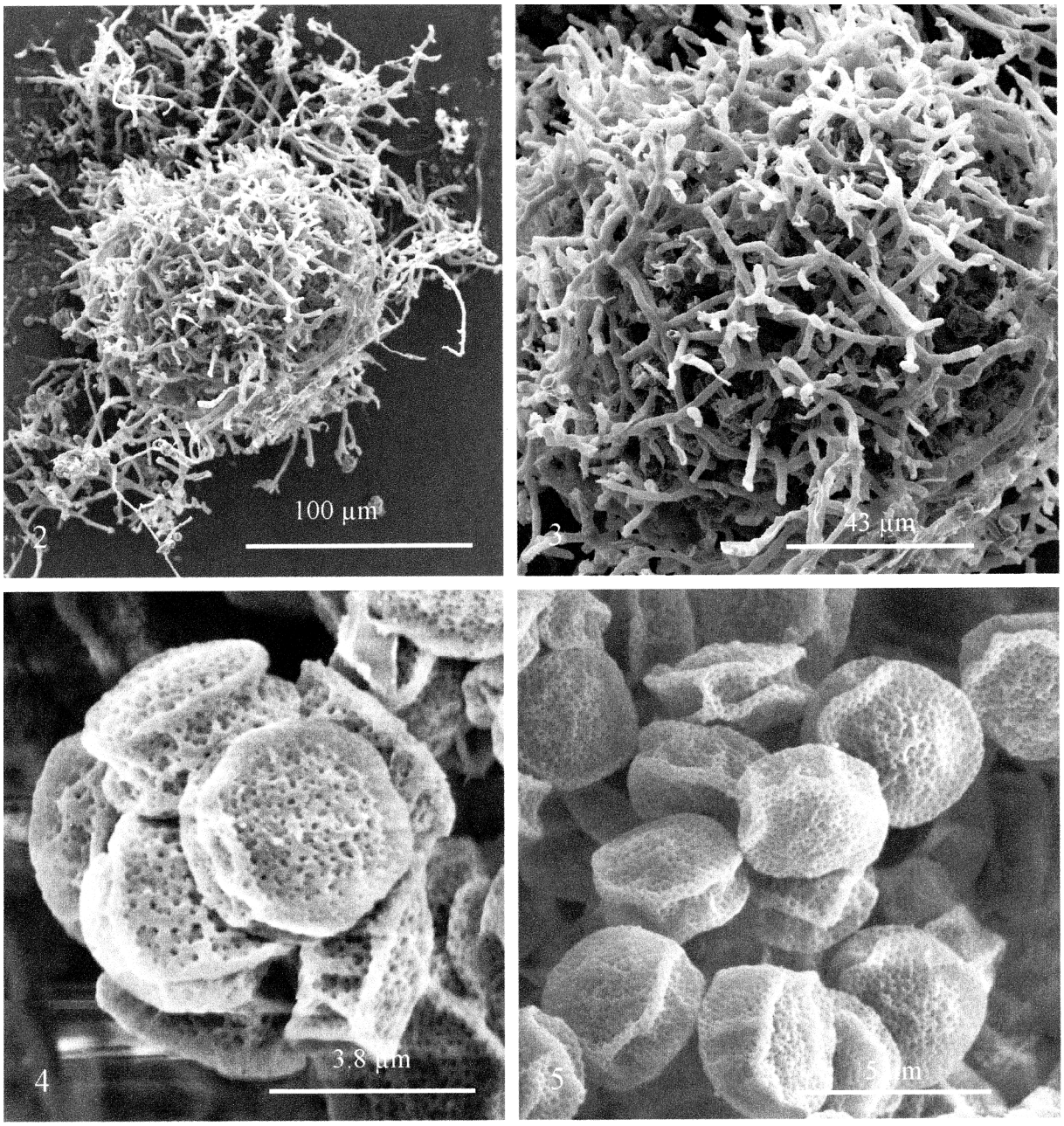
