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Isolation and Characterization of a New Fungal Species, *Chrysosporium ophiodiicola*, from a Mycotic Granuloma of a Black Rat Snake (*Elaphe obsoleta obsoleta*)[▽]

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Isolation and characterization of the new species *Chrysosporium ophiodiicola* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*) are reported. Analysis of the sequences of different fragments of the ribosomal genes demonstrated that this species belongs to the Onygenales and that this species is genetically different from other morphologically similar species of *Chrysosporium*. This new species is unique in having both narrow and cylindrical-to-slightly clavate conidia and a strong, pungent odor.

CASE REPORT

A black, male rat snake (*Elaphe obsoleta obsoleta*) of undetermined age was presented with a history of prolonged anorexia and slow-growing facial masses. The snake was found as an adult at an old home site in an old barn near Sparta, GA, by the current owner, a wildlife educator. The snake had been in his possession for 4 years and was frequently used in public educational performances in the southeast. Upon presentation, the snake had a 1-cm by 1.5-cm subcutaneous, longitudinally ovoid swelling overlying his right ventral mandible area (Fig. 1A). He also had a 1-cm swelling overlying his right eye and extending down into the orbit, displacing the eyeball laterally and displacing the palate and dorsal limit of the choana ventrally. The masses were lobular, whitish in appearance, and enclosed in a thin capsule. The submandibular mass was removed in its entirety, as its capsule was very discrete. The other mass was very friable and locally extensive. Both masses were surgically removed and submitted for histopathological examination and culture. Not all portions of the second mass could be completely removed, due to the location of this mass, but the area enclosing it was debrided. At the time of surgery, the snake was treated with meloxicam (Metacam; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) at a dose of 0.2 mg/kg of body weight once a day and enrofloxacin (Baytril; Bayer HealthCare, LLC, Animal Health Division, Shawnee Mission, KS) at a dose of 5 mg/kg twice a day. This was continued until the histopathology report indicating a fungal infection was received. Enrofloxacin was discontinued, and ketoconazole was initiated. A single oral administration of ketoconazole (Apotex, Inc., Toronto, Ontario, Canada) at 50 mg/kg was administered daily. The snake was kept at 29.5°C and was tube fed Hill's a/d prescription diet (Hill's Pet Nutrition, Inc., Topeka, KS) A/D at 25 ml every 3 days. There was a moderate amount of postoperative swelling at the incision over the orbit. This was treated with warm wet compresses daily, and the swelling decreased. The snake passed away 2 months after surgery.

The histopathological evaluation and primary culture were performed at the University of Georgia, Veterinary Diagnostic and Investigational Laboratory, Tifton, GA. Both masses consisted of multifocal-to-coalescing granulomas. The granulomas had central regions of amorphous eosinophilic and occasional cellular debris, surrounded by an inflammatory cell infiltrate consisting of histiocytes, lymphocytes, and occasional heterophils (Fig. 1B). Mild concentric fibrosis surrounding these areas was observed. Moderate numbers of hyphae and closely segmented arthroconidiating hyphae were found primarily within the centers of the granulomas. The hyphae were 3 to 7 μm broad, parallel walled, segmented, and occasionally branching. Similar fungal structures were also observed with the use of a Grocott-Gomori methenamine silver stain (Fig. 1C).

Routine bacterial and fungal cultures were performed with the tissue sample. For fungal culture, a portion of the sample was inoculated on Sabouraud dextrose agar (Remel, Lenexa, KS) and incubated at 29°C for 4 weeks. Bacterial cultures were negative. A moderate-to-heavy and pure growth of a fungus was observed on fungal medium. Colonies were white and had sterile septate hyphae, and no fruiting bodies were present. The fungus was unidentifiable by conventional laboratory techniques. The isolate was forwarded to the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, TX, for morphological iden-

