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Original Articles

Isolation and characterization of a new fungal genus and species, *Aphanoascella galapagosensis*, from carapace keratitis of a Galapagos tortoise (*Chelonoidis nigra microphyes*)

D. A. SUTTON*, Y. MARÍN#, E. H. THOMPSON*, B. L. WICKES†, J. FU†, D. GARCÍA#, A. SWINFORD‡, T. DE MAAR§ & J. GUARRO#

*Departments of *Pathology & †Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA, ‡Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas, USA, §Gladys Porter Zoo, Brownsville, Texas, USA, and #Mycology Unit, Medical School, Universitat Rovira i Virgili, Reus, Spain*

A new fungal genus and species, *Aphanoascella galapagosensis,* recovered from carapace keratitis in a Galapagos tortoise residing in a south Texas zoological collection, is characterized and described. The presence of a pale peridium composed of textura epidermoidea surrounded by scarce Hülle cell-like chlamydospores, and the characteristic reticulate ascospores with an equatorial rim separates it from other genera within the Onygenales. The phylogenetic tree inferred from the analysis of D1/D2 sequences demonstrates that this fungus represents a new lineage within that order. As D1/D2 and ITS sequence data also shows a further separation of *Aphanoascus* spp. into two monophyletic groups, we propose to retain the generic name *Keratinophyton* for species whose ascospores are pitted and display a conspicuous equatorial rim, and thereby propose new combinations in this genus for four *Aphanoascus* species.

Keywords *Onygenales, Aphanoascella, Aphanoascella galapagosensis*, Galapagos tortoise, *Keratinophyton*

Introduction

In recent years there have been anecdotal reports of a form of keratitis affecting carapaces (shells) of dry land tortoises kept in captivity and residing in the wild on the Galapagos Islands. The syndrome is commonly seen in tortoises living in zoological gardens in Florida and the Gulf Coast states and may be associated with prolonged exposure to moisture. In these cases, the keratin in the scute sutures of the carapaces turns white and powdery, and is easily scraped away to reveal normal black keratin or underlying bone. It has been hypothesized that the etiology is potentially mycotic.

A wild caught, Volcan Darwin Tortoise, also called a Galapagos Tortoise (*Chelonoidis nigra microphyes*), estimated to be approximately 50 years old and residing in a zoological collection in south Texas, was observed to develop white discoloration in lower areas of the carapace. The lesions were first noted during a summer with significant rainfall. Only one animal in eight was affected. These lesions slowly expanded over the next 3 years across the lower quadrants of the costal scutes, upper quadrants of the marginal scutes and the plastral bridge (Fig. 1). Lesions were most severe along scute sutures, areas of the newest keratin growth. All lesions were situated below the high water mark of the animal's mud wallow. The affected keratin developed an eroded shale-like pattern. Scraping of the crumbling surface revealed healthy keratin or bone underneath. Samples of the scraped material were harvested and submitted to a veterinary diagnostic laboratory for fungal culture.

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Correspondence: Deanna A. Sutton, Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA. E-mail: suttond@uthscsa.edu

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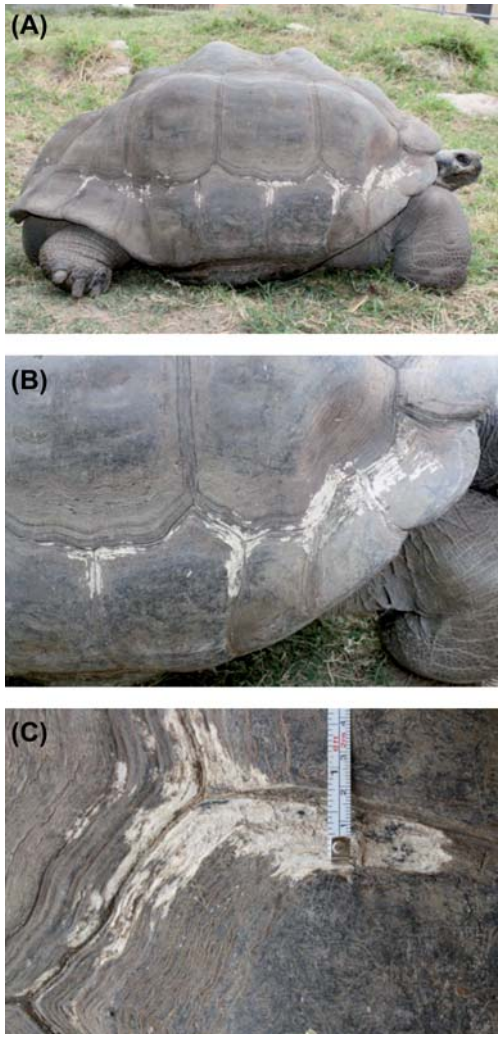

