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Occurrence of *Ochroconis* and *Verruconis* Species in Clinical Specimens from the United States

Alejandra Giraldo,[a] Deanna A. Sutton,[b] Kittipan Samerpitak,[c,d] G. Sybren de Hoog,[c] Nathan P. Wiederhold,[b] Josep Guarro,[a] Josepa Gené[a]

Unitat de Micologia, Facultat de Medicina i Ciències de la Salut and IISPV, Universitat Rovira i Virgili, Reus, Spain[a]; Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA[b]; CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands[c]; Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand[d]

***Ochroconis* is a dematiaceous fungus able to infect immunocompetent people. Recently, the taxonomy of the genus has been reevaluated, and the most relevant species, *Ochroconis gallopava*, was transferred to the new genus *Verruconis*. Due to the important clinical implications of these fungi and based on the recent classification, it was of interest to know the spectra of *Ochroconis* and *Verruconis* species in clinical samples received in a reference laboratory in the United States. A set of 51 isolates was identified morphologically and molecularly based on sequence analyses of the nuclear ribosomal RNA (nrRNA), actin, and β-tubulin genes. *Verruconis gallopava* was the most common species (68.6%), followed by *Ochroconis mirabilis* (21.5%). One isolate of *Ochroconis cordanae* was found, being reported for the first time in a clinical setting. The most common anatomical site of isolation was the lower respiratory tract (58.8%), followed by superficial and deep tissues at similar frequencies (21.6 and 19.6%, respectively). Interestingly, three new species were found, which are *Ochroconis olivacea* and *Ochroconis ramosa* from clinical specimens and *Ochroconis icarus* of an environmental origin. The *in vitro* antifungal susceptibilities of eight antifungal drugs against the *Ochroconis* isolates revealed that terbinafine and micafungin were the most active drugs.**

Ochroconis is a dematiaceous anamorphic genus described by de Hoog and von Arx (1) to accommodate species with slow to moderate growth, brown to olivaceous colonies, brownish conidiophores, and septate, dark-pigmented, and rough-walled conidia, which are produced by sympodial conidiogenesis and liberated rhexolytically (1–3). The species of the genus have a cosmopolitan distribution and are isolated from different sources, i.e., soil, decaying vegetable material (4, 5), indoor and outdoor environments (6), cave rocks, and Paleolithic paintings (7, 8). Due to the thermotolerance of some species, it is common to find them in thermal soils (9–12), hot spring effluents (11, 13), sewage from nuclear power plants, coal waste piles (14–16), and broiler-house litters (17, 18). Some species have been reported to be opportunistic pathogens in humans, producing localized infections in the brain and lungs, as well as subcutaneous and systemic infections, sometimes with fatal outcomes (13, 19–23). These organisms also cause infections in birds (18, 24–27), cats (28, 29), and dogs (30). The type species of the genus, *Ochroconis constricta*, was initially described by Abbott (31) as a species of *Scolecobasidium*. However, Samerpitak et al. (32) reviewed these fungi, and *Scolecobasidium* was abandoned, since the original material of the type species, *Scolecobasidium terreum* (strain CBS 203.27), was found to be of doubtful identity. In the same study, the thermophilic species with light to dark brown and verrucose to coarsely ornamented conidia, such as *Ochroconis gallopava*, *Ochroconis calidifluminalis*, and *Ochroconis verrucosa*, were transferred to the new genus *Verruconis*. The mesophilic species with subhyaline, smooth-walled to verruculose conidia and commonly associated with infections in cold-blooded animals were retained in *Ochroconis* (32). Both *Verruconis* and *Ochroconis* were located within the *Sympoventuriaceae* family in the recently described order *Venturiales* (*Dothideomycetes*) (33).

Due to the clinical relevance of these fungi and the recent taxonomical studies involving them, it was of interest to assess the spectra of the species of *Verruconis* and *Ochroconis* in clinical samples received by a reference center in the United States. Because little is known about the antifungal susceptibility of *Ochroconis*, we have determined the *in vitro* activity to the clinically available antifungal drugs against the *Ochroconis* species identified in the present study.

MATERIALS AND METHODS

Fungal isolates. Fifty-one clinical isolates (Table 1) received at the Fungus Testing Laboratory at the University of Texas Health Science Center (UTHSC) at San Antonio, TX, were investigated in this study. Several type and reference strains provided by the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) were also included in the study.

Phenotypic studies. Morphological characterization of the isolates was done on oatmeal agar (OA) (30 g of filtered oat flakes after 1 h of simmering, 20 g of agar, distilled water to final volume of 1,000 ml), potato carrot agar (PCA) (20 g each of filtered potatoes and carrots, 20 g of agar, distilled water to final volume of 1,000 ml), 2% malt extract agar (MEA 2%) (10 g of malt extract, 20 g of agar, distilled water to final volume of 1,000 ml), and potato dextrose agar (PDA) (Pronadisa, Madrid, Spain). The cultures were incubated at 25°C in the dark and examined after 4 weeks. The colony diameters were measured after 14 days of growth, and colony colors were determined using the color charts of Kornerup and Wanscher (34). In addition, the ability of the isolates to grow at 4, 15, 30, 32, 33, 35, 37, 40, and 42°C was tested on PDA. Micro-

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Address correspondence to Josepa Gené, josepa.gene@urv.cat.