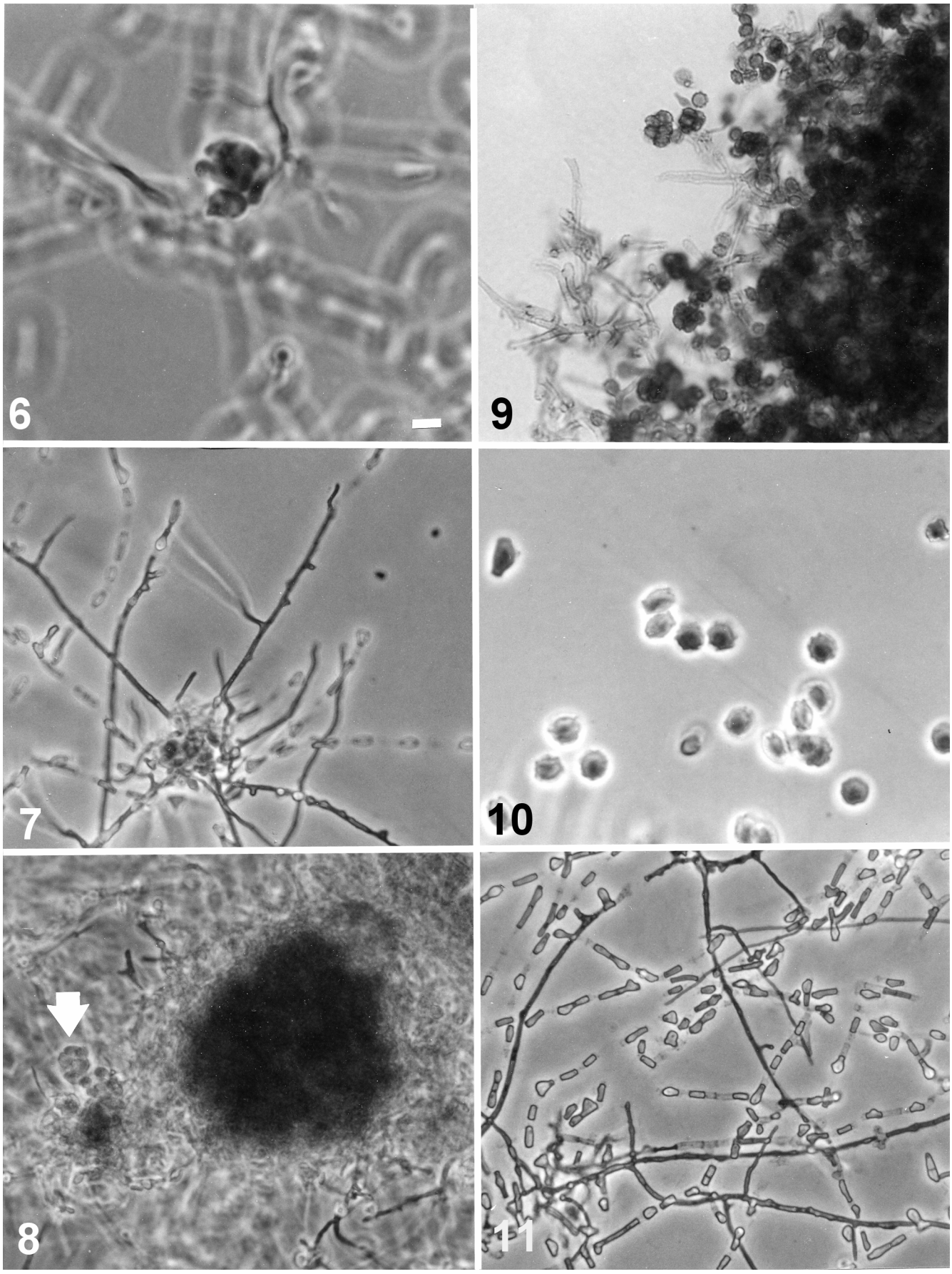