Fig. 1 Lesions on the carapace of a 50-year-old Galapagos Tortoise (*Chelonoidis nigra microphyes*) due to *Aphanoascella galapagosensis*. Extent of lesions (A), close up of right side (B), and depth of erosion (C).

Materials and methods

Fungal isolation and initial identification

The carapace scrapings were inoculated onto Sabouraud dextrose, potato dextrose, Mycobiotic, and dermatophyte test medium agars (Remel, Lenexa, KS) and incubated at 25°C for 3 weeks. Heavy growth of a white to buff-colored fungus was observed on all media. The colony morphology of the isolate was downy to cottony and resembled a dermatophyte, but could not be identified by conventional laboratory methods, and was forwarded to the Fungus Testing Laboratory, Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, TX, for further attempts at identification. There the isolate, Fig. 2, was accessioned into their collection as UTHSC 11-1518 and tentatively identified as an *Aphanoascus* species based on ascomata and ascospore formation resembling those seen in this genus. However, the isolate was subsequently referred to Spain for more in-depth study when a BLAST search of the ITS and D1/D2 sequence data in GenBank failed to return an identification with any significant percent identity.

Fig. 2 Colony of *Aphanoascella galapagosensis* on potato flakes agar after 3 weeks incubation at 25°C, measuring approximately 20 µm in diameter.

Morphologic identification. The fungal isolate was grown on oat meal agar (OA), potato carrot agar (PCA), potato dextrose agar (PDA) and Czapek agar (Cz) plates at 15°C, 25°C and 35°C. Color notations in parentheses are from Kornerup and Wanscher [1]. The fungal structures were measured after 2 months of growth on Cz using lactophenol-stained mounts (Fig. 3). Photomicrographs were obtained with a Leitz Dialux 20 EB microscope. Scanning electron microscopy techniques were described previously by Figueras and Guarro [2].

DNA extraction and sequencing. Template DNA was prepared from a 24 h culture of UTHSC 11-1518 (= R-4747) grown on potato dextrose agar at 30°C as previously described [3]. PCR reactions were then performed in a 50 µl volume using 3 µl of template DNA, 5 µl 10 × PCR buffer, 5 µl of a 10 µM stock solution of each primer (ITS-1 forward primer [4] and NL-4 reverse primer [5,6]), 1.5 µl of 10 mM dNTP (Invitrogen, Carlsbad, CA), and 2.5 U of *Taq* Extender (Fisher Scientific, Pittsburgh, PA). PCR reactions were performed in an Eppendorf Master Thermocycler (Eppendorf) and were run with a temperature profile of 2 min at 94°C followed by 30 cycles of 20 s at 94°C, 20s at 60°C, and 1 min at 72°C. The 30 cycles were followed by 5 min at 72°C.