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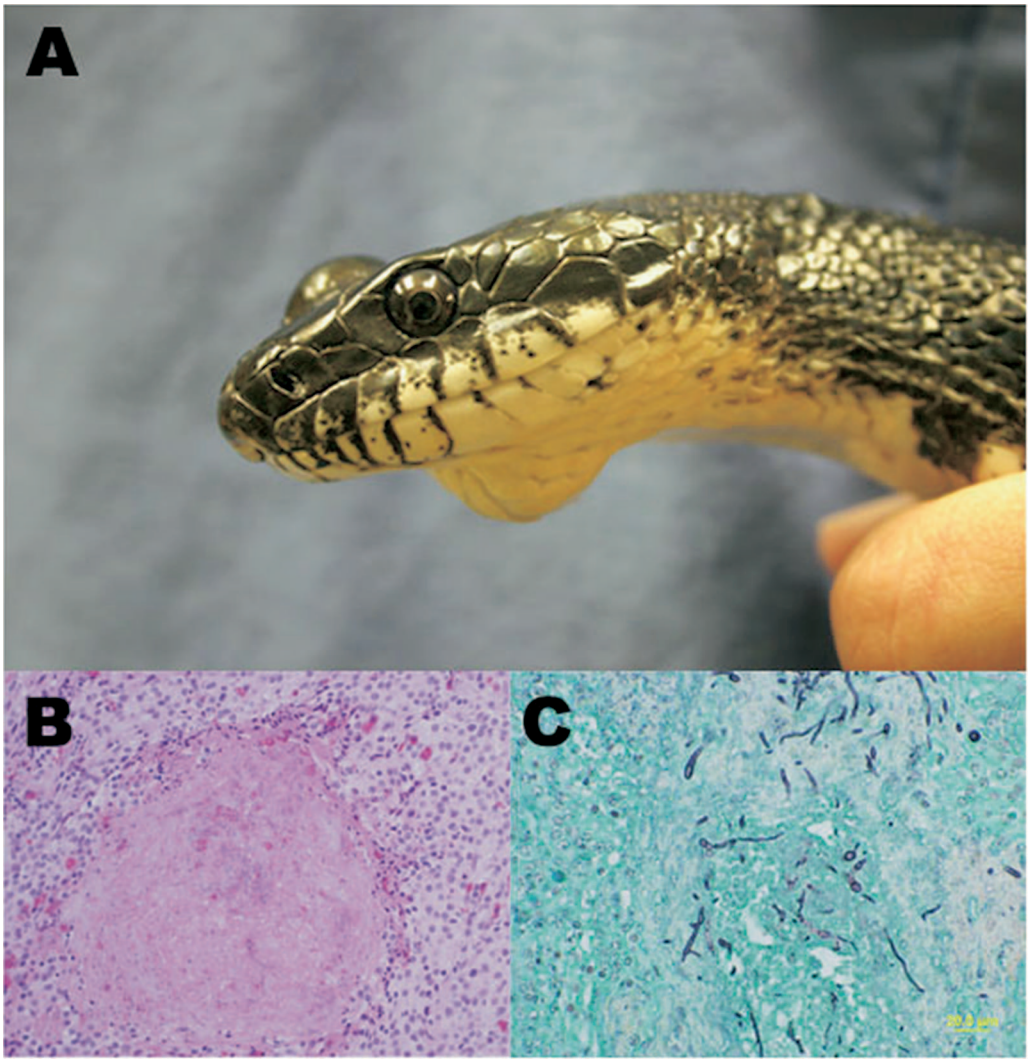

FIG. 1. (A) Cutaneous masses; (B) hematoxylin and eosin-stained section of the lesion; (C) Grocott-Gomori methenamine silver-stained section of the lesion.

tification and accessioned into the culture collection as UTHSC 07-604.

On potato flake agar (prepared in-house) (10) at 23°C, the colonies were white to pale yellow, with a similarly colored reverse side; were velvety to granular with age; resembled *Chrysosporium* colonies microscopically; displayed conidia borne on stalks as well as arthroconidia; and produced a strong, pungent odor. As the isolate did not appear to morphologically match any known *Chrysosporium* species, it was submitted to the Department of Microbiology and Immunology for molecular characterization under accession number R-3923.

