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MYCOLOGY

Emydomyces testavorans, a New Genus and Species of Onygenalean Fungus Isolated from Shell Lesions of Freshwater Aquatic Turtles

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ABSTRACT The fungal order *Onygenales* includes many pathogens of humans and animals, and recent studies have shown some onygenalean fungi to be significant emerging pathogens of reptiles. Although many of these fungi have similar morphological features in histologic tissue sections, recent molecular analyses have revealed a genetically complex and diverse group of reptile pathogens comprising several genera, most notably *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis*. Infections by members of these genera have been previously reported in a variety of reptile species, including crocodilians, lizards, snakes, and tuataras, with negative impacts on conservation efforts for some reptiles. Despite the well-documented pathogenicity of these fungi in all other extant reptile lineages, infection has not yet been reported in aquatic turtles. In this study, we report the isolation of an onygenalean fungus associated with shell lesions in freshwater aquatic turtles. The morphologic and genetic characteristics of multiple isolates ($n = 21$) are described and illustrated. Based on these features and results of a multigene phylogenetic analysis, a new genus and species, *Emydomyces testavorans*, are proposed for these fungi isolated from turtle shell lesions.

KEYWORDS CANV, chrysosporium anamorph, emerging fungal pathogen, *Nannizziopsidaceae*, shell disease, turtle, 2 new taxa

Onygenalean fungi, including members of the genera *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis*, have been identified in recent years as primary reptile pathogens that have significantly impacted *in situ* conservation efforts (1–6). Many of these fungal infections in reptiles had been previously reported as the *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV), but recent studies have shown this group of fungi to be genetically and morphologically diverse (5–7). These keratinophilic fungi of the order *Onygenales* are known to exist as environmental saprophytes (2). Infection in many reptile species typically results in ulcerative dermatitis that progresses to localized cellulitis and osteomyelitis and, in some cases, disseminated mycotic infection (4, 8). Disease has been reported in a variety of captive and free-ranging reptile species, including crocodilians (order *Crocodilia*), tuataras (order *Rhynchocephalia*), and lizards and snakes (order *Squamata*) (1, 9–11). In addition, another onygenalean fungus, *Aphanoascella galapagosensis*, has been isolated from a subspecies of Galapagos tortoise with carapace keratitis (12).

To date, there have been no published reports of these or other onygenalean fungi

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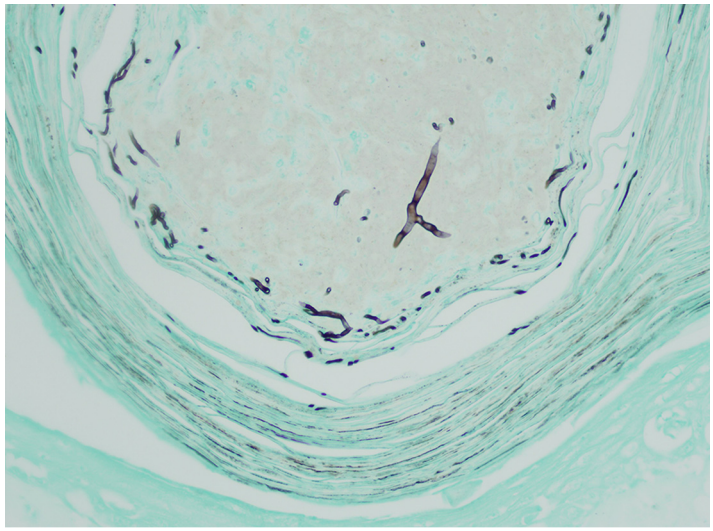

FIG 1 Photomicrograph of shell lesion of a Mata Mata (*Chelus fimbriatus*) with *Emydomyces testavorans* hyphae noted at the leading edge. *Emydomyces testavorans* was confirmed in this case via ITS sequence analysis. Grocott-Gomori's methenamine silver stain, 600×.

causing disease in aquatic turtles (order *Testudines*). However, in recent years an unusual pattern of ulcerative shell disease associated with fungal infection has been observed by a variety of zoological institutions housing aquatic turtle species, as well as in free-ranging populations of western pond turtles (*Actinemys marmorata*) in the state of Washington. Initial routine diagnostic testing identified fungi morphologically comparable to previously described onygenalean fungal pathogens at the leading edge of shell lesions (Fig. 1). Molecular testing performed on a subset of these cases detected fungal DNA that was most similar to previously published sequences of various members of *Onygenaceae*; however, the sequence homology was too low (<92%) for precise identification. Previous attempts at isolation of these fungi were unsuccessful, and failure of isolation was frequently attributed to rapid overgrowth of bacteria or other fungi interpreted as environmental contaminants or nonpathogenic components of the shell microflora.

In the present study, the isolation of a novel onygenalean fungus from turtle shell lesions is described, as are the morphological and genetic characteristics of the fungal isolates. Based on morphological features of isolates and multilocus phylogenetic analyses, a novel genus and species are proposed for this fungus.

MATERIALS AND METHODS

Clinical specimens. All turtle tissues used for this study were clinical samples submitted to the University of Illinois Zoological Pathology Program for diagnostic purposes. Samples from 70 individual turtles were examined in total, consisting of refrigerated (4°C) shell tissue ($n = 59$), archival frozen (−80°C) shell tissue ($n = 10$), and a refrigerated (4°C) cutaneous swab (AST-RC34RV5B). Samples were collected from taxonomically diverse turtle species, including *Actinemys marmorata* ($n = 62$), *Apalone spinifera* ($n = 2$), *Chelus fimbriatus* ($n = 1$), *Emydura subglobosa* ($n = 1$), *Graptemys oculifera* ($n = 1$), *Hydromedusa tectifera* ($n = 1$), *Macrochelys temminckii* ($n = 1$), *Trachemys scripta elegans* ($n = 1$), *Podocnemis unifilis* ($n = 1$), and *Podocnemis vogli* ($n = 2$).

Fungal isolation. Since previous culture attempts from clinical specimens had been unsuccessful, a modified isolation medium was developed based on adaptation of Sabouraud dextrose agar, Emmons modification (SDA). Basal SDA medium consisted of 2% dextrose (Oxoid), 1% peptone (Bacto), and 2% agar (Acros Organics). To encourage the growth of keratinophilic fungi, keratin scutes were collected from archived frozen normal shells of red-eared sliders (*Trachemys scripta elegans*) and incorporated into the medium. Scutes were autoclaved at 121°C for 20 min, dried at ambient temperature and humidity, and finely ground with an electric blade mill grinder. The powered shell keratin was added to the SDA medium, and the mixture was autoclaved at 121°C for 20 min. After autoclaving, chloramphenicol (50 mg/liter; Fisher Scientific), gentamicin sulfate (2.9 mg/liter; Sigma-Aldrich), and cycloheximide (400 mg/liter; Acros Organics) were added to inhibit growth of bacteria and saprophytic fungi. Approximately 25 ml of medium was poured into sterile plastic petri plates (100 by 15 mm; Fisherbrand) and allowed to gel at ambient temperature prior to inoculation.