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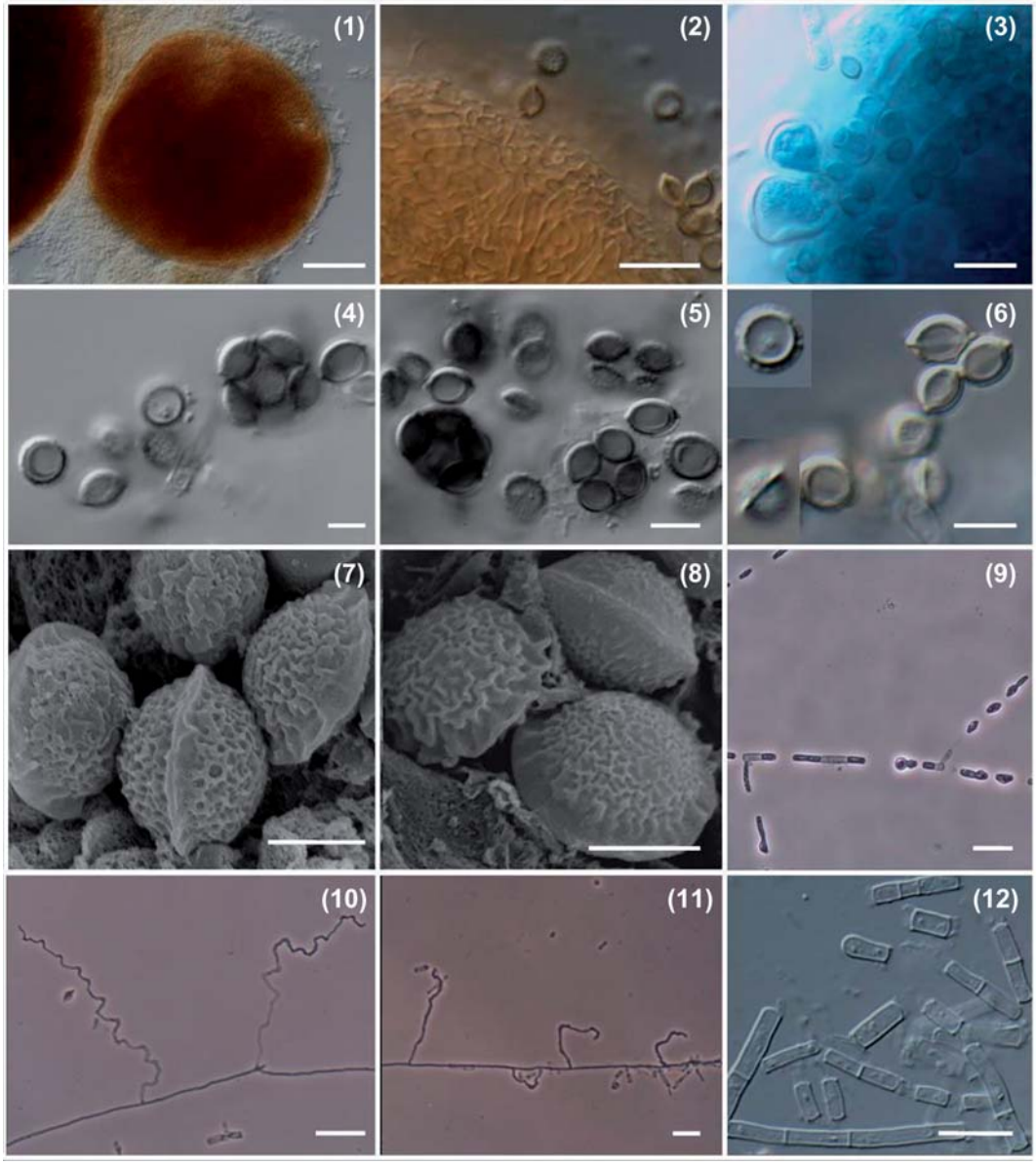

Fig. 3 Microscopic features of *Aphanoascella galapagosensis* (Czapek, 2 months, 25°C). (1) Non-ostiolate, spherical ascoma, bar = 50 µm; (2) *textura epidermoidea*, bar = 10 µm; (3) Hülle cells (chlamydospore-like) covering the ascoma, bar = 10 µm; (4, 5 & 6) asci and ascospores, bar = 5 µm; (7 & 8) scanning electron microscopy of oblate ascospores demonstrating irregular reticulate wall, anastomosing ridges and an equatorial ridge, bar = 2.5 µm; (9) intercalary arthroconidia, bar = 20 µm; (10 and 11) arthroconidia borne on straight primary hyphae or on short loosely curved or sinuous lateral branches, bar = 20 µm; (12) alternating or adjacent cylindrical to slightly barrel-shaped arthroconidia, bar = 20 µm.

PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced on both strands using the two flanking primers (ITS-1 and NL-4), as well as two internal primer runs (ITS-4 and NL-1) [5,6]. Sequencing was performed at the UTHSCSA Advanced Nucleic Acids Core Facility and data were edited using Sequencing Analysis Software v5.3.1 (Applied Biosystems, Foster City, CA).

Sequence analysis. The sequence data were assembled and analyzed using MacVector software (MacVector, Inc, Cary, NC) and then searched using the ITS-1 and ITS-4 primer sequences to delineate the ITS region, as well as the NL-1 and NL-4 sequences to delineate the D1/D2 region. The ITS and D1/D2 regions were then used in separate BLASTn searches of GenBank at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

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Sequence-based identities with a cutoff of 97% or greater and query length of 90% or greater were considered significant.

Alignment and phylogenetic reconstruction. Phylogenetic analyses of the two regions selected for study were performed using the neighbor-joining (NJ) method with the MEGA 2.1 computer program. The NJ tree was constructed using maximum composite likelihood method [7] with the pairwise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1000 replicates.

Nucleotide sequence accession numbers. GenBank nucleotide sequence accession numbers for the case isolate and morphologically similar species are listed in Table 1.

Results

A BLAST search using the ITS region (18S partial, ITS1, 5.8S, ITS2, 28S partial) provided only insignificant hits from GenBank, the top three of which were *Aphanoascus foetidus* (accession# AJ439448.1, 88% identity), *Chrysosporium lucknowense* (accession# AJ131682.1, 88% identity), and *Chrysosporium mephiticum* (accession# AJ131683.1, 87% identity). None of which were considered significant for a conspecific isolate (>97% identity). The D1/D2 region (28S partial sequence) also did not return a significant BLAST hit, with the top three closest identities being *Chrysosporium keratinophilum* (accession# AB359446.1, 94% identity), *Chrysosporium keratinophilum* (accession# AB359445.1, 94% identity), and *Aphanoascus verrucosus* (accession# AB075348.1,

Table 1 LSU and ITS DNA sequences included in the phylogenetic analyses.

		GenBank no.	
Family	Species	LSU	ITS
Arthrodermataceae	*Arthroderma ciferrii*	EF413625	
	Ctenomyces serratus	AY176733	
Gymnoascaceae	*Gymonascus aurantiacus*	AY176747	
	Gymnoascus littoralis	FJ35827	
	Gymnoascus ruber	AY176746	
Onygenaceae	*Amauroascus niger*	AY176706	
	Aphanoascus canadensis		AJ439435
	Aphanoascus clathratus		AJ439436
	Aphanoascus cubensis		AJ439432
	Aphanoascus durus	AB075345	AJ439434
	Aphanoascus foetidus		AJ439448
	Aphanoascus fulvescens	JN941548	AF038357
	Aphanoascus hispanicus		AJ439438
	Aphanoascus keratinophilus		AJ133436
	Aphanoascus mephitalis	AY176725	AJ439439
	Aphanoascus orissi		AJ315843
	Aphanoascus pinarensis		AJ439433
	Aphanoascus punsolae		AJ439440
	Aphanoascus reticulisporus	JN941550	JN943435
	Aphanoascus saturnoideus	AB075347	AJ439442
	Aphanoascus terreus	JN941552	JN943438
	Aphanoascus verrucosus	JN941554	JN943439
	Aphanoascella galapagosensis	JQ864082	JQ864081
	Chrysosporium sp.		AJ439445
	Chrysosporium evolceanui		AJ005368
	Chrysosporium fluviale		AJ005367
	Chrysosporium indicum		AJ439446
	Chrysosporium keratinophilum		AJ131681
	Chrysosporium lucknowense		AJ131682
	Chrysosporium minutisporosum		AJ131689
	Chrysosporium siglerae		AJ131684
	Chrysosporium submersum		AJ131686
	Chrysosporium tropicum		AJ131685
	Chrysosporium zonatum		AJ390393
	Uncinocarpus queenslandicus	AB075358	AB361646
	Uncinocarpus reesii	AY176724	
Trichocomaceae	*Byssochlamys nivea*	AY176750	
	Eurotium herbariorum	AY176751	
Sordariaceae	*Neurospora nigeriensis*	FR774265	