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TABLE 1 Strains included in the study

Species	Strain[a]	Origin[b]	GenBank accession no.[c]				
			ITS	LSU-D1/D2	*ACT1*	*BT2*	SSU
Ochroconis anellii	CBS 284.64[T]	Stalactite, Italy	FR832477	KF156138	KF155912	KF156184	KF156070
Ochroconis anomala	CBS 131816[T]	Lascaux Cave, France	HE575201	KF156137	KF155935	KF156194	KF156065
Ochroconis constricta	CBS 202.27[T]	Soil, USA	AB161063	KF156147	KF155942	KF156161	KF156072
	CBS 211.53	Soil, Canada	HQ667519	KF156148			
	FMR 3906	Goat dung, Spain	**LM644509**	**LM644552**			
Ochroconis cordanae	CBS 475.80[T]	Dead leaf, Colombia	KF156022	KF156122	HQ916976	KF156197	KF156058
	CBS 780.83	*Podocarpus* litter, Japan	HQ667539	KF156120			
	UTHSC 10-1875	Tissue of left thigh, USA	**LM644510**	**LM644553**			
Ochroconis gamsii	CBS 239.78[T]	Plant leaf, Sri Lanka	KF156019	KF156150	KF155936	KF156190	KF156088
Ochroconis humicola	CBS 116655[T]	Peat soil, Canada	HQ667521	KF156124	KF155904	KF156195	KF156068
Ochroconis icarus (= *Ochroconis* sp. III)	CBS 423.64	Rhizosphere of *Solanum tuberosum*, the Netherlands	HQ667523	KF156131	KF155943	KF156173	KF156085
	CBS 536.69[T]	Forest soil, Canada	HQ667524	KF156132	KF155944	KF156174	KF156084
	CBS 116645	Sandy soil, Canada	HQ667525		**LM644599**	**LM644604**	KF156083
Ochroconis lascauxensis	CBS 131815[T]	Stain in Lascaux Cave, France	FR832474	KF156136	KF155911	KF156183	KF156069
Ochroconis longiphorum	CBS 435.76	Soil, Canada	KF156038	KF156135	KF155908	KF156182	KF156060
Ochroconis minima	CBS 510.71[T]	Rhizosphere, Nigeria	HQ667522	KF156134	KF155945	KF156172	KF156087
	CBS 119792	Soil, India	KF156027	KF156133	KF155946	KF156175	KF156086
Ochroconis mirabilis	CBS 729.95[T]	Regulator of diver, the Netherlands	KF156029	KF156144	KF155948	KF156171	KF156082
	UTHSC 01-1570	Nail, USA	**LM644511**	**LM644554**			
	UTHSC 03-1114	BAL fluid, USA	**LM644512**	**LM644555**			
	UTHSC 04-2378	BAL fluid, USA	**LM644513**	**LM644556**			
	UTHSC 02-232	BAL fluid, USA	**LM644514**	**LM644557**			
	UTHSC 03-3089	Skin with impetigo, USA	**LM644515**	**LM644558**			
	UTHSC 05-1500	BAL fluid, USA	**LM644516**	**LM644559**			
	UTHSC 07-3073	Toenail, USA		**LM644560**			
	UTHSC 08-1958	Toenail, USA	**LM644517**	**LM644561**			
	UTHSC 10-1519	Skin, USA	**LM644518**				
	UTHSC 11-2020	BAL fluid, USA	**LM644519**	**LM644562**			
	UTHSC 11-3523	BAL fluid, USA	**LM644520**	**LM644563**			
Ochroconis olivacea (= *Ochroconis* sp. I)	UTHSC 10-2009[T]	BAL fluid, USA	**LM644521**	**LM644564**	**LM644600**	**LM644605**	**LM644548**
Ochroconis ramosa (= *Ochroconis* sp. II)	UTHSC 03-3677	Skin, USA	**LM644522**	**LM644565**	**LM644601**	**LM644606**	**LM644549**
	UTHSC 04-2729	BAL fluid, USA	**LM644523**	**LM644566**	**LM644602**	**LM644607**	**LM644550**
	UTHSC 12-1082[T]	Nail, USA	**LM644524**	**LM644567**	**LM644603**	**LM644608**	**LM644551**
Ochroconis sexualis	CBS 135765[T]	Domestic, South Africa	KF156018	KF156118	KF155902	KF156189	KF156089
Ochroconis tshawytschae	CBS 100438[T]	Fish, USA	HQ667562	KF156126	KF155918	KF156180	KF156062
Ochroconis verrucosa	CBS 225.77	Leaf, Burma	HQ667564	KF156130	KF155909	KF156186	KF156066
	CBS 383.81[T]	Soil, India	KF156015	KF156129	KF155910	KF156185	KF156067
Ochroconis sp.	CBS 119644	Indoor sample, Germany	KF961086	KF961097	KF956086	KF961065	KF961108
Scolecobasidium excentricum	CBS 469.95[T]	Leaf litter, Cuba	HQ667543	KF156105			
Veronaeopsis simplex	CBS 588.66	Leaf litter, South Africa	KF156041	KF156103			
Verruconis calidifluminalis	CBS 125817	Hot spring river, Japan	AB385699	KF156107			
	CBS 125818[T]	Hot spring river, Japan	AB385698	KF156108	KF155901	KF156202	KF156046

(Continued on following page)

TABLE 1 (Continued)