For tissue, approximately 25 mg was transferred to a sterile 1.5-ml microcentrifuge tube, macerated, and suspended in 0.2 ml of sterile distilled H_2O. Resulting fluid was adsorbed onto a sterile cotton-tipped swab and streaked on to culture plates for isolation. The single swab specimen (AST-RC34RV5B) was directly plated for isolation in the same fashion. Culture plates were incubated at 30°C and monitored daily for colony growth. Individual fungal colonies were subcultured as they appeared and maintained as slant cultures on SDA at ambient temperature and lighting in Rubbermaid plastic storage containers.

Culture morphology. Culture studies were conducted with isolates on three types of media: water agar (WA; Difco Bacto), cornmeal agar (CMA; Difco), and potato dextrose agar (PDA; Difco). To standardize growth rates, isolates were initially transferred to PDA in 60-mm-diameter plastic petri plates (Fisherbrand). After 3 to 4 weeks, 5-mm-diameter plugs were removed from the margin of each isolate and placed inverted in the center of 60-mm-diameter plastic petri plates containing WA, CMA, or PDA. The plates were incubated at room temperature (21 to 25°C) under ambient light in Rubbermaid plastic storage containers. Growth rates and colony characteristics were recorded every 7 days for 28 days. Asexual microscopic features were observed in water mounts after 28 days and under slide culture mounts grown on peptone-yeast extract agar (PYE; Fisherbrand) according to the techniques of Riddell (13). Color terms are used as presented by Kornerup and Wanscher (14).

Mycelia were squash mounted in water or observed under slide cultures, and images of micromorphological structures were captured with a QImaging QColor 3 digital camera mounted on an Olympus BX51 compound microscope using differential interference microscopy. Images were processed using Adobe Photoshop 7.0 (Adobe Systems, Inc., Mountain View, CA). A minimum of 30 measurements were taken for all morphological structures using NIH Image 1.63 (National Institutes of Health, Bethesda, MD). Means and standard deviations were calculated for conidia.

Genetic sequencing. For each isolate, total genomic DNA was extracted from approximately 25 mg of mycelium using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. To enhance the recovery of fungal DNA, an additional step was performed in which 12 μl of yeast lytic enzyme (25 U/ml; MP Biomedicals) was added, and samples were incubated at 37°C for 60 min prior to proteinase K digestion. DNA extract quality was verified by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). For archival turtle specimens from which fungal isolates could not be obtained, total genomic DNA was similarly extracted from approximately 25 mg of frozen (−80°C) lesional tissue.

DNA fragments of the internal transcribed spacer (ITS) region, 18S small subunit rRNA (SSU), D1-D2 domain of the 28S large subunit rRNA (LSU), and α-actin (ACT) genes were PCR amplified using previously published generic fungal primer sets (15–17). All PCRs were performed in 0.2 ml thin-walled PCR tubes (Fisherbrand) with a 25-μl reaction mixture consisting of 2.5 μl of GeneAmp 10× PCR buffer with 15 mM $MgCl_2$ (Applied Biosystems), 0.5 μl of 10 mM deoxynucleotide triphosphate mix (Thermo Scientific), 0.25 μl of AmpliTaq Gold polymerase (Applied Biosystems), 0.25 μl each of 25 μM concentrations of forward and reverse primers, 18.75 μl of diethyl pyrocarbonate-treated water (Ambion), and 2.5 μl of DNA extract as the template. PCR amplification was performed on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) with a 10 min 95°C activation step, followed by 35 cycles of 94°C denaturation for 1 min, 55°C (ITS, SSU, and ACT) or 50°C (LSU) annealing for 1 min, and 72°C extension for 1 min, with a final 72°C extension for 5 min. Target amplification was confirmed via 1.5% agarose gel electrophoresis. PCR amplicons were purified using ExoSAP-IT (Applied Biosystems) according to the manufacturer's instructions and submitted to a commercial laboratory for Sanger sequencing (ABI 3730; University of Chicago Cancer Sequencing Facility). The resulting forward and reverse sequencing reads were pairwise aligned, manually edited if necessary, and consensus sequences generated using Geneious v10.2.3 software (18).

Phylogenetic analyses. Sequences obtained from turtle clinical tissues and fungal isolates, as well as sequences publicly available in the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov/genbank), were analyzed to examine relationships between these fungi associated with turtle shell lesions and other fungi in the *Onygenales* (19).

Multiple sequence alignments were created for each gene individually with MAFFT v7.310 using the L-INS-i strategy (20). Alignments were visualized and manually trimmed using AliView v1.18 so that final alignments included only the target regions amplified by primer sets as previously described (21). These trimmed MAFFT alignments of the SSU, LSU, and ACT genes were used for further analyses. For the ITS MAFFT alignment, ambiguous regions were removed using Gblocks v0.91b under the following parameters: minimum number of sequences for a conserved position = 56, minimum number of sequences for a flanking position = 94, maximum number of contiguous nonconserved positions = 8, minimum length of a block = 10, and gap positions allowed in resulting blocks for any number of taxa (22). Each individual gene alignment was analyzed with jModelTest v2.1.10, and in all cases the general-time-reversible (GTR) model of nucleotide substitution with a GAMMA model of rate heterogeneity and a proportion of invariable sites (GTRGAMMAI approximation) was selected as the most appropriate model for phylogenetic analyses (23). Individual sequence alignments were concatenated in the order SSU-ITS-LSU-ACT to produce a final alignment for phylogenetic estimation.