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94% identity). The phylogenetic tree inferred from the analysis of the D1/D2 sequences showed a clear genetic separation between *Aphanoascus* spp. and other genera of Onygenales included in the study (Fig. 4). It also demonstrated that the species of *Aphanoascus* included in the study were grouped in two highly supported clades (98% and 94%, respectively). One clade included those species displaying reticulate-walled ascospores without an equatorial rim, and the other clade consisted of isolates with pitted ascospores with a prominent equatorial rim. The new fungus described here represents a new lineage phylogenetically distant from the two mentioned clades. The ITS phylogenetic tree (Fig. 5) showed a similar topology, confirming that there is insufficient evidence to place the currently accepted *Aphanoascus* spp. into the same genus and that the new fungus is not related with any of the two clades of *Aphanoascus*.

Taxonomy

Aphanoascella D.A. Sutton, Y. Marín, E.H. Thompson et Guarro, gen. nov.

Anamorph: *Malbranchea* sp.
Etym: Similar to *Aphanoascus*
MycoBank: MB 564389

Ascomata superficialia, sphaerica, non-ostiolata, cum chlamydosporae 'hülle' cellulis similis, aurantiaca vel brunnea; peridium cum textura epidermoidea. Asci octospori, subglobosi vel ellipsoidei, muris evanescentibus. Ascosporae unicellulares, oblatae, reticulatae, cum crista equatoriali, subhyalinae vel aurantiaca. Anamorphosis: Arthroconidia hyalina, tenuitunicata, laevia.
Species typica: *Aphanoascella galapagosensis* D.A. Sutton, Y. Marín, E.H. Thompson et Guarro

Ascomata superficial, spherical, non-ostiolate, orange to brown at maturity, surrounded by Hülle cell-like chlamydospores; peridium pale, with textura epidermoidea. Asci 8-spored, subglobose to oblate, evanescent. Ascospores one-celled, oblate, reticulate, with an equatorial rim, subhyaline to orange in mass. Anamorph: Arthroconidia hyaline, thin-walled, smooth.
Type species: *Aphanoascella galapagosensis* D.A. Sutton, Y. Marín, E.H. Thompson & Guarro

Aphanoascella galapagosensis D.A. Sutton, Y. Marín, E.H. Thompson et Guarro sp. nov.

Anamorph: *Malbranchea* sp.
Etym.: *galapagosensis* latinized from the name Galapagos Islands referring to the type locality.
MycoBank: MB 564390

Hyphae vegetativae hyalinae, ramosae, 2–4 crassae. Ascomata superficialia, sphaerica, non-ostiolata, cum chlamydosporae 'hülle' cellulis similis, 170–270 µm diam, aurantiaca vel brunnea; peridium 4–7 µm crassi,