The internal transcribed spacer (ITS) and D1-D2 regions were amplified using a DNA preparation methodology, primers, and PCR conditions as previously described (5, 12). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced at the Advanced Nucleic Core Facility of the University of Texas. Each sequence was then used to search GenBank, using the BLASTn algorithm at http://www.ncbi.nlm.nih.gov/. Sequencing of the ITS (614 bp in length; GenBank accession no. EU715819) and D1-D2 (486 bp in length; GenBank accession no. EU715820) regions failed to provide an unequivocal identification, as the closest D1-D2 maximum identity was 93% (*Onygena corvina*; GenBank accession no. AB075355) and the closest ITS maximum identity was 84% (*Arthroderma multifidum*; GenBank accession no. AB361651). However, sequence data confirmed the association of the clinical isolate with the onygenalean fungi. As the percentages of similarity with all the sequences deposited in GenBank were very low, a conclusive identity could not be made. The isolate was forwarded to the Mycology Unit at Rovira i Virgili University in Reus, Spain, where further extensive morphological and molecular phylogenetic studies were undertaken to characterize this fungus.

The morphological description of the present isolate is as follows. Colonies on potato carrot agar (PCA; 20 g potato, 20 g

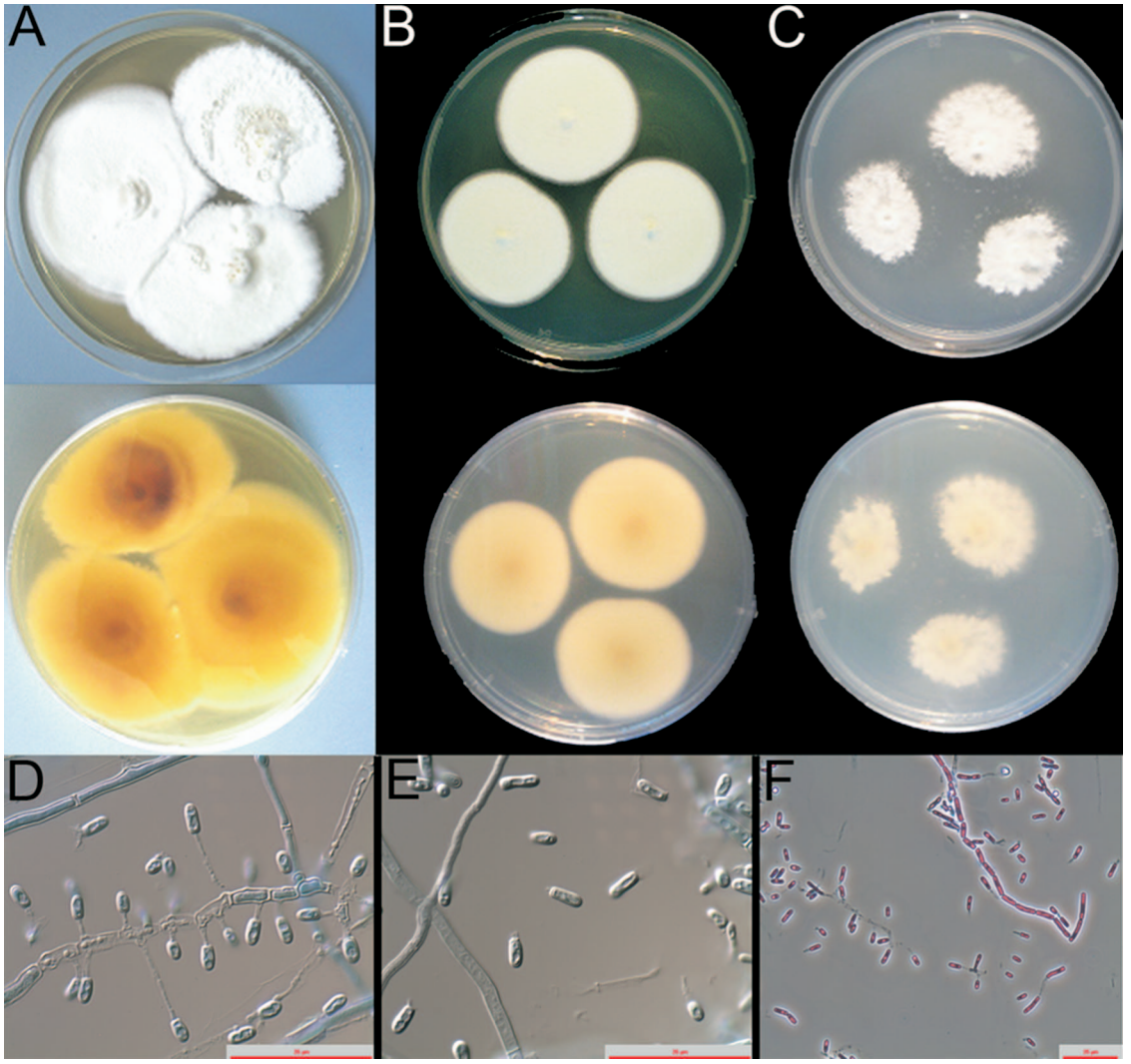

FIG. 2. Colonial and microscopic morphology of *Chrysosporium ophiodiicola* R-3923. (A) PYE, front and reverse; (B) potato dextrose agar, front and reverse; (C) PCA, front and reverse; (D) fertile hyphae and conidia; (E) conidia showing remnants of wall following rhexolytic dehiscence; (F) fertile hyphae with arthroconidia and terminal and lateral conidia.