Maximum-likelihood (ML) analysis was performed on the concatenated sequence alignment using RAxML v8.2.11 with the GTRGAMMAI approximation model. Nodal support was determined by rapid bootstrapping with 1,000 replicates (24). Bayesian inference (BI) was performed with MrBayes v3.2.6 as a supplement to ML analysis to determine posterior probability support for clades generated in the ML analysis (25). BI was executed with GTRGAMMAI approximation, and the Markov-chain Monte Carlo (MCMC) algorithm was run until the average standard deviation of split frequencies fell below 0.01 (4,000,000 generations). Representative members of the *Eurotiales* (*Aspergillus fumigatus* and *Penicillium chrysogenum*) were designated as outgroup taxa to root the resulting tree. Clades with ML bootstrap support

TABLE 1 Accession numbers for fungal genetic sequences deposited in GenBank[a]

Isolate ID(s)	Host species	Date isolated	Host location (U.S. state)	Genetic locus			
				SSU	ITS	LSU	ACT
16-2883 (T) (ILLS 81479, ATCC TSD-145)	*Actinemys marmorata*	20 May 2016	Washington	MG780542	MG780489	MG780517	MG815660
13-1796 (ILLS 81480)	*Apalone spinifera*	20 March 2017	Illinois	MG780559	MG780506	MG780534	MG815676
AST-RC34RV5B (ILLS 81481)	*Macrochelys temminckii*	13 December 2016	Illinois	MG780558	MG780505	MG780533	
16-2881	*Actinemys marmorata*	26 May 2016	Washington	MG780541	MG780488	MG780516	MG815659
16-2886	*Actinemys marmorata*	26 May 2016	Washington	MG780543	MG780490	MG780518	MG815661
16-2887	*Actinemys marmorata*	23 May 2016	Washington	MG780544	MG780491	MG780519	MG815662
16-2888	*Actinemys marmorata*	02 June 2016	Washington	MG780545	MG780492	MG780520	MG815663
16-2895	*Actinemys marmorata*	01 June 2016	Washington	MG780546	MG780493	MG780521	MG815664
16-2897	*Actinemys marmorata*	06 June 2016	Washington	MG780547	MG780494	MG780522	MG815665
16-2899	*Actinemys marmorata*	08 June 2016	Washington	MG780548	MG780495	MG780523	MG815666
16-2901	*Actinemys marmorata*	07 June 2016	Washington	MG780549	MG780496	MG780524	MG815667
16-2905	*Actinemys marmorata*	06 June 2016	Washington	MG780550	MG780497	MG780525	MG815668
16-2916	*Actinemys marmorata*	20 September 2016	Washington	MG780551	MG780498	MG780526	MG815669
16-2921	*Actinemys marmorata*	23 November 2016	Washington	MG780552	MG780499	MG780527	MG815670
16-2923	*Actinemys marmorata*	23 November 2016	Washington	MG780553	MG780500	MG780528	MG815671
16-2924	*Actinemys marmorata*	23 November 2016	Washington	MG780554	MG780501	MG780529	MG815672
16-2925	*Actinemys marmorata*	23 November 2016	Washington	MG780555	MG780502	MG780530	MG815673
16-2927	*Actinemys marmorata*	23 November 2016	Washington	MG780556	MG780503	MG780531	MG815674
ST-T1	*Trachemys scripta elegans*	14 July 2016	Washington	MG780557	MG780504	MG780532	MG815675
17-2847	*Actinemys marmorata*	18 May 2017	Washington	MG780560	MG780507	MG780535	MG815677
17-2848	*Actinemys marmorata*	18 May 2018	Washington	MG780561	MG780508	MG780536	MG815678
01-1919 (tis.)	*Podocnemis unifilis*	–	–	–	MG780509	MG780537	–
03-1714 (tis.)	*Emydura subglobosa*	–	–	–	MG780510	MG780538	MG815679
10-1838 (tis.)	*Hydromedusa tectifera*	–	–	–	MG780511	–	–
10-1915 (tis.)	*Podocnemis vogli*	–	–	–	MG780512	MG780539	MG815680
10-1941 (tis.)	*Podocnemis vogli*	–	–	–	MG780513	–	MG815681
11-1748 (tis.)	*Apalone spinifera*	–	–	–	MG780514	MG780540	MG815682
13-1705 (tis.)	*Chelus fimbriatus*	–	–	–	MG780515	–	–

[a]Each isolate ID corresponds to sequences of fungi isolated from lesional tissues of individual turtles, or if followed by (tis.), sequences obtained directly from tissues with no isolate available. (T), type strain for *Emydomyces testavorans*. Genes for which a sequence was not obtained or available are indicated with a dash (–). SSU, 18S small subunit rRNA gene; ITS, internal transcribed spacer region; LSU, 28S large subunit rRNA gene D1-D2 domain; ACT, α-actin; ATCC, American Type Culture Collection; ILLS, Illinois Natural History Survey Fungarium.

values of ≥70% and BI posterior probability values of ≥0.95 were considered significant. Trees were visualized and formatted using Dendroscope v3.5.7 and Adobe Acrobat Pro DC (Adobe Systems) (26).

Antifungal susceptibility testing. Three fungal isolates (16-2883, 13-1796, and AST-RC34RV5B) were submitted to a reference laboratory (UT Health San Antonio Fungus Testing Laboratory) for antifungal susceptibility testing. Broth dilution antifungal susceptibility testing was performed on each isolate for fluconazole, itraconazole, posaconazole, voriconazole, and terbinafine in accordance with Clinical and Laboratory Standards Institute reference method M38-A2 (27).

Data availability. Sequences obtained from fungal isolates and lesional tissue were deposited in the GenBank database and are listed with accession numbers in Table 1 (19). In some cases where fungal isolates were not obtained, direct gene amplification from lesional tissue was not successful for all genes. Accession numbers for sequences of other fungi within *Onygenales* that were used in these analyses are listed in Table 2.

RESULTS

Fungal isolation. Twenty-one distinct fungal isolates were recovered from examined shells using the isolation methods described, with each tissue specimen originating from a unique individual turtle. Of these, the vast majority (n = 18/21) were recovered from shell tissue of western pond turtles (*Actinemys marmorata*). Additional isolates were recovered from lesional shell tissue of a spiny softshell turtle (n = 1, *Apalone spinifera*, 13-1796) and a red-eared slider (n = 1, *Trachemys scripta elegans*, ST-T1), as well as one cutaneous swab of an alligator snapping turtle (n = 1, *Macrochelys temminckii*, AST-RC34RV5B, collected under Illinois Department of Natural Resources permit 14-046). All isolates recovered from turtles were morphologically similar (see "Culture morphology" above) and based on multilocus sequence analyses (see "Phylogenetic analyses" above) were more closely related to each other than to other members of *Onygenales* with publicly available sequence data.