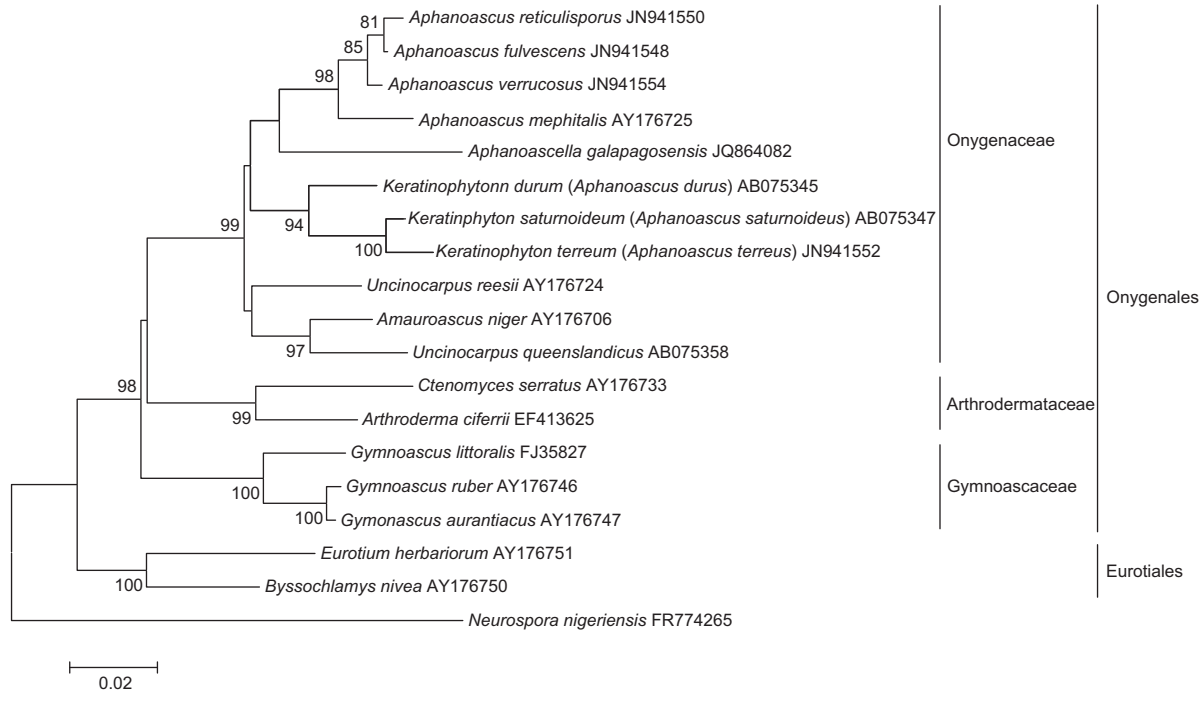

Fig. 4 NJ tree based on LSU rDNA sequences, including 19 taxa belonging to Onygenales, two taxa belonging to Eurotiales and *Neurospora nigeriensis* as outgroup. Bootstrap values of 70% or greater are indicated above the internodes.

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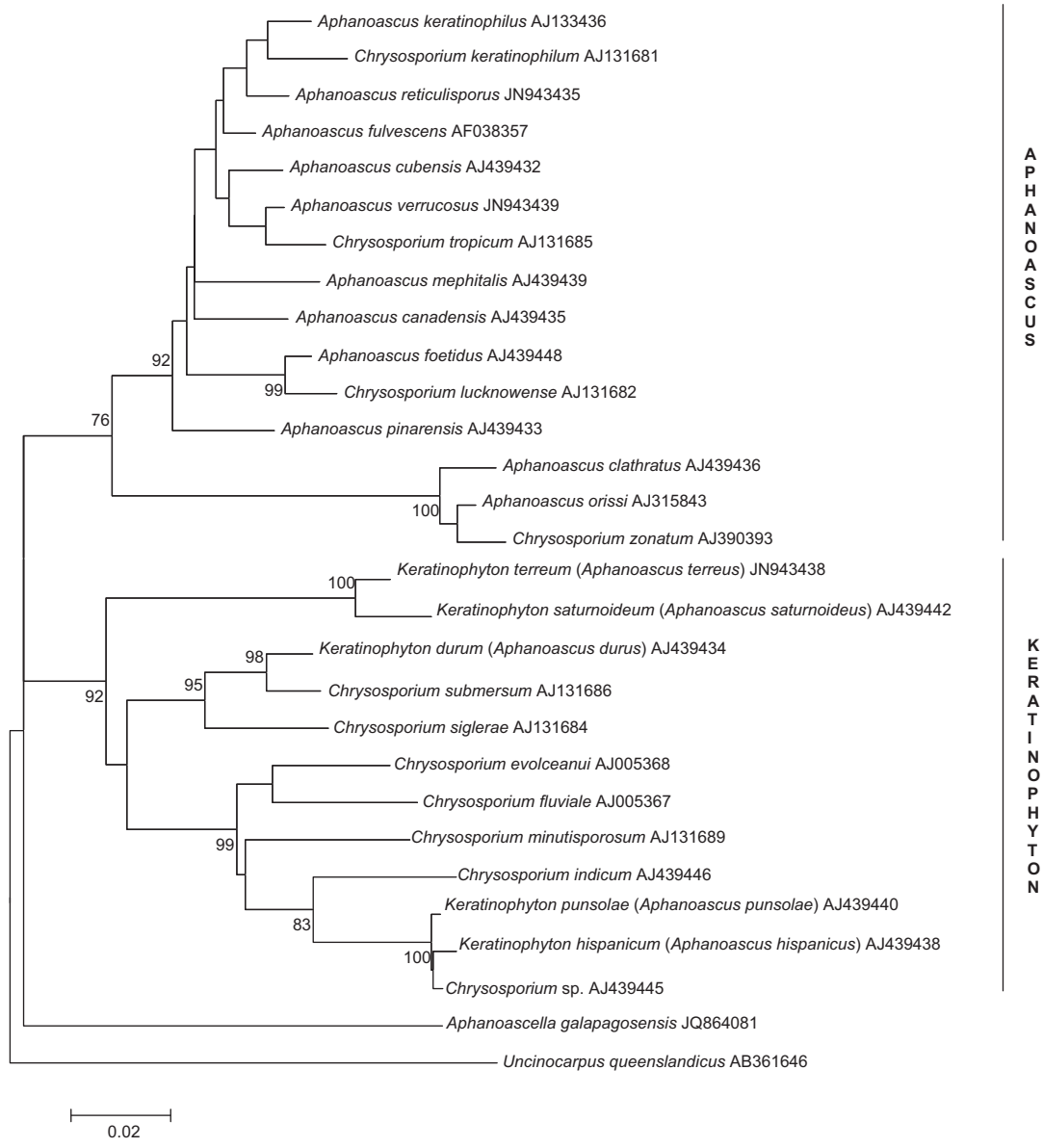