carrot, 15 g agar, 1 liter water) (Fig. 2C) attained 27- to 29-mm diameters in 14 days at 25°C and were white, with an uncolored reverse side. They were felty, plane, and fimbriate, with a poorly defined margin. Sparse tufts of aerial mycelium were present on the submarginal zone. Vegetative hyphae were hyaline, branched, septate, smooth, and thin walled. They were 1.5 to 2.5 μm wide and often disarticulated at maturity to form cylindrical, 7.5- to 10- by 2- to 2.5 (3)-μm arthroconidia adjacent to each other. Fertile hyphae arise as lateral branches. Terminal and lateral conidia were borne on straight or flexuous side branches of variable length (4.5 to 16 μm) or were sometimes sessile. Conidia were unicellular, solitary, thin walled, smooth, hyaline to pale yellow, and cylindrical to slightly clavate (4.0 to 6.5 (9) by 2.0 to 3.0 μm) and were released by rhexolytic dehiscence, with broad and long basal scarring (Fig. 2D to F). Intercalary, solitary conidia were often present, similar to the terminal and lateral ones. Racquet hyphae were scarce, and chlamydospores were not observed. On potato dextrose agar (Difco Laboratories, Detroit, MI), the fungus grew more quickly and produced denser colonies, 31 to 35 mm in diameter, in 14 days at 25°C (Fig. 2B). They were white to pale yellow, buff after 1 month, and powdery, with droplets of colorless or light yellow exudates at the periphery. On phytone-yeast extract agar (PYE; BBL, Cockeysville, MD), the colonies had 32- to 39-mm diameters in 14 days at 25°C (Fig. 2A), and they were white and light yellow at the center, powdery, and dense, with the presence of droplets of colorless exudate at the center and a light brown reverse side. On oatmeal agar (30 g oat flakes, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 15 g agar, 1 liter tap water), the colonies were similar to those on PCA. The fungus had a very restricted growth at 15°C (5-mm diameter in 14 days). At 37°C, there was no growth. The colonies produced a strong, pungent (skunklike) odor after 1 month of incubation in all the media tested. Attempts to induce the teleomorph on oatmeal agar and sterile garden soil to which horse hair had been added were unsuccessful after 2