TABLE 2 Accession numbers of fungal sequences retrieved from GenBank for inclusion in phylogenetic analyses[a]

Species	Strain ID	Genetic locus			
		SSU	ITS	LSU	ACT
Aphanoascella galapagosensis (T)	UAMH 11703	–	NR_132869	NG_057013	–
Aphanoascus cinnabarinus	CBS 267.72	AB015788	JN899376	–	–
Aphanoascus reticulisporus (T)	NBRC 32372	JN941599	JN943434	JN941549	–
Aphanoascus terreus (T)	NBRC 31781	JN941597	JN943436	JN941551	–
Aphanoascus verrucosus	NBRC 32382	JN941595	JN943439	JN941554	–
Arachnomyces peruvianus	CBS 113.54	–	MF572319	MF572324	–
Arthroderma ciferrii (T)	CBS 272.66	AB015769	AJ877217	AB040681	–
Aspergillus fumigatus (T)	ATCC 1022	–	HQ026746	AY660917	–
Auxarthron alboluteum (T)	UAMH 2846	AY124494	AY177303	AB359411	–
Auxarthron compactum (T)	CBS 200.64	AB015767	AJ271574	AB040692	–
Auxarthron filamentosum (T)	UAMH 4097	AY124501	AY177298	–	–
Auxarthron kuehnii (T)	CBS 539.72	AB015766	AJ271417	AB040691	–
Auxarthron umbrinum (T)	UAMH 3952	AY124498	AY177309	–	–
Auxarthron zuffianum (T)	UAMH 1875	AY124493	AY177306	–	–
Castanedomyces australiensis	FMR 5484	AJ131786	AJ131785	–	–
Chrysosporium evolceanui (T)	RV 26475	–	AJ005368	–	–
Chrysosporium fluviale	FMR 6005	–	AJ005367	–	–
Chrysosporium keratinophilum (T)	IFM 55159	–	AB361656	AB359444	–
Chrysosporium siglerae (T)	UAMH 6541	–	AJ131684	–	–
Chrysosporium submersum	IMI 379911	–	AJ131686	–	–
Chrysosporium tropicum	UAMH 691	–	AJ131685	–	–
Coccidioides immitis	RS	XR_001099880	KJ783447	XR_001099877	NW_004504310
Coccidioides posadasii (T)	ATCC 28868	X58571	U18360	–	–
Ctenomyces serratus	CBS 187.61	AB015771	AJ877222	AB040683	–
Emmonsia crescens	UAMH 1067	U29390	AF038346	–	–
Emmonsia sola	NCPF 4289	–	HF563673	HF563677	HF563666
Gymnascella hyalinospora (T)	CBS 548.72	AB015775	AJ315826	AB040687	–
Gymnoascus aurantiacus	ATCC 22394	–	HM991267	AY176747	–
Gymnoascus littoralis (T)	CBS 454.73	FJ358340	AJ315833	FJ358272	–
Gymnoascus petalosporus	IFM 47417	–	AB361639	AB359429	–
Histoplasma capsulatum	UAMH 7141	–	AF038353	–	–
Kraurogymnocarpa trochleospora (T)	UAMH 10101	AY177295	KF477238	AB075344	–
Microsporum canis	ATCC 36299	EF631605	EF631605	–	EF631626
Nannizziopsis arthrosporioides (T)	UTHSC R-4263	–	HF547872	HF547857	HF547885
Nannizziopsis barbatae (T)	UAMH 11185	KF466861	JF323871	–	–
Nannizziopsis chlamydospora	UTHSC 06-1419	–	HF547871	HF547855	HF547881
Nannizziopsis chlamydospora (T)	UTHSC 04-2056	–	HF547870	HF547854	HF547879
Nannizziopsis crocodili	UAMH 9908	–	KF477205	–	–
Nannizziopsis crocodili (T)	UAMH 9666	KF466860	KF477204	–	–
Nannizziopsis dermatitidis	UAMH 7861	–	KF477201	–	–
Nannizziopsis dermatitidis (T)	UAMH 7583	KF466859	KF477200	–	–
Nannizziopsis draconii (T)	CCFVB CH12	–	EU883993	HF547856	HF547883
Nannizziopsis guarroi	UAMH 10171	KF466862	KF477206	–	–
Nannizziopsis guarroi	UTHSC 06-3993	–	HF547875	HF547866	HF547897
Nannizziopsis guarroi (T)	CBS 124553	–	EU018451	FJ839684	HF547895
Nannizziopsis hominis	UAMH 9852	–	KF477216	–	–
Nannizziopsis hominis (T)	UAMH 7859	KF466864	KF477215	–	–
Nannizziopsis infrequens (T)	UAMH 10417	KF466863	AY744467	–	–
Nannizziopsis pluriseptata (T)	UTHSC 10-1045	–	HF547874	HF547859	HF547889
Nannizziopsis vriesii	UAMH 3526	–	KF477197	–	–
Nannizziopsis vriesii (T)	UAMH 3527	KF466858	KF477198	AY176715	HF547893
Onygena equine	ATCC 22731	–	–	AY176717	–
Ophidiomyces ophiodiicola	UAMH 10717	KF466871	KF477233	–	–
Ophidiomyces ophiodiicola	UAMH 6642	KF466869	KC884267	–	–
Ophidiomyces ophiodiicola	UAMH 6688	KF466870	KF477228	–	–
Ophidiomyces ophiodiicola (T)	CBS 122913	–	EU715819	EU715820	HF547891
Paranannizziopsis australasiensis	UAMH 10439	KF466866	KF477218	–	–
Paranannizziopsis australasiensis	UAMH 11665	–	KF477221	–	–
Paranannizziopsis australasiensis (T)	UAMH 11645	–	KF477220	–	–
Paranannizziopsis californiensis	UAMH 10692	–	KF477223	–	–
Paranannizziopsis californiensis (T)	UAMH 10693	KF466867	KF477224	–	–
Paranannizziopsis crustacea (T)	UAMH 10199	KF466868	KF477225	–	–
Paranannizziopsis longispora (T)	UTHSC R-4380	–	HF547873	HF547858	HF547887
Paraphyton cookei (T)	CBS 228.58	–	KT155835	KT155174	–

(Continued on next page)

TABLE 2 (Continued)

Species	Strain ID	Genetic locus			
		SSU	ITS	LSU	ACT
Pectinotrichum chinense	LC 5811	–	KU746695	KU746741	–
Pectinotrichum llanense	CBS 882.71	AB015783	NR_119467	AB040698	–
Penicillium chrysogenum	CBS 306.48	GU733359	AY213669	AY213615	–
Spiromastigoides princeps (T)	IMI 169642	–	AJ315840	–	–
Spiromastigoides warcupii (T)	CBS 576.63	AB015768	LN867609	AB040679	LN867616
Uncinocarpus queenslandicus	CBS 280.77	–	AB361646	AB359434	–
Uncinocarpus queenslandicus (T)	IMI 121675	AJ315173	AJ390394	–	–
Uncinocarpus reesii	CBS 121.77	–	AJ271566	AY176724	–

[a]Genes for which sequence data were not available are indicated by a dash (–). ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures (now Westerdijk Fungal Biodiversity Institute); FMR, Facultad de Medicina Reus; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses; IMI, International Mycological Institute; LC, Laboratory of Cryptogamy; NBRC, National Institute of Technology and Evaluation (NITE) Biological Resource Center; NCPF, National Collection of Pathogenic Fungi; RV, Collection of Leptospira Strains, Istituto Superiore di Sanita; UAMH, University of Alberta Microfungus Collection and Herbarium; UTHSC, University of Texas Health Science Center. Type strains, as reported, are denoted by "(T)."