Fig. 5 NJ tree based on ITS sequences from *Aphanoascus* and *Chrysosporium* species and our isolated *Uncinocarpus queenslandicus* as outgroup. Bootstrap values of 70% or greater are indicated above the internodes.

et 3–4 stratiorum compositum, ex textura epidermoidea. Asci octospori, subglobosi vel ellipsoidei, 10–14.5 × 7–11 µm, evanescentes. Ascosporae oblatae, reticulatae, cum crista equatoriali, hyalinae vel aurantiaca in massa, 5–6 × 3–4.5 µm. Arthroconidia cylindrica vel subdoliformia, laevia, hyalina, 4–11(–13) × 2–4 µm, in senectute inflata.

Colonies on potato carrot agar (PCA) reaching 12–13 mm in diameter after 14 days at 25°C, white, velvety to cottony, margins fringed; reverse uncolored. Growth at 15 and 35°C was very restricted on all media tested. Vegetative hyphae hyaline, branched, smooth, septate, 2–4 µm broad, thin-walled. Ascomata superficial, spherical, non-ostiolate, surrounded by Hülle cell-like chlamydospores, 170–270 µm diam, orange to brown at maturity, ascoma wall pale, 4–7 µm thick, composed of 3–4 layers of flattened cells, *textura epidermoidea.* Asci numerous, 8-spored, subglobose to ellipsoidal, 10–14.5 × 7–11 µm, evanescent. Ascospores oblate, with an irregularly reticulate wall formed by inconspicuous and anastomosed ridges, with an equatorial rim 0.5–1 µm broad, sub-hyaline to orange in

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mass, 5–6 × 3–4.5 µm (including rim). Arthroconidia borne on the straight primary hyphae or on short loosely curved or sinuous lateral branches, separated by one or more alternate empty cells, or rarely, formed immediately adjacent to each other. Arthroconidia cylindrical or barrel-shaped, slightly broader than the width of the interconnecting hyphae, hyaline, smooth, 4–11(–13) × 2–4 µm.

Holotype: CBS H-20943 (ex-type strains CBS 132345, FMR12019, UTHSC 11-1518)