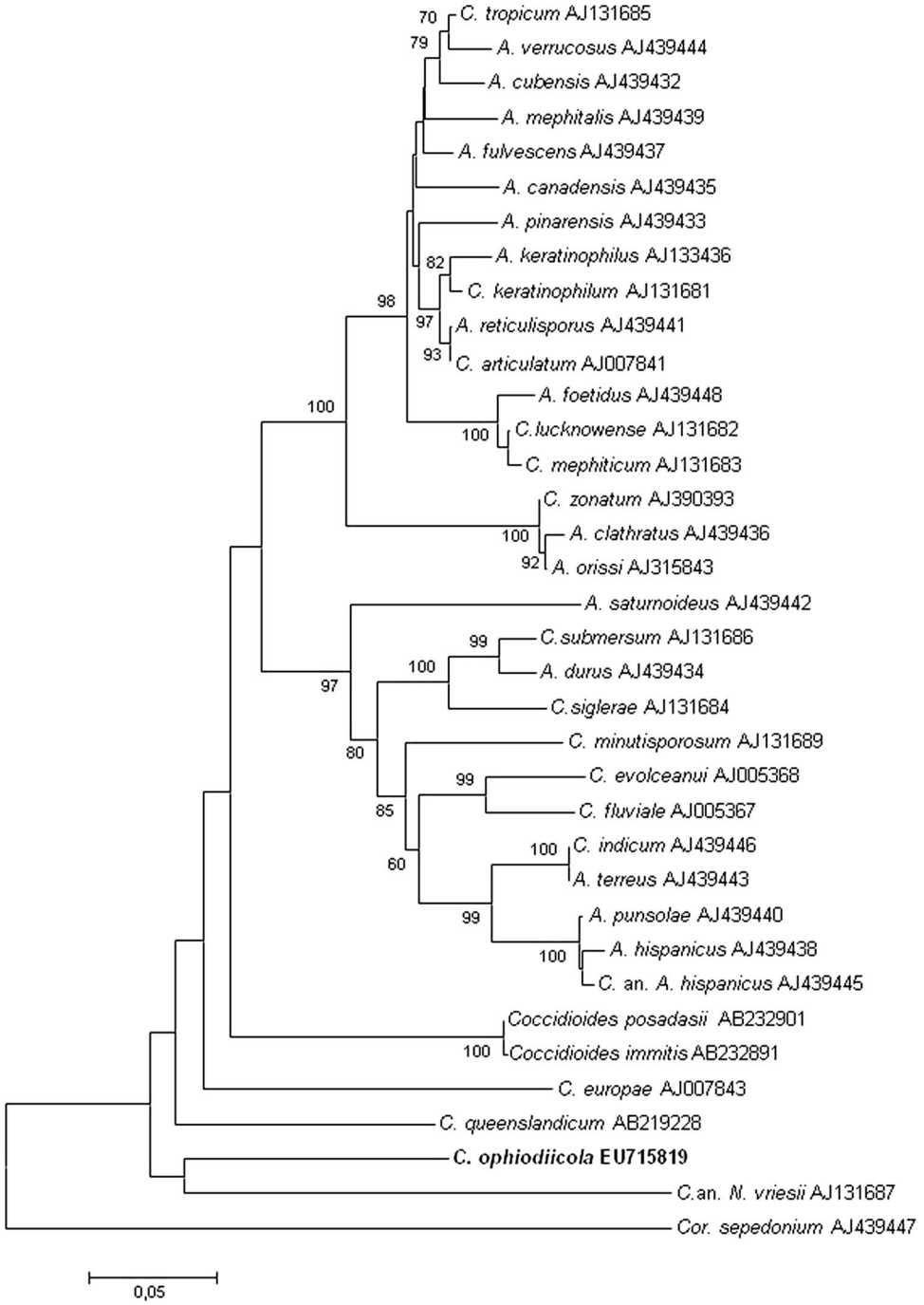

FIG. 3. Neighbor-joining tree based on Kimura two-parameter corrected nucleotide distances among ITS1-5.8s. ITS2 ribosomal DNA sequences of the species are compared with those of *Chrysosporium ophiodiicola*. Branch lengths are proportional to distance. Bootstrap replication frequencies over 70% (1,000 replications) are indicated on the nodes. Abbreviations: *A.*, *Aphanoascus*; *C.*, *Chrysosporium*; *Cor.*, *Corynascus*; *N.*, *Nannizziopsis*; an., anamorph.

months of incubation at 25°C. However, a strong keratinolytic activity was noticed.