Species	Strain[a]	Origin[b]	GenBank accession no.[c]				
			ITS	LSU-D1/D2	*ACT1*	*BT2*	SSU
Verruconis gallopava	CBS 437.64[T]	Turkey brain, USA	HQ667553	KF156112	HQ916989	KF156203	KF156053
	UTHSC 04-1355	Sputum, USA					
	UTHSC 03-447	Sputum, USA	**LM644525**	**LM644568**			
	UTHSC 04-43	Bronchial wash, USA	**LM644526**	**LM644569**			
	UTHSC 04-236	Brain, USA	**LM644527**	**LM644570**			
	UTHSC 04-539	Canine liver, USA	**LM644528**	**LM644571**			
	UTHSC 04-2693	Brain abscess, USA		**LM644572**			
	UTHSC 05-1018	Brain abscess, USA		**LM644573**			
	UTHSC 06-513	Sputum, USA	**LM644529**	**LM644574**			
	UTHSC 06-541	Sputum, USA					
	UTHSC 06-3565	Canine abdominal fluid, USA	**LM644530**	**LM644575**			
	UTHSC 06-4445	Right hand abscess, USA	**LM644531**	**LM644576**			
	UTHSC 07-153	Left lower lobe lung Bx, USA		**LM644577**			
	UTHSC 07-212	Induced sputum, USA	**LM644532**	**LM644578**			
	UTHSC 07-623	Canine L2-L3 disc, USA	**LM644533**	**LM644579**			
	UTHSC 07-2994	Sputum, USA	**LM644534**	**LM644580**			
	UTHSC 08-40	Bronchial wash, USA	**LM644535**	**LM644581**			
	UTHSC 08-112	Sputum, USA	**LM644536**	**LM644582**			
	UTHSC 08-657	Feline wound, USA	**LM644537**	**LM644583**			
	UTHSC 08-810	Sputum, USA	**LM644538**	**LM644584**			
	UTHSC 08-1340	Bronchial wash, USA	**LM644539**	**LM644585**			
	UTHSC 08-1625	Right hip, USA		**LM644586**			
	UTHSC 08-1756	Sputum, USA					
	UTHSC 08-3158	Bronchial wash, USA		**LM644587**			
	UTHSC 09-1229	Ear/mastoid, USA	**LM644540**	**LM644588**			
	UTHSC 09-3111	Lung, USA		**LM644589**			
	UTHSC 10-510	Brain abscess, USA	**LM644541**	**LM644590**			
	UTHSC 10-1509	Bronchial wash, USA	**LM644542**	**LM644591**			
	UTHSC 10-1541	Bronchial wash, USA	**LM644543**	**LM644592**			
	UTHSC 10-3013	Sputum, USA	**LM644544**	**LM644593**			
	UTHSC 11-315	Sputum, USA		**LM644594**			
	UTHSC 11-509	Bronchial wash, USA	**LM644545**	**LM644595**			
	UTHSC 11-2401	Bronchial wash, USA					
	UTHSC 11-2569	Bronchial wash, USA		**LM644596**			
	UTHSC 12-69	Bronchial wash, USA	**LM644546**	**LM644597**			
	UTHSC 12-549	Bronchial wash, USA	**LM644547**	**LM644598**			
Verruconis verruculosa	CBS 119775	Plant root, *Hevea* sp., Malaysia	KF156041	KF156103	KF155919	KF156193	KF156055

[a] CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; FMR, Faculty of Medicine Reus, Spain; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; [T], type strain.
[b] BAL, bronchoalveolar lavage; Bx, biopsy.
[c] The accession numbers of sequences newly determined in this study are indicated in bold type. ITS, internal transcribed spacer regions of the nuclear ribosomal DNA (nrDNA) and intervening 5.8S nrDNA; LSU, large subunit of the nrDNA; *ACT1*, partial actin gene; *BT2*, β-tubulin gene; SSU, small subunit of the nrDNA.

scopic features were examined and measured by making direct wet mounts with 85% lactic acid or by slide cultures on OA and PCA using the light microscope Olympus CH-2 (Olympus Corporation, Tokyo, Japan). Photomicrographs were made with a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany), using Nomarski differential interference contrast. The 95% confidence intervals were derived from 50 observations (×1,000 magnification), with the extremes given in parentheses. The ranges of the dimensions of other characters are given in the descriptions of new taxa.

DNA extraction, amplification, and sequencing. The isolates were grown on YES agar (20 g of yeast extract, 150 g of sucrose, 20 g of agar, distilled water to final volume of 1,000 ml) for 10 days at 25°C. DNA extraction was done by using FastDNA kit protocol (MP Biomedicals, Solon, OH), with the homogenization step done with a FastPrep FP120 cell disrupter (Thermo Savant, Holbrook, NY). The 18S nuclear small subunit (nuSSU), the internal transcribed spacer regions (ITS), including the 5.8S small subunit gene, and the D1/D2 domains of the 28S nuclear large subunit (nuLSU) were amplified with the primer pairs NS1/NS4, ITS5/ITS4, and NL1/NL4b or LR0R/LR5, respectively (35–37). The fragments of the actin (*ACT1*) and β-tubulin (*BT2*) genes were amplified using the primers ACT-512F/ACT-783R and Bt1a/Bt1b, respectively (38, 39). The amplified fragments were purified and sequenced at Macrogen Corp. Europe (Amsterdam, the Netherlands) with a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequencing was performed with the same primers used for amplification to ensure good-quality sequences over the total length of the amplicon. Consensus sequences were obtained using SeqMan version 7.0.0 (DNAStar, Madison, WI). Some ITS, D1/D2, *ACT1*, and *BT2* sequences corresponding to several species of *Ochroconis* or *Verruconis* were retrieved from GenBank (32, 33) and included in the phylogenetic study (Table 1).

Phylogenetic analysis. Multiple sequence alignments using the Clustal W and MUSCLE applications (40, 41) were made in MEGA version 5.05 (42), in which the best substitution models were searched for each locus and for the combined data set, and maximum likelihood (ML) and maximum parsimony (MP) analyses were also performed. For ML, gaps and missing data were treated as a partial deletion with a site coverage cutoff 95%, and nearest-neighbor interchange (NNI) was used as the heuristic method. Internal branch support was assessed by a search of 1,000 bootstrapped sets of data. A bootstrap support (BS) of ≥70 was considered significant. The phylogenetic distance values between the isolates were estimated with Kimura 2-parameter as a nucleotide substitution model under MEGA version 5.05. For the multilocus analysis, a phylogenetic analysis using a Markov chain Monte Carlo (MCMC) algorithm was done with MrBayes version 3.1.2 (43) on the CIPRES portal (http://www.phylo.org), with two simultaneous runs for 10 million generations, with a sampling frequency of 1,000 trees. A burn-in tree sample of 10% was discarded. Bayesian posterior probabilities (PP) were obtained from the 50% majority-rule consensus of trees. A PP value of ≥0.95 was considered significant. The congruencies of the sequence data sets for the separate loci were determined using tree topologies of 70% reciprocal neighbor-joining (NJ) bootstrap trees with maximum likelihood distances, which were compared visually to identify conflicts between the partitions (44). *Scolecobasidium excentricum* strain CBS 469.95 and *Veronaeopsis simplex* strain CBS 588.66 were used as outgroups.