Growth characteristics and morphologic measurements of fungal isolates recovered from turtles are presented in Table 3, and representative images of colony morphology and microscopic features are presented in Fig. 2. Isolates were slow growing on all media at ambient temperatures (21 to 25°C). All isolates exhibited only the asexual (anamorphic) state with no sexual (teleomorphic) state observed during the course of the study.

Genetic sequencing. DNA sequences were obtained from a total of 21 fungal isolates and 7 turtle shells. Sequences were genetically more similar to each other than to other publicly available sequences; however, there was significant genetic diversity between isolates at all loci. Sequence homology ranged from 98.8 to 100% for SSU, 80 to 100% for ITS, 92.1 to 100% for LSU, and 94.6 to 100% for ACT genes.

The concatenated sequence alignment comprised 106 taxa and 2,580 characters. The most likely tree resulting from the RAxML analysis is presented in Fig. 3. All sequences obtained from turtle lesional tissues or fungal isolates from affected turtles occurred in a well-supported (ML bootstrap = 100, BI posterior probability = 1.00) monophyletic clade distinct from representative taxa within the order *Onygenales*. Other genera within the *Onygenales*, including *Auxarthron*, *Nannizziopsis*, *Ophidiomyces*, *Paranannizziopsis*, and *Spiromastigoides*, also occurred in well-supported monophyletic clades, in accordance with previous reports. Higher-order relationships between these genera and fungi recovered from turtles were not well supported, and thus, family-level taxonomy remains uncertain in the present analysis. Based on this phylogeny in combination with morphological features, a novel genus and species are proposed for the fungus infecting freshwater aquatic turtles.

Taxonomy. *Emydomyces* A.N. Mill. & D.B. Woodburn gen. nov. (28). MycoBank accession no. MB827506. Etymology: *Emydo* refers to a freshwater tortoise and *myces* to fungus. Colonies are slow growing, silky to wooly, translucent to white (1A1); margin even, appressed and translucent; reverse same as the mat. Hyphae largely undifferen-

TABLE 3 Microscopic characteristics of *Emydomyces testavorans* compared to those previously reported (5, 6) for selected reptile pathogens on PDA medium

Species (reference)	Size (μm)			Presence or absence of chlamydospores
	Hyphae	Aleurioconidia	Arthroconidia	
Emydomyces testavorans	1–4	5.5–27 × 3.5–11.2	5.4–52.9 × 1.4–4.2	Absent
Nannizziopsis arthrosporioides (6)	1–4	2.5–7 × 1.5–3	5–15 × 1.4–4	Absent
Nannizziopsis chlamydospora (6)	1–3	3–9 × 1.5–2	4–10 × 2–4	Present
Nannizziopsis draconi (6)	1–3	4–7 × 1.5–2	5–9 × 1.5–2.5	Absent
Nannizziopsis guarroi (5)	NR[a]	3.2–6.5 × 1.5–2.5	2.8–7 × 2–3.7	Absent
Nannizziopsis vriesii (5)	NR	2.5–6 × 1.5–2.7	2.7–7.3 × 1.7–2.7	Absent
Paranannizziopsis australiensis (5)	NR	3.5–8 × 1.5–2.7	NR	Absent
Paranannizziopsis californiensis (5)	NR	4–8.5 × 1.8–2.6	NR	Absent
Ophidiomyces ophiodiicola (5)	1.5–2.5	2.5–7.5 × 1.5–2.5	3–12.5 × 1.5–3.5	Absent

[a]NR, not reported in original published description of species.

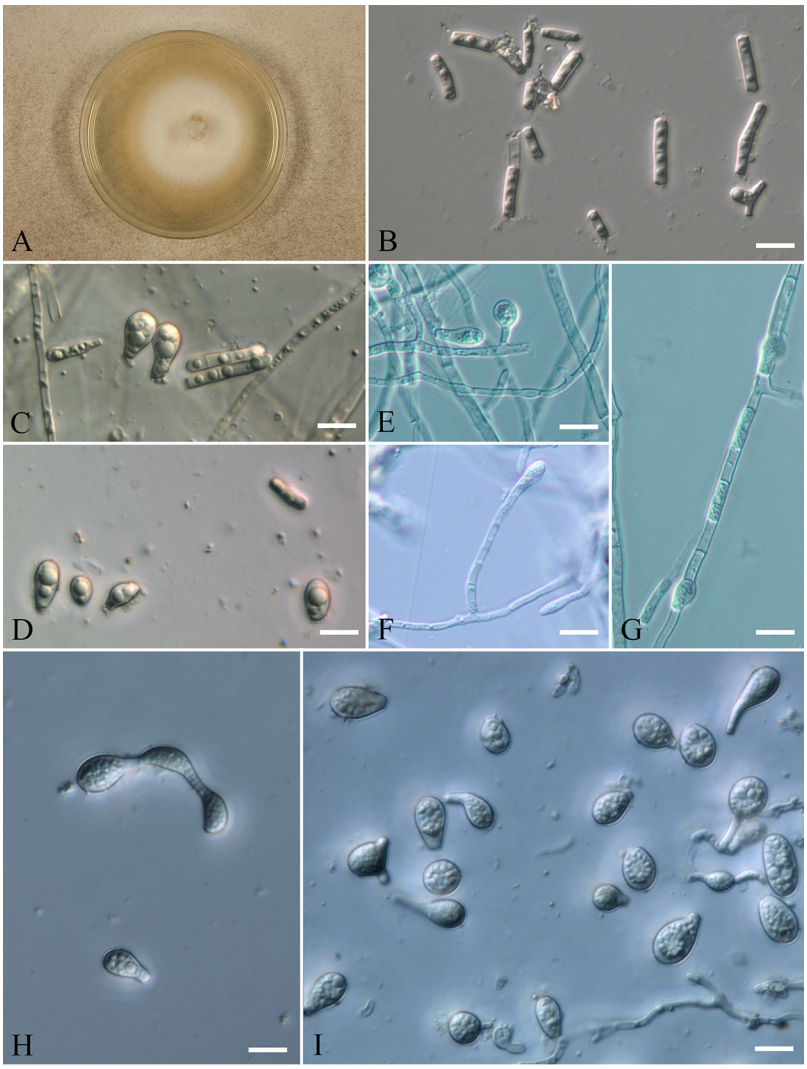

FIG 2 Colony of *Emydomyces testavorans* after 28 days of incubation on CMA at 22°C (A). Microscopic morphology of *Emydomyces testavorans* showing fission arthroconidia on WA (B), aleurioconidia and arthroconidia on CMA (C), aleurioconidia on WA (D), a single aleurioconidium produced laterally on PYE (E), a single aleurioconidium produced apically on long lateral branch on PYE (F), intercalary arthroconidia on PYE (G), variously shaped aleurioconidia on PDA (H and I). Images A to D are from holotype (ILLS81479); images E to I from paratype (ILLS81480). Scale bars, 10 μm.