Fig. 1. Maximum parsimony analysis of the SSU data matrix using the heuristic search algorithm of PAUP* v. 4.ob8 yielded a single most parsimonious tree (376 steps, CI = 0.537). Bootstrap values above 70% are given adjacent to the corresponding node. The newly sequenced strains are indicated in bold type and their GenBank accession numbers are AY177297 for UAMH 9990, AY177295 for UAMH 10101 and AY177296 for UAMH 3543. Provenance of other strains, including GenBank accession number and the corresponding culture collection number, is noted on the Fig. Numbers in brackets are UAMH strain deposition numbers. "T" refers to ex-type strain; "NT" to ex-neotype strain; "MT" to mating type strain.



Figures 2 - 5. *Chlamydosauromyces punctatus* viewed by scanning electron microscopy (ex-type strain UAMH 9990). **Fig.** 2. Ascoma (gymnothecium) composed of poorly differentiated, narrow hyphae. **Fig**. 3. Branched, thin walled peridial hyphae. **Figs.** 4 - 5. Oblate ascospores with puncta and two equatorial rims.



Figures 6-11. *Chlamydosauromyces punctatus* viewed by light microscopy (ex-type strain UAMH 9990). **Figs.** 6 - 7. Ascomatal initials and early development of gymnothecium. **Fig.** 8. Immature gymnothecium and ascus (arrow). **Fig.** 9. Thin-walled peridial hyphae and ascospores. **Fig.** 10. Oblate ascospores with two equatorial rims. **Fig.** 11. Slide culture preparation showing alternate arthroconidia..

Figures 12-14. *Chlamydosauromyces punctatus* in culture after 6 weeks at 30°C. **Fig.** 12. Potato dextrose agar.**Fig.** 13. Phytone yeast extract agar (top), Mycosel agar (bottom) showing tolerance of cycloheximide. **Fig.** 14. Gymnothecia forming on Takashio agar

In the phylogenetic analysis, UAMH 9990 grouped with members of the *Onygenaceae*, with *Renispora flavissima* AB015784 as a sister taxon, although bootstrap support for the relationship was low at 73%.These taxa are dissimilar in morphology of both ascospores and anamorphs. *Arachniotus ruber* clustered with *Gymnascella aurantiaca* AB015772, ex-type strain of *Arachiotus verruculosus* with strong bootstrap support (94%) in a clade corresponding to the *Gymnoascaceae*. *Kraurogymnocarpa trochleospora*, formerly considered synonymous with *Arachniotus ruber*, was basal within that clade. The *Onygenales* received strong bootstrap support (100%) and three families of onygenalean fungi, *Arthrodermataceae*, *Gymnoascaceae* and *Onygenaceae*, and the genus *Ajellomyces*, represented by its anamorphs *Histoplasma* and *Blastomyces* were strongly supported as monophyletic groups within the order.

Taxonomy

CHLAMYDOSAUROMYCES Sigler, Hambleton & Paré, *gen. nov*.

Ascomycota, Onygenales, Onygenaceae
Ascomata gymnothecia, discreta, globosa; hyphae peridiales parum distinctae; ramosae, septatae, leves, tenuiter tunicatae, subhyalinae, angustae; appendices elongatae absunt; in initiis cellulae anastomosae et tumidae; asci octospori, evanescentes; ascosporae moniliaceae, nonseptatae, oblatae cum oris aequatorialibus, superficies punctata; anamorphosis alterna arthroconidia cum dehiscentia lytica.

Ascomata gymnothecia discrete, globose, peridial hyphae poorly differentiated, branched, septate, smooth, thin-walled, subhyaline, narrow hyphae; elongate appendages absent; initials of anastomosed, slightly swollen cells; asci 8-spored, evanescent; ascospores moniliaceous, nonseptate, oblate with equatorial rims, surface punctate; anamorph of alternate arthroconidia having lytic dehiscence.

Type species**:** Chlamydosauromyces punctatus Sigler, Hambleton & Paré, sp. nov.

Chlamydosauromyces punctatus Sigler, Hambleton & Paré, *sp. nov*. Figs. 2-13

Ascomata gymnothecia (150) 200 – 600 (900) µm, discreta, globosa; hyphae peridiales parum distinctae; composita de hyphis ramosis, septatis, angustatis, subhyalinis vel flavis, levibus, tenuiter tunicatis, 1.5 – 2.5 µm latis; appendices elongatae absunt; in initiis cellulae anastomosae et tumidae; asci 7 - 10 x 7 – 9 µm , ascosporae flavae in massa, oblatae cum duabus oris aequatorialibus, superficies punctata, 4 – 4.5 x 3 – 3.5 µm.; anamorphosis alterna arthroconidia formantia in hyphis rectis, cylindrica vel irregularia, tumentia in lateribus alteris vel ambis, 4 – 8 x 2 – 4 µm.
Holotypus: UAMH 9990 colonia exsiccata.
Etymology: *Chlamydosauromyces* after the genus of lizard (*Chlamydosaurus*) from which the fungus was isolated; punctatus, after the ascospore wall ornamentation.