Antifungal susceptibility. The *in vitro* activities of amphotericin B (AMB), itraconazole (ITC), posaconazole (PSC), voriconazole (VRC), anidulafungin (AFG), caspofungin (CFG), micafungin (MFG), and terbinafine (TBF) were determined for all the clinical isolates of *Ochroconis*, according to the methods outlined in the CLSI document M38-A2 (46) but with an incubation temperature of 30°C. The minimal effective concentration (MEC) was determined at 48 h for the echinocandins, and the MICs at 48 h and 72 h were determined for the remaining drugs. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth for AMB, ITC, PSC, and VRC and an 80% reduction in growth for TBF. *Paecilomyces variotii* strain ATCC MYA-3630 and *Aspergillus fumigatus* strain ATCC MYA-3626 were used as quality control strains. Statistical analyses of the data were done using the Kruskal-Wallis test in GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as a P value of ≤0.05 (two-tailed).

Nucleotide sequence accession numbers. All novel DNA sequences were deposited in GenBank under accession numbers shown in bold type in Table 1, and taxonomic novelties were deposited in MycoBank (http://www.MycoBank.org) (45).

RESULTS

Phylogenetic analysis. The D1/D2 analysis (Fig. 1) showed that 68.6% and 31.4% of the isolates studied were distributed in two main groups corresponding to *Ochroconis* and *Verruconis*, respectively. The *Verruconis* isolates grouped with high support in the same clade as the ex-type strain of *V. gallopava* (CBS 437.64). Eleven of the clinical isolates of *Ochroconis* were nested with the ex-type strain of *O. mirabilis* (CBS 729.95) and one with the ex-type strain of *O. cordanae* (CBS 475.80). Three clinical isolates (UTHSC 03-3677, UTHSC 12-1082, and UTHSC 04-2729), morphologically similar to *Ochroconis minima*, constituted a clade that was phylogenetically distant from the type strain of that species. Although the isolate UTHSC 10-2009 clearly showed the differential features of *Ochroconis*, it did not group with any of the species of that genus included in this study, including the reference strain CBS 536.69, which was received as *O. minima*.

With the aim of confirming and clarifying the data obtained in the D1/D2 analysis, a multilocus analysis of five concatenated gene regions (nuSSU, ITS, nuLSU, *ACT1*, and *BT2*) was performed, including the type and some reference strains of the currently accepted *Ochroconis* species. The concatenated sequence consisted of 4,188 bp (Fig. 2), of which 1,000 were parsimony informative (51 nuSSU, 127 LSU, 432 ITS, 175 *ACT1*, and 215 *BT2*) and revealed the formation of 17 lineages, 13 of them corresponding to known *Ochroconis* species and three representing putative new species (*Ochroconis* sp. I, *Ochroconis* sp. II, and *Ochroconis* sp. III). *Ochroconis* sp. I (strain UTHSC 10-2009) was located in the same clade as that of two reference strains of *Ochroconis verrucosa*, including the ex-type strain (CBS 383.81), although with significant phylogenetic distance. The clade corresponding to *Ochroconis* sp. II consisted of three clinical isolates (UTHSC 03-3677, UTHSC 12-1082, and UTHSC 04-2729), while *Ochroconis* sp. III included three environmental reference strains all previously identified as *O. minima* (CBS 423.64, CBS 536.69, and CBS 116645). The clades representing *Ochroconis* sp. II and *Ochroconis* sp. III were phylogenetically related (6.5% phylogenetic distance in the combined data set) although distinct from the sequences of *O. minima* type strain CBS 510.71 (5.7% and 7.0% phylogenetic distances with *Ochroconis* sp. III and *Ochroconis* sp. II, respectively).

Most of the clinical isolates included in this study were of respiratory origin (58.8%), mainly obtained from bronchoalveolar lavage (BAL) fluid and sputum samples, followed by superficial tissue samples (21.6%), principally from the nails and skin (Table 2). The remaining 19.6% of the isolates were from miscellaneous deep tissue or sterile fluid specimens, with most of them collected from the lungs and brain. *V. gallopava* was recovered from a wide range of clinical specimens, being isolated from all the deep tissues and in equal numbers from sputum and bronchial wash fluids. The isolates of *Ochroconis* spp. were exclusively recovered from respiratory samples (BAL fluid), skin, and nails.

Phenotypic studies. Most of the isolates belonging to the *V. gallopava* clade showed the typical phenotypic characteristics described for the species, i.e., colonies on PDA at 25°C with moderate growth (up to 58 mm diameter after 14 days), brownish gray (5E2) with a diffusible brown pigment, poorly differentiated conidiogenous cells, and pale brown clavate 1-septum conidia constricted at the septum that were (7)10 to 21 μm long by 2.5 to 4.5 μm wide. All the strains showed optimum growth at 42°C (up to 80 mm at 14 days). Some strains showed atypical features not previously reported for the species. Isolate UTHSC 07-623 produced yellowish white (4A2) colonies on all media tested and hyaline conidia. The isolates UTHSC 07-212 and UTHSC 12-549 showed slow growth at 25°C (17 to 20 mm and 7 to 10 mm in 14 days, respectively), and their colonies were radially striate, with a lobulate edge, and moist, with a cerebriform aspect, respectively. Several isolates, apart from the clavate conidia, produced some ellipsoidal conidia with an apiculated base (UTHSC 03-447, UTHSC 04-43, UTHSC 04-236, UTHSC 06-513, UTHSC 07-153, UTHSC 08-112, UTHSC 08-810, UTHSC 10-510, UTHSC 11-509, and UTHSC 12-549). The isolates that grouped in the *O. mirabilis* clade produced slow-growing (20 to 32 mm diameter in 14 days), olive brown (4D4), and velvety colonies on PDA and dark brown (5F8) and dry colonies on OA and PCA; the conidiophores were cylindrical, thick walled, and with denticles distributed sympodially along the conidiophore; the conidia were cylindrical or ellipsoidal 1-septum, slightly constricted at the septum, and pale brown with rugose walls. The isolate UTHSC 10-1875, clustered in the *O. cordanae* clade, displayed long (up to 40 μm), brown, and simple conidiophores, some of them with several