tiated, occasionally branched, regularly septate, thin walled, hyaline. Aleurioconidia produced holoblastically, apically, laterally or intercalary, clavate to pyriform, widely variable in shape, thin-walled, one-celled, occasionally two-celled with septa near base, occasionally stalked. Arthroconidia produced via fission or intercalary, barrel shaped to cylindrical, thin walled, usually one celled, occasionally two celled with median septa. No sexual morph observed.

Type species: *Emydomyces testavorans* A.N. Mill. & D.B. Woodburn. *Emydomyces testavorans*. A.N. Mill. & D.B. Woodburn sp. nov. (28). MycoBank accession no. MB827507. Etymology: *testa* for shell and *vorans* for devouring. Colonies on WA slow growing, 23 to 30 mm diameter in 28 days, slow growing on CMA and PDA, 25 to 37 mm diameter in 28 days, silky on WA, wooly on CMA and PDA, mat appressed on WA, aerial on CMA and PDA, translucent on WA, white (1A1) on CMA and PDA; margin even, appressed and translucent in all media; reverse same as the mat in all media; asexual state not observed before 28 days. Hyphae largely undifferentiated, occasionally branched, regularly septate, 1 to 4 μm wide, thin walled, hyaline, rarely becoming

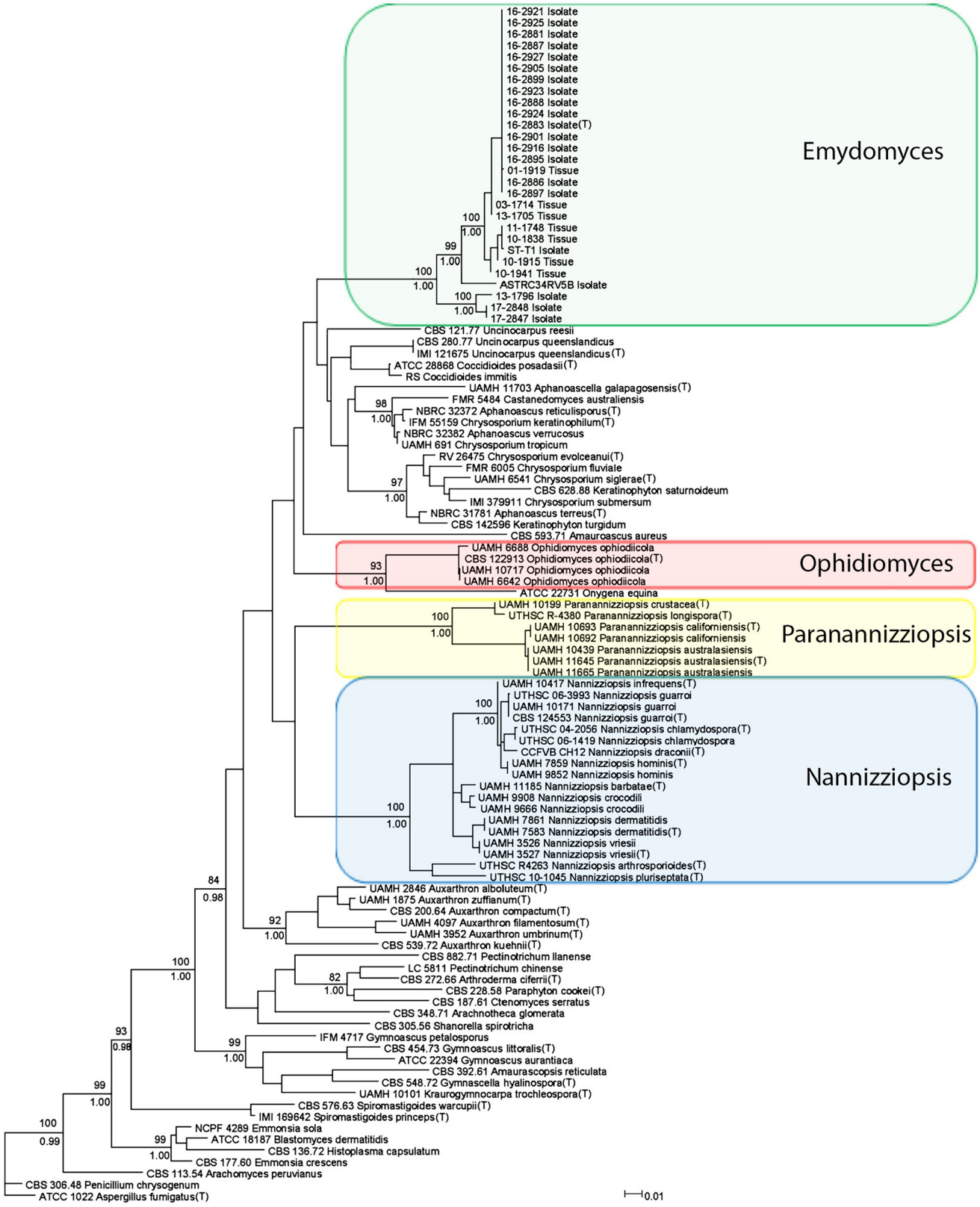

FIG 3 Most likely phylogenetic tree derived from RAxML analysis of concatenated SSU, ITS, LSU, and ACT nucleotide sequences. Nodes with significant support in both ML and BI are labeled above branches with ML bootstrap (BS) support values (≥70%) and below branches with Bayesian posterior probability (PP) values (≥0.95). Branch lengths are proportional to genetic distance, as measured by the average number of substitutions per site (a scale bar is at the bottom right). The tree is rooted with representative taxa of order *Eurotiales*. Clades are colored for *Emydomyces* (green), *Ophidiomyces* (red), *Paranannizziopsis* (yellow), and *Nannizziopsis* (blue). "(T)" denotes taxa reported as type or ex-type specimens.