The main characteristics of the snake isolate were the presence of numerous narrow, cylindrical-to-slightly clavate conidia and the strong, pungent odor of the colonies. This odor is not rare in the Onygenales, since strains of other species, such as *Chrysosporium mephiticum* Sigler and *Aphanoascus mephitalis* (Malloch & Cain) Cano & Guarro, show similar characteristics (12). However, these species can be easily differentiated from the present fungus by their morphology; *C. mephiticum* has pyriform-to-subglobose conidia occurring more or less synchronously, and *A. mephitalis* usually produces the teleomorph in culture and has a *Malbranchea* anamorph. In addition, these species show very different ITS sequences (4, 6,

14) (Fig. 3). Narrow cylindrical conidia are also produced by *Chrysosporium europae* Sigler, Guarro & Punsola. But this species can be easily differentiated from the new species by its characteristic vinaceous-buff-pigmented colonies on PYE and the absence of a strong, pungent odor (11).

The combined morphological, cultural, and molecular characteristics of the snake isolate do not correspond to any of the species within the genus *Chrysosporium* described to date. Thus, the following new species is proposed.

Chrysosporium ophiodiicola Guarro, D. A. Sutton, Wickes, and Rajeev, sp. nov. Etymology: from the Greek ophio, snake. Ad fungos conidiales, hyphomycetes pertinens. Coloniae in agaro cum decocto tuberorum et carotarum post 14 dies ad 25°C, 27- ad 29-mm diametro celeriter crescentes, planae, albae; reversum hyalinae. Coloniae in agaro cum decocto tuberorum post 14 dies ad 25°C, 31- ad 35-mm diametro; in agaro phytone extracto levedinis post 14 dies at 25°C, 32- ad 39-mm diametro. Ad 37°C incrementum nullum. Odor foetidus. Hyphae hyalinae vel subhyalinae, leviter ramosae, septatae, 1.5- ad 2.5-μm latae. Conidia terminalia et lateralia sessilia vel in ramae laterales, cylindrica vel clavatae, hyalina vel lutea, leviatunicata, 4.0 ad 6.5 (9) per 2.0 ad 3.0 μm; arthroconidia hyalina vel lutea, leviatunicata, cylindrica, 7.5 ad 10 per 2 ad 2.5 (3) μm. Chlamydosporae absunt. Teleomorphosis ignota. Species keratinolytica cultura typica: ex ophio pelle. In collectione fungorum CBS 122913 deposita est. Isotypus FMR 9510, UTHSC 07-604.

Phylogenetic analysis of the ITS region of *C. ophiodiicola* and other related onygenalean fungi was performed with MEGA 2.1 software (7), using the neighbor-joining method and basing the analysis on Kimura's two-parameter corrected nucleotide distances. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* was the species nearest to *C. ophiodiicola* in the ITS neighbor-joining tree (Fig. 3). Both species are associated with infections in reptiles.

Chrysosporium ophiodiicola was isolated from a subcutaneous granuloma of a snake, which is not an unusual source for recovering chrysosporia. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* has been isolated from cases of dermatitis in snakes (2, 15), chameleons (9), crocodiles (13), and bearded dragons (3) and from a nasal granuloma in an Ameiva lizard (8). In a recent report, a *Chrysosporium* species related to *Nannizziopsis vriesii* was isolated from a case of cutaneous hyalohyphomycosis from two green iguanas (1). Phenotypically, *C. ophiodiicola* can be separated from the *Chrysosporium* anamorph of *Nannizziopsis vriesii* by the absence of asperulate fertile hyphae and globose-to-pyriform conidia sometimes grouped in clusters and the presence of an odor in the colonies of the former.

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