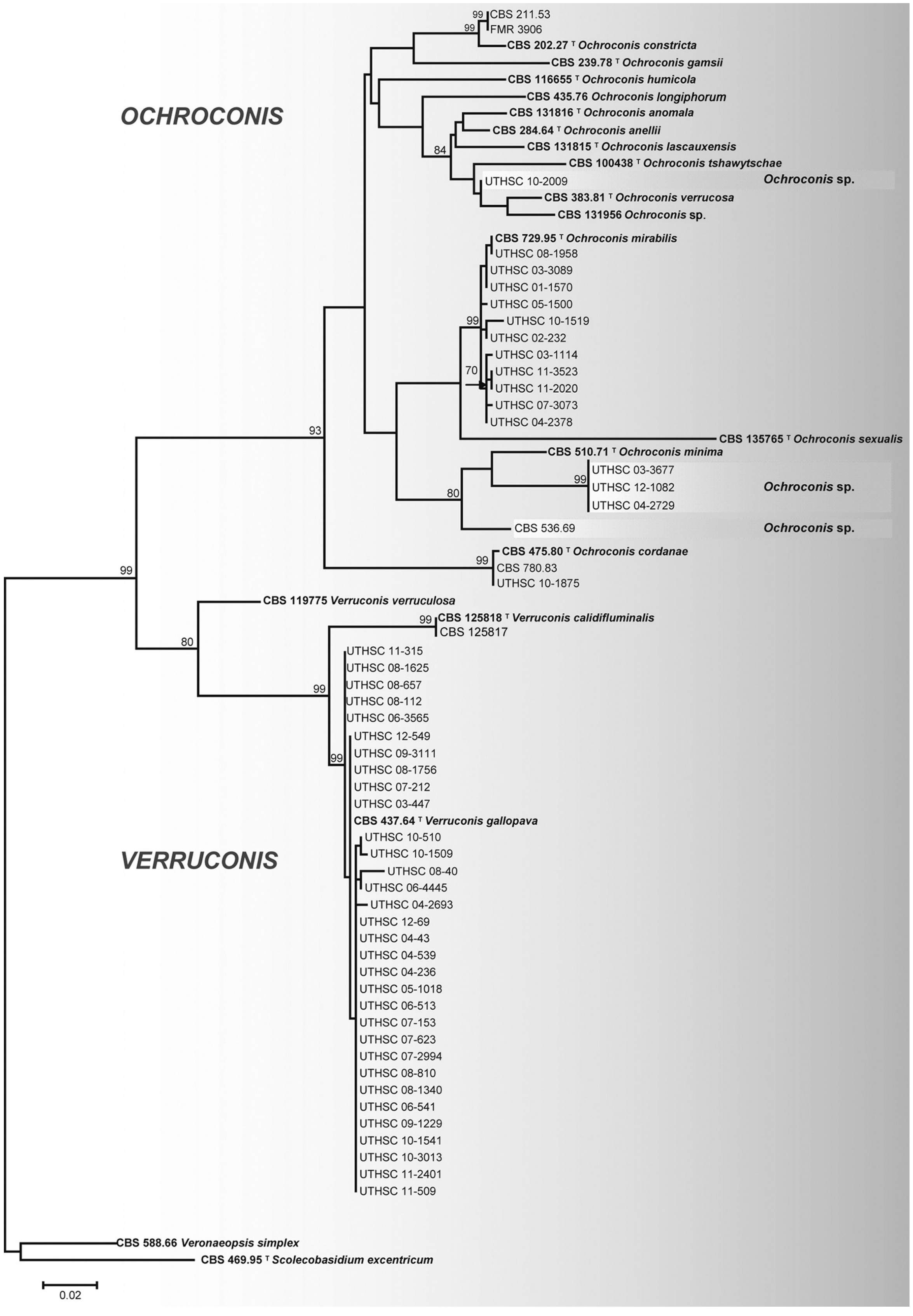

FIG 1 Maximum-likelihood (ML) tree constructed with sequences of the D1/D2 domains of the 28S rRNA gene. Bootstrap support (BS) values of >70% are shown at the nodes. *V. simplex* CBS 588.66 and *S. excentricum* CBS 469.95 were used as outgroup taxa. T, ex-type strains.