Discussion

The ascomycete genus *Aphanoascus* (Onygenaceae, Onygenales) encompasses a large number of species characterized by spherical, pale to dark brown ascomata, lenticular ascospores, either discoid or oblate, with or without an equatorial rim, pale to dark brown with a reticulate, pitted or verrucose wall, and with anamorphs belonging to the genera *Chrysosporium* or *Malbranchea*. Members of the genus are found in soil or dung. While some species are keratinophilic [8,9,10], rarely do they cause human infections. Although poorly supported genetically, two morphologically well-differentiated groups were defined within the genus, i.e., one comprising species with reticulate ascospores and without a rim, and a second with pitted ascospores and with an equatorial rim [9]. This second group included two species that previously belonged to the genus *Keratinophyton*, i.e. *A. terreus* and *A. durum*. The new fungus shows unique morphological characteristics which are intermediate between the two mentioned groups and is also genetically unrelated. The ascospores of *Aphanoascus pinarensis* and *Aphanoascus cubensis*, when observed under light microscopy, appear to have equatorial crests, but when examined by SEM, such structures are in fact prolongations of the reticules of the surface. The genus *Aphanoascella* is characterized by the presence of a pale peridium composed of textura epidermoidea surrounded by scarce Hülle cell-like chlamydospores, and by its characteristic reticulate ascospores with an equatorial rim. These features separate it from other genera within the Onygenales.

In Onygenales ribosomal genes have been commonly used to infer molecular phylogenies [11–15]. In general, the genetic distances among the genera of Onygenales are considerably large and probably numerous species could be proposed as new genera, although most of them would be monotypic. The percent similarity between the type strains of *Aphanosacus fulvescens* and *Aphanoascus terreus* (*Keratinophyton terreum*), which are the type species of *Aphanoascus* and *Keratinophyton*, is 81.3%. Between these species and the new fungus, the percent similarity is 78.7% and 80.35%, respectively.

Reconsidering the phenotypic and molecular data concerning these fungi we believe it is more appropriate to maintain the generic name *Keratinophyton* for those species with ascospores with a pitted wall and a conspicuous equatorial rim, which were clearly separated from the clade where the type species of *Aphanoascus*, *A. fulvescens*, was nested. Therefore, the new combinations are proposed.

Keratinophyton multiporum (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus multiporus* Cano & Guarro, Mycol. Res. 366. 1990.

MycoBank: MB 800127

Keratinophyton hispanicum (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus hispanicus* Cano & Guarro, Mycol. Res. 94: 364. 1990.

MycoBank: MB 800128

Keratinophyton punsolae (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus punsolae* Cano & Guarro, Mycotaxon 38: 162. 1990.

MycoBank: MB 800129

Keratinophyton saturnoideum (Cano & Guarro) Guarro & Y. Marín, comb. nov.

Basyonym *Aphanoascus saturnoideus* Cano & Guarro, Mycol. Res. 94: 370. 1990.

MycoBank: MB 800130

Various disease syndromes involving the shells of tortoises and turtles (*Chelonia*) have been described since the 1980s [16,17]. The chelonian shell is composed of bony plates with intercalating areas of epidermis (scutes). Just as the integumentary systems of other animals can become diseased due to a wide range of causes, from nutritional deficiencies to infectious agents, the shells of tortoises can be similarly afflicted, though the etiologies are less well-documented. Two studies of populations of wild desert tortoises in California suffering from high morbidity rates and shell disease [18,19] failed to demonstrate a single definitive cause for necrotic lesions, despite extensive pathological and microbiologic evaluations. The authors speculated that in these tortoises, the shell lesions might be attributable to chronic toxicoses or nutrient deficiencies. Conversely, in a study of Texas tortoises (*Gopherus berlandieri*) demonstrating scute necrosis, the fungus *Fusarium semitectum* was isolated and believed to have been the etiologic agent [20].

It is unknown at this time if the newly-characterized fungal isolate in this report, *Aphanoascella galapagosensis*, is a primary pathogen of the scute disease observed in this

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tortoise, or represents an opportunistic, secondary pathogen. Lesions similar to the ones seen in this tortoise have been observed in multiple tortoises of another species at the same zoological park, and a similar fungus was isolated. These animals demonstrate a different pattern of lesion but share the possibility of prolonged exposure to moisture. Attempts are currently underway to identify these strains and determine potential commonality with our case isolate in the Galapagos tortoise.

While there is no present indication that this fungus affects mortality in tortoises, any disease process causes some morbidity. Morbidity may decrease resistance to other diseases, longevity or reproductive success. In the husbandry and breeding of endangered species any increase in morbidity may affect the progress of the species' recuperation. Further research into the identity, prevalence, epidemiology and possible treatment of this fungus is proposed to reduce any negative effects on these rare animals.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the writing and content of the paper.

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