TABLE 4 MICs of antifungal drugs for three *Emydomyces* isolates—16-2883 (T), 13-1796, and AST-RC34RV5B—as determined by broth dilution antifungal susceptibility testing performed at the UT Health San Antonio Fungus Testing Laboratory[a]

	MIC (μg/ml)		
Drug	**16-2883**	**13-1796**	**AST-RC34RV5B**
Fluconazole	2	1	*
Itraconazole	0.25	*	0.06
Posaconazole	0.125	<0.03	<0.03
Voriconazole	<0.03	*	<0.03
Terbinafine	0.06	0.015	0.03

[a]*, no MIC was determined as no isolate growth was detected under standard assay conditions.

severely undulated with age on CMA and PDA. Aleurioconidia and arthroconidia produced on all media. Aleurioconidia produced holoblastically, apically, occasionally laterally directly from hyphae, or rarely intercalary, clavate to pyriform, widely variable in shape, thin-walled, one-celled, occasionally two-celled with septa near base, occasionally stalked, with one to several oil droplets, if more than one oil droplet then decreasing in size, 5.5–27.2 × 3.5–11.2 (13.5 ± 3.5 × 7.0 ± 1.2) μm. Arthroconidia produced via fission or intercalary, barrel shaped to cylindrical, thin walled, usually one celled, occasionally two celled with median septa, with one to several equally sized oil droplets, 5.4–52.9 × 1.4–4.2 (16.4 ± 8.7 × 3.0 ± 0.6) μm. No sexual morph observed. Holotype: 16-2883, isolated from shell tissue of *Actinemys marmorata*, 20 May 2016, WA, USA, preserved as a dried culture (ILLS 81479), ex-type culture (ATCC TSD-145). Other material examined: 13-1796, isolated from shell tissue of *Apalone spinifera*, 20 March 2017, IL, USA, preserved as a dried culture (ILLS 81480); AST-RC34RV5B, isolated from cutaneous swab of *Macrochelys temminckii*, 13 December 2016, IL, USA, preserved as a dried culture (ILLS 81481). Notes: Morphologically similar hyphae were observed histologically at the leading edges of ulcerative shell and cutaneous lesions of host turtles for isolates 16-2883, 16-2886, 16-2887, 16-2888, 16-2895, 16-2897, 16-2899, 16-2901, 16-2916, 16-2921, 16-2923, 16-2924, 16-2925, 16-2927, AST-RC34RV5B, and 13-1796. All living isolates are deposited at ILLS in long-term storage.

Antifungal susceptibility testing. MIC values as determined by broth dilution antifungal susceptibility testing are presented in Table 4. Isolates 16-2883, 13-1796, and AST-RC34RV5B were selected for testing based on phylogenetic analyses (Fig. 3). MIC values for all antifungals tested were determined for isolate 16-2883. The MIC value of fluconazole for isolate AST-RC34RV5B, as well as MIC values of itraconazole and voriconazole for isolate 13-1796, could not be determined due to failure of the isolates to grow in those assays.

DISCUSSION

The morphological and genetic features of fungal isolates examined in this study support the designation of a novel genus and species, *Emydomyces testavorans*, for these fungi isolated from turtle shell lesions. Recent studies utilizing genetic sequencing and phylogenetic analyses have identified multiple genera of fungal reptile pathogens within the group formerly designated as the *Chrysosporium* anamorph of *Nannizziopsis vriesii*, including *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis*, though all are morphologically similar in histologic section and produce aleurioconidia and fission arthroconidia (Fig. 2) (5, 6, 29). *Emydomyces testavorans* demonstrates morphological and genetic features different from these other members of the *Onygenales*. In histologic section, members of *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis* produce narrow, septate hyphae, often accompanied by fission arthroconidia. While the hyphae of *Emydomyces* in shell lesions or tissue are septate and narrow (Fig. 2), arthroconidia have not been observed. Similarly in culture, conidia of *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis* are smaller than those of *Emydomyces*, which are more variably shaped (Table 3 and Fig. 2). In the present study, no chlamydospores or

sexual structures were observed in *Emydomyces* isolates, as have been reported in *N. chlamydospora* and *N. vriesii*, respectively (Table 1) (5, 6).

The monophyly of *Emydomyces* isolates described in the present study is well supported by multilocus phylogenetic analyses. Other recent studies investigating the phylogenetic relationships of reptilian fungal pathogens have also identified monophyletic clades corresponding to the genera *Nannizziopsis*, *Ophidiomyces*, and *Paranannizziopsis* (5, 6). Higher-order phylogenetic relationships are beyond the scope of the present study, although the discovery of *Emydomyces* as a genus adds to the increasingly recognized diversity of onygenalean reptile pathogens. Additional phylogenetic analyses including more genes or even whole-genome sequences are warranted to better understand taxonomic relationships among genera and families within the *Onygenales*.

Reported MIC values suggest that *Emydomyces* is susceptible to antifungal drugs commonly used in the treatment of reptilian fungal infections, such as itraconazole, voriconazole, and terbinafine (4, 30). However, only a few studies evaluating the safety, efficacy, and pharmacokinetics of these antifungal drugs have been performed in a limited selection of reptile species (30–32). Toxicity associated with voriconazole administration has been reported in at least one species of snake with clinically relevant dosages (30). Despite anecdotal evidence supporting the use of these drugs in reptilian fungal infections, little is known regarding their safety and efficacy in aquatic turtles. While the more lipophilic compounds have been shown in other species to accumulate above plasma concentrations in bone and keratinaceous tissues, similar studies have not been conducted in turtles (33). Therefore, interpretation of the significance of these antifungal susceptibility results is limited until such pharmacokinetic studies are performed.

Although a causative relationship has yet to be confirmed, ulcerative shell disease has been histologically associated with the fungus described in this report. Identification and characterization of this fungus are a crucial first step to better understanding this potential emerging threat to turtle health and conservation.