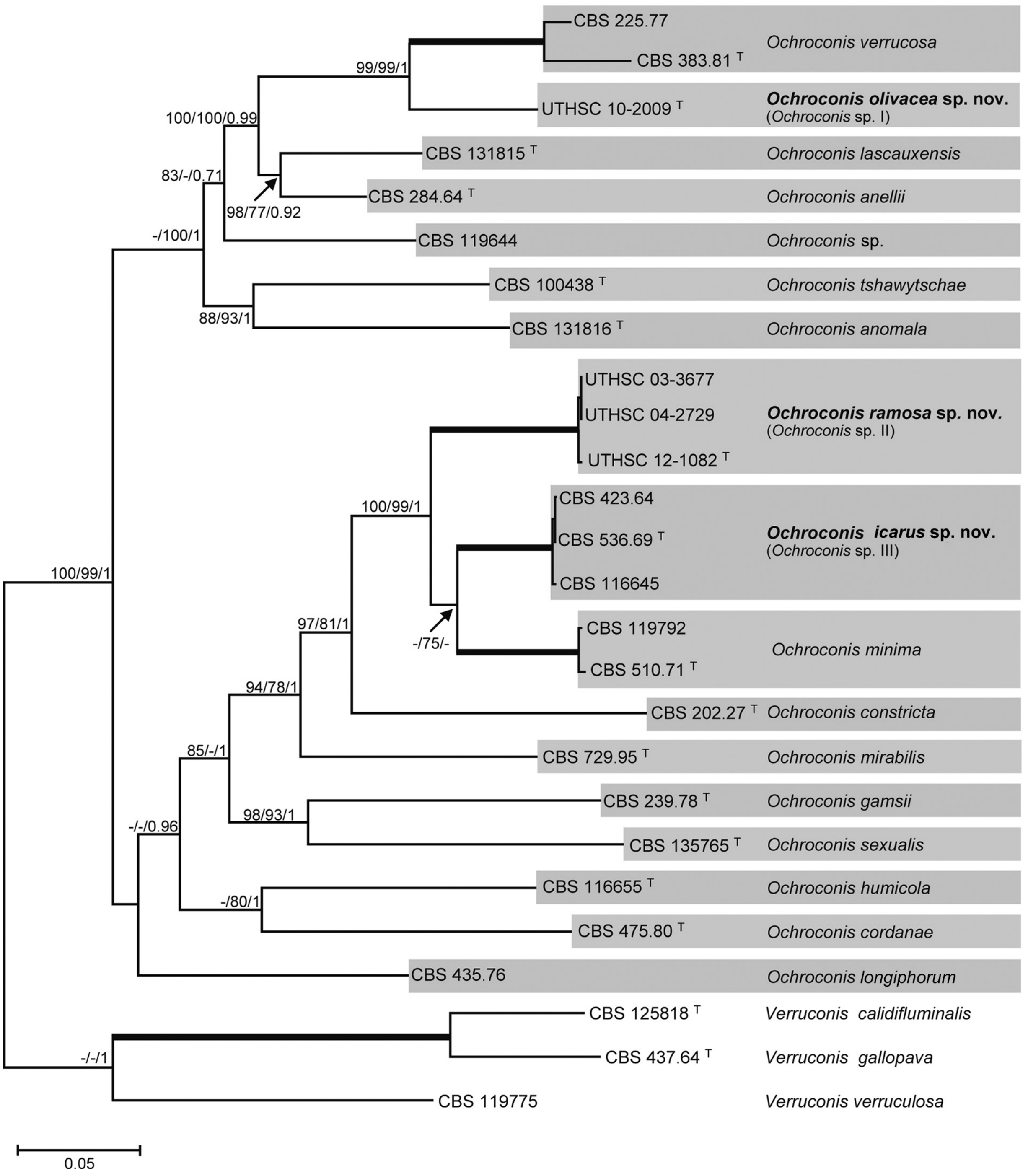

FIG 2 Bayesian tree from a concatenated data set, including the five regions nuSSU, ITS, nuLSU, *ACT1*, and *BT2*. Bootstrap support obtained with maximum-likelihood (left) and maximum parsimony (middle) of >70% and Bayesian posterior probability (right) values of >0.95 are shown at the nodes. T, ex-type strains. Full supported branches are depicted as thick lines.

septa, and were thick walled, with denticles on the apical region; the conidia were ellipsoidal, slightly constricted at the septum, smooth or finely verruculose, and 7 to 13 μm by 3 to 3.5 μm. *Ochroconis* sp. I (isolate UTHSC 10-2009) was mainly characterized by the production of long conidiophores (up to 60 μm) and verrucose broadly ellipsoidal conidia, sometimes slightly apiculated at the base (Fig. 3C and F). Both *Ochroconis* sp. II (Fig. 4) and *Ochroconis* sp. III (Fig. 5) showed phenotypic characteristics similar to those of *O. minima* but with some differences, i.e., in *Ochroconis* sp. II, the conidia had a rough surface (Fig. 4F and G), the conidiophores reached up to 20 μm long, the chlamydospores were smaller (3 to 3.5 μm by 3 to 3.5 μm), and the maximum and minimum temperatures for growth were 35°C and 15°C, respectively; in *Ochroconis* sp. III, the conidia were slightly smaller, with

TABLE 2 Anatomical sources of isolates of *Verruconis* and *Ochroconis* spp. from clinical samples

	No. of isolates obtained from samples from:															
	Superficial tissue (n = 11)						Lower respiratory tract (n = 30)			Deep tissue (n = 10)						
Species	Nails	Skin	Tissue of left thigh	Hand abscess	Wound	Ear	Bronchial wash	BAL fluid[a]	Sputum	Brain	Liver	Lung biopsy sample	Intervertebral disc	Hip	Abdominal fluid	Total no. (%)
V. gallopava	0	0	0	1	1	1	11	0	11	4	1	2	1	1	1	35 (68.6)
O. mirabilis	3	2	0	0	0	0	0	6	0	0	0	0	0	0	0	11 (21.5)
O. ramosa	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	3 (5.9)
O. olivacea	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1 (2)
O. cordanae	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1 (2)
Total	4	3	1	1	1	1	11	8	11	4	1	2	1	1	1	51 (100)

[a] BAL, bronchoalveolar lavage.

a narrower lower cell (up to 2.5 μm wide), and the maximum and minimum temperatures for growth were 33°C and 4°C, respectively.

Antifungal susceptibility. The results of the *in vitro* activities of the antifungal drugs tested are summarized in Table 3. No statistical difference in antifungal activities were observed among the *Ochroconis* spp. studied. Terbinafine was the most active drug against all the species tested, followed by MFG, with MICs and MECs of 0.02 μg/ml and 0.25 μg/ml, respectively. AMB showed poor *in vitro* activity against all the isolates tested, with geometric mean (GM) MICs and MIC_{90}s of 24.5 μg/ml and 32 μg/ml, respectively. Similarly, the three azoles showed very little activity, with elevated MICs.

TAXONOMY

Based on the mentioned phylogenetic data, which correlated with the phenotypic features observed, we concluded that *Ochroconis* sp. I, *Ochroconis* sp. II, and *Ochroconis* sp. III are different from the taxa currently accepted in this genus and are therefore described here as new.

Ochroconis icarus Samerpitak, Giraldo, Guarro, & de Hoog, sp. nov., MycoBank accession no. MB809376 (Fig. 5). Etymology: the conidia look like the mythological figure Icarus, son of Daedalus, who made wings to reach the sun. Diagnosis: it differs from *O. minima* by the production of smaller conidia with a narrower lower cell and a maximum growth temperature of 33°C and from *O. ramosa* mainly by having longer denticles and smooth-walled conidia, with a wider upper cell.