Ascomata gymnothecia (150) 200 – 600 (900) µm diam, discrete, globose; peridial hyphae poorly differentiated; composed of branched, septate, smooth, thin walled, subhyaline to yellow, narrow hyphae, 1.5 – 2.5 µm wide; elongate appendages absent; initials of anastomosed, slightly swollen cells; asci 7 - 10 x 7 – 9 µm, ascospores yellow in mass, oblate with two equatorial rims, punctate, 4 – 4.5 x 3 – 3.5 µm; anamorph forming arthroconidia in alternate cells along the length of straight hyphae; arthroconidia cylindrical to irregular with one or both sides swollen, 4 – 8 x 2 – 4 µm.

Colonies on PDA after 22 days at 30°C were 5 cm in diam, greyish yellow (4B3) in the centre and unevenly raised, flat and paler at the periphery, velvety, reverse yellowish brown. After 6 weeks, colonies were 7 cm diam, greyish orange (5B4), with yellow exudate droplets forming centrally and with a diffusing yellow pigment. Colonies on OAT and TAK after in 22 days at 30°C were similar in size (5 cm

diam) and the topography being flat, with thin aerial mycelium. Ascomata were discrete, well developed and abundant on OAT at 22 d, but less so on TAK. By 6 weeks, ascomata were numerous on both media and were often associated with exudate droplets. There was good growth on medium with cycloheximide, no growth at 35°C, and moderate digestion of hairs without perforating bodies, after 14 days.

Discussion

Chlamydosauromyces punctatus is distinguished by ascomata composed of narrow, thin-walled hyphae, punctate oblate ascospores with equatorial rims, an anamorph of alternate arthroconidia, and an ability to digest hairs *in vitro*. In the phylogenetic analysis, it grouped with *Renispora flavissima* Sigler, Gaur, Lichtw. & J.W. Carm., a heterothallic species that is the only member of the genus *Renispora* Sigler & J.W. Carm. (Sigler *et al*., 1982). *Renispora flavissima* has punctate ascospores and ascomata composed of hyaline to yellow thin-walled, narrow hyphae, but ascospores are reniform, and the conidia of its *Chrysosporium* anamorph are large, spiny to tuberculate aleurioconidia formed on stalks.

The ascomata composed of poorly differentiated peridial hyphae and ascospores with distinct rims initially suggested a possible relationship to the genus *Arachniotus* J. Schröt. Two representatives of *Arachniotus* were therefore included in the phylogenetic analysis (Fig. 1). *Arachniotus ruber* (UAMH 3543) has smooth "pulley-wheel" shaped ascospores, i.e., having a shallow equatorial furrow bordered with distinct rims. This was the only species accepted in the genus *Arachniotus* by Currah (1985) who also placed *Pseudoarachniotus trochleosporus* Kuehn & G.F. Orr (UAMH 10101) in synonymy. Udagawa (1997), however, accepted the latter as a distinct species and proposed a transfer as *Arachniotus trochleosporus* (Kuehn & G. F. Orr) Udagawa. Subsequently, the species was redisposed as *Kraurogymnocarpa trochleospora* (Kuehn & G.F. Orr) Udagawa & Uchiy. based on ascospore similarity with the type species of *Kraurogymnocarpa*, *K. lenticulospora* Udagawa & Uchiy. (Udagawa & Uchiyama, 2001). Scanning electron microscopy revealed that ascospores of *K. trochleospora* have two equatorial crests and a lobate-tuberculate ornamentation to the convex surface (Udagawa & Uchiyama, 2001). However, the two species currently accepted in *Kraurogymnocarpa* differ from each other in peridial morphology. *K. lenticulospora* has a well-developed mesh-like reticuloperidium (Udagawa & Uchiyama, 1999) whereas *K. trochleospora* has a poorly differentiated peridium of thin and smooth-walled hyphae (Udagawa & Uchiyama, 2001). Our SSU analysis provides support for the distinction of *Pseudoarachniotus trochleosporus* from *A. ruber*, and for its disposition in a separate genus, but does not clarify the relationship with *K. lenticulospora* because a living culture of the latter was unavailable for molecular analysis. However, the authors did provide a portion of the holotype for morphological assessment.

Chlamydosauromyces punctatus was isolated from skin shed from both the head and tail of an apparently healthy 7-year-old male frilled lizard housed at the San Diego zoo. The source of the fungus is likely bedding or climbing material in the lizard's enclosure, but no attempt was made to reisolate the fungus from the animal's zoo habitat. Two other skin samples obtained from different reptile species and submitted by the San Diego zoo did not yield the same fungus. *Chlamydosaurus kingii* belongs to the *Agamidae*. It is primarily arboreal and is found in Australia in the Northern Territory and Queensland, as well as in Papua-New Guinea.

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