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REFERENCES

1. Allender MC, Dreslik M, Wylie S, Phillips C, Wylie DB, Maddox C, Delaney MA, Kinsel MJ. 2011. *Chrysosporium* sp. infection in eastern Massasauga rattlesnakes. Emerg Infect Dis 17:2383–2384. https://doi.org/10.3201/eid1712.110240.
2. Allender MC, Raudabaugh DB, Gleason FH, Miller AN. 2015. The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. Fungal Ecol 17:187–196. https://doi.org/10.1016/j.funeco.2015.05.003.
3. Barten SL. 2006. Shell damage, p 893–899. *In* Mader DR (ed), Reptile medicine and surgery, 2nd ed. Saunders Elsevier, St. Louis, MO.
4. Paré JA, Sigler L. 2006. Fungal diseases, p 217–226. *In* Mader DR (ed), Reptile medicine and surgery, 2nd ed. Saunders Elsevier, St. Louis, MO.
5. Sigler L, Hambleton S, Paré JA. 2013. Molecular characterization of reptile pathogens currently known as members of the *Chrysosporium* anamorph of *Nannizziopsis vriesii* complex and relationship with some human-associated isolates. J Clin Microbiol 51:3338–3357. https://doi.org/10.1128/JCM.01465-13.
6. Stchigel AM, Sutton DA, Cano-Lira JF, Cabañes FJ, Abarca L, Tintelnot K, Wickes BL, García D, Guarro J. 2013. Phylogeny of chrysosporia infecting reptiles: proposal of the new family *Nannizziopsiaceae* and five new species. Persoonia 31:86–100. https://doi.org/10.3767/003158513X669698.
7. Abarca ML, Castella G, Martorell J, Cabanes FJ. 2010. *Chrysosporium guarroi* sp. nov. a new emerging pathogen of pet green iguanas (*Iguana iguana*). Med Mycol 48:365–372. https://doi.org/10.3109/13693780903173401.
8. Robertson J, Chinnadurai SK, Woodburn DB, Adkesson MJ, Landolfi JA. 2016. Disseminated *Ophidiomyces ophiodiicola* infection in a captive eastern Massasauga (*Sistrurus catenatus catenatus*). J Zoo Wildl Med 47:337–340. https://doi.org/10.1638/2014-0222.1.
9. Bowman MR, Paré JA, Sigler L, Naeser JP, Sladky KK, Hanley CS, Helmer P, Phillips LA, Brower A, Porter R. 2007. Deep fungal dermatitis in three inland bearded dragons (*Pogona vitticeps*) caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii*. Med Mycol 45:371–376. https://doi.org/10.1080/13693780601188610.
10. Thomas AD, Sigler L, Peucker S, Norton JH, Nielan A. 2002. *Chrysosporium* anamorph of *Nannizziopsis vriesii* associated with fatal cutaneous mycoses in the salt-water crocodile (*Crocodylus porosus*). Med Mycol 40:143–151. https://doi.org/10.1080/mmy.40.2.143.151.
11. Humphrey S, Alexander H, Ha H. 2016. Detection of *Paranannizziopsis*

australasiensis in tuatara (*Sphenodon punctatus*) using fungal culture and a generic fungal PCR. N Z Vet J 64:298–300. https://doi.org/10.1080/00480169.2016.1177472.
12. Sutton DA, Marín Y, Thompson EH, Wickes BL, Fu J, García D, Swinford A, De Maar T, Guarro J. 2013. Isolation and characterization of a new fungal genus and species, *Aphanoascella galapagosensis*, from carapace keratitis of a Galapagos tortoise (*Chelonoidis nigra microphyes*). Med Mycol 51:113–120. https://doi.org/10.3109/13693786.2012.701767.
13. Riddell RW. 1950. Permanent stained mycological preparations obtained by slide culture. Mycologia 42:265–270. https://doi.org/10.1080/00275514.1950.12017830.
14. Kornerup A, Wanscher JH. 1967. Methuen handbook of colour, 2nd ed. Eyre Methuen, London, England.
15. Kwiatkowski NP, Babiker WM, Merz WG, Carroll KC, Zhang SX. 2012. Evaluation of nucleic acid sequencing of the D1/D2 region of the large subunit of the 28S rDNA and the internal transcribed spacer region using Smartgene IDN software for identification of filamentous fungi in a clinical laboratory. J Mol Diagn 14:393–401. https://doi.org/10.1016/j.jmoldx.2012.02.004.
16. Voigt K, Wöstemeyer J. 2000. Reliable amplification of actin genes facilitates deep-level phylogeny. Microbiol Res 155:179–195. https://doi.org/10.1016/S0944-5013(00)80031-2.
17. White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315–322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press, London, England.
18. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. https://doi.org/10.1093/bioinformatics/bts199.
19. NCBI Resource Coordinators. 2017. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 45:D12–D17. https://doi.org/10.1093/nar/gkw1071.
20. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066. https://doi.org/10.1093/nar/gkf436.
21. Larsson A. 2014. AliView: a fast and lightweight alignment viewer and editor for large data sets. Bioinformatics 30:3276–3278. https://doi.org/10.1093/bioinformatics/btu531.
22. Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17:540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334.
23. Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772. https://doi.org/10.1038/nmeth.2109.
24. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. https://doi.org/10.1093/bioinformatics/btu033.
25. Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755. https://doi.org/10.1093/bioinformatics/17.8.754.
26. Huson DH, Scornavacca C. 2012. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst Biol 61:1061–1067. https://doi.org/10.1093/sysbio/sys062.
27. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard, 2nd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
28. McNeill J, Barrie FR, Buck WR, Demoulin V, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Marhold K, Prado J, Prud'homme van Reine WF, Smith GF, Wiersema JH, Turland NJ. 2012. International code of nomenclature for algae, fungi and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011 (Regnum Vegetabile 154). Koeltz Scientific Books, Koenigstein, Germany.
29. Rajeev S, Sutton DA, Wickes BL, Miller DL, Giri D, Van Meter M, Thompson EH, Rinaldi MG, Romanelli AM, Cano JF, Guarro J. 2009. Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola*, from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). J Clin Microbiol 47:1264–1268. https://doi.org/10.1128/JCM.01751-08.
30. Lindemann DM, Allender MC, Rzadkowska M, Archer G, Kane L, Baitchman E, Driskell EA, Chu CT, Singh K, Hsiao SH, Sykes JM, IV, Cox S. 2017. Pharmacokinetics, efficacy, and safety of voriconazole and itraconazole in healthy cottonmouths (*Agkistrodon piscivorus*) and Massasauga rattlesnakes (*Sistrurus catenatus*) with snake fungal disease. J Zoo Wildl Med 48:757–766. https://doi.org/10.1638/2016-0179.1.
31. Kane LP, Allender MC, Archer G, Leister K, Rzadkowska M, Boers K, Souza M, Cox S. 2017. Pharmacokinetis of nebulized and subcutaneously implanted terbinafine in cottonmouths (*Agkistrodon piscivorus*). J Vet Pharmacol Ther 40:575–579. https://doi.org/10.1111/jvp.12406.
32. Van Waeyenberghe L, Baert K, Pasmans F, van Rooij P, Hellebuyck T, Beernaert L, de Backer P, Haesebrouck F, Martel A. 2010. Voriconazole, a safe alternative for treating infections caused by the *Chrysosporium* anamorph of *Nannizziopsis vriesii* in bearded dragons (*Pogona vitticeps*). Med Mycol 48:880–885. https://doi.org/10.3109/13693781003743122.
33. Felton T, Troke PF, Hope WH. 2014. Tissue penetration of antifungal agents. Clin Microbiol Rev 27:68–88. https://doi.org/10.1128/CMR.00046-13.