Colonies on OA and PDA at 25°C attaining 16 to 20 mm and 17 to 24 mm diameter after 14 days, respectively; chocolate brown (6F4), flat, slightly curled, velvety at center, membranous at periphery. Colonies on PCA at 25°C attaining 21 to 24 mm diameter after 14 days, dark brown (7F4), flat, membranous becoming velvety. On MEA 2% at 25°C, reaching 13 to 17 mm diameter after 14 days, yellowish brown (5E5), flat, woolly at center. Vegetative hyphae septate, pale brown, smooth and thin walled, 1 to 2 μm wide; anastomosing and coiled hyphae usually present. Conidiophores poorly differentiated, arising laterally from vegetative hyphae, flexuose, clavate, or cylindrical, 15 to 20 μm by 1.5 to 2 μm, pale brown, thin and smooth walled, bearing one or more denticles in the apical region; denticles cylindrical, subhyaline to pale brown, up to 2 μm long. Conidia abundant on OA, moderate on MEA 2%, and scarce on PCA, mostly two celled, trilobate, T or Y shaped, 8 to 12 μm long, lower cell 1.5 to 2.5 μm wide, upper cell up to 4 to 8 μm wide, pale brown, smooth and thin walled, released by rhexolytic secession. Chlamydospores abundant on PCA, moderate on OA and MEA 2%, growing directly on vegetative hyphae, terminal or lateral, sessile, solitary, unicellular, globose to slightly subglobose, 4 to 5 μm in diameter, brown, smooth and thick walled. Sexual morph not observed. Cardinal temperatures for growth: optimum 24 to 27°C, maximum 33°C, minimum 4°C.

Specimens examined: Canada, Ontario, from forest soil, 1969, G. L. Barron (holotype CBS H-21643; cultures ex-type CBS 536.69 = MUCL 15054 = OAC 10212). From sandy soil, 1963, G. L. Barron (CBS 116645 = ATCC 16074 = MUCL 102118 = OAC 10094). The Netherlands, Wageningen, from rhizosphere of *Solanum tuberosum*, 1964, J. H. van Emden (CBS 423.64 = MUCL 10610).

Ochroconis olivacea Giraldo, Gené, Deanna A. Sutton, & Guarro, sp. nov., MycoBank accession no. MB809377 (Fig. 3).

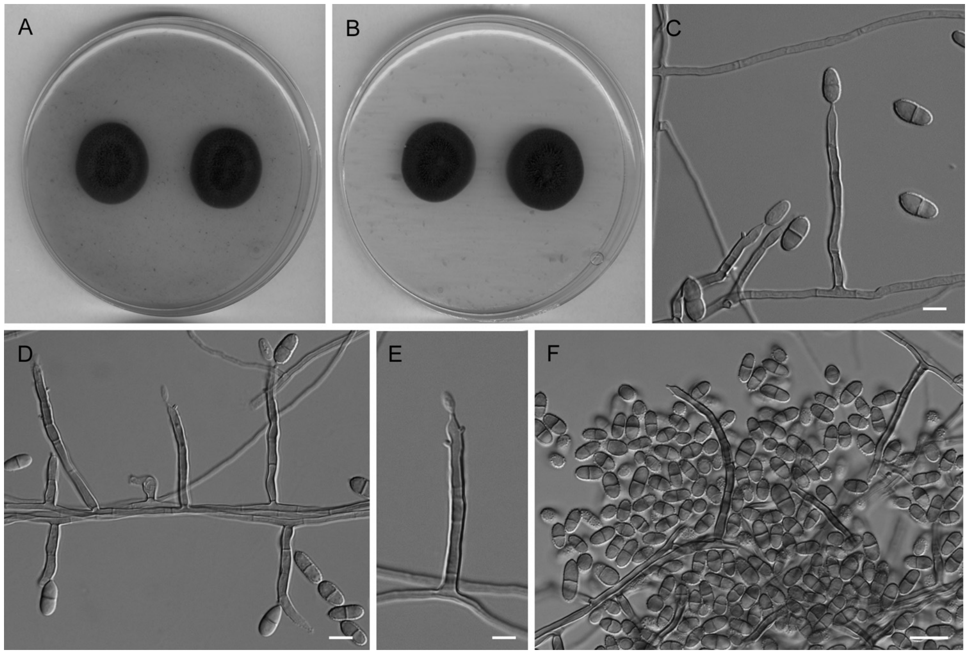

FIG 3 *Ochroconis olivacea* sp. nov. UTHSC 10-2009. (A and B) Colonies on OA and PCA, respectively, at 25°C after 14 days. (C, D, and F) Simple conidiophores arising directly from vegetative hyphae and conidia. (E) Young conidium growing on the apex of a conidiophore. Scale bars = 10 μm.

Etymology: referring to the colony color. Diagnosis: it differs from *Ochroconis humicola* mainly by having slower growth, shorter conidiophores, and verrucose conidia, and from *O. verrucosa* by the production of solitary two-celled conidia.

Colonies on OA at 25°C attaining 23 to 24 mm diameter after 14 days, from olive (1F4) to olive brown (4E5), flat, felty. Colonies on PCA at 25°C reaching 18 to 23 mm after 14 days, olive (2F8), flat, woolly at center, becoming felty toward the periphery. Colonies on MEA 2% and PDA attaining 15 to 17 mm and 22 to 23 mm diameter in 14 days, respectively, olive brown (4E6), radially folded, velvety. Vegetative hyphae septate, pale brown, smooth and thin walled, 1.5 to 2 μm wide. Conidiophores differentiated, arising directly from vegetative hyphae, erect, straight or slightly bent, simple, with 0 to 2 septa, cylindrical, (14)21 to 42(60) μm by

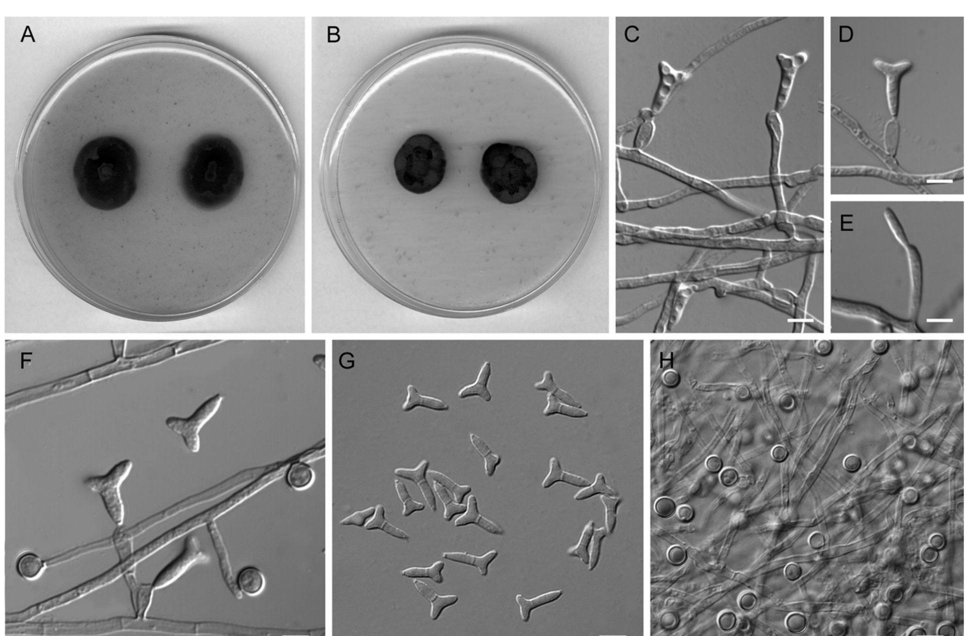

FIG 4 *Ochroconis ramosa* sp. nov. UTHSC 12-1082 (A and B), UTHSC 04-2729 (D and F to H), UTHSC 03-3677 (C and E). (A and B) Colonies on OA and PCA, respectively, at 25°C after 14 days. (C to F) Conidiophores producing trilobate conidia. (G) Trilobate conidia. (H) Chlamydospores growing directly on vegetative hyphae. Scale bars = 10 μm.

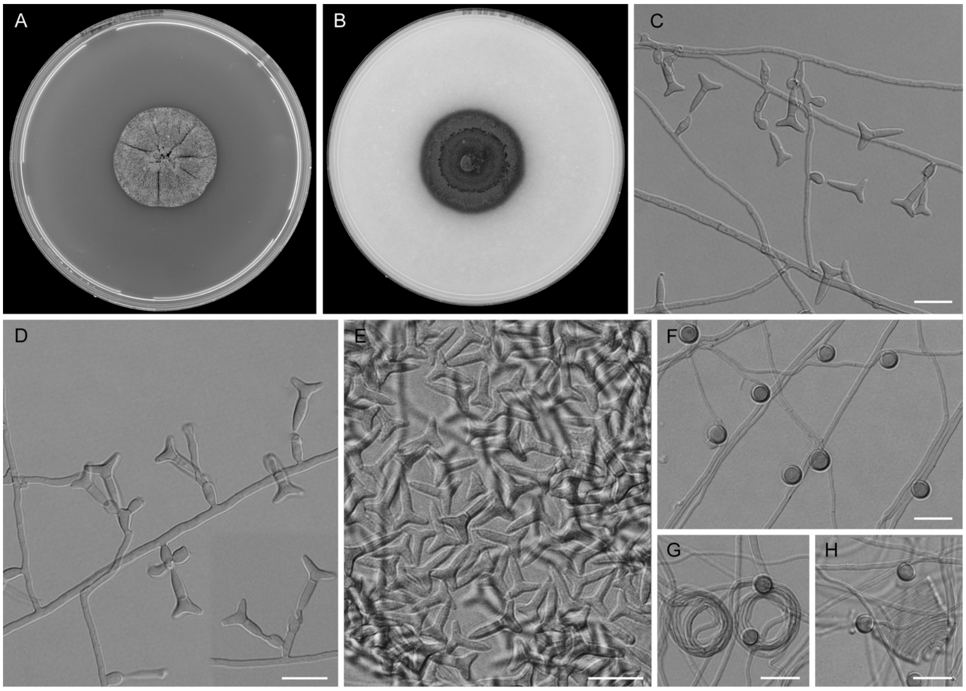

FIG 5 *Ochroconis icarus* sp. nov. CBS 536.69. (A and B) Colonies on MEA 2% and OA, respectively, at 25°C after 21 days. (C and D) Conidiophores bearing trilobate conidia. (E) Trilobate conidia. (F to H) Chlamydospores and coiled hyphae. Scale bars = 10 μm.

2 to 3 μm, brown, thick and smooth walled, producing conidia sympodially on long open denticles; denticles cylindrical, pale brown, up to 1 μm long. Conidia abundant on OA and PCA, absent on PDA and MEA 2%, mostly two celled, cylindrical or broadly ellipsoidal, 6 to 9.5 μm by 1.9 to 4.5 μm, sometimes slightly apiculate at the base and constricted at the septum, pale brown, verrucose and thick walled, released by rhexolytic secession. Chlamydospores and sexual morph not observed. Cardinal temperatures for growth: optimum 20 to 25°C, maximum 35°C, minimum 15°C.

Specimen examined: USA, Utah, from bronchoalveolar lavage fluid, 2010, D. A. Sutton (CBS H-21779 holotype; cultures ex-type CBS 137170 = FMR 12509 = UTHSC 10-2009).

Ochroconis ramosa Giraldo, Gené, Deanna A. Sutton & Guarro, sp. nov., MycoBank accession no. MB809378 (Fig. 4). Etymology: referring to branched conidia. Diagnosis: it differs from *O. minima* by the production of smaller and narrower conidia and a maximum growth temperature of 35°C and from *O. icarus* mainly by having shorter denticles and rough-walled conidia with a narrower upper cell.

Colonies on OA at 25°C attaining 22 to 24 mm diameter in 14 days, chocolate brown (6F4), flat, felty at center, membranous toward the periphery. Colonies on PCA at 25°C reaching 17 to 20 mm after 14 days, brownish black (6H8), flat, woolly at center, membranous toward the periphery. Colonies on MEA 2% and PDA attaining 13 to 16 mm and 20 to 24 mm diameter in 14 days,

TABLE 3 Results of *in vitro* antifungal susceptibility testing of the 16 clinical isolates of *Ochroconis* spp. included in the study

Species (no. of isolates tested)	GM or MIC data[a]	MIC or MEC (μg/ml) for[b]:							
		AMB	ITC	PSC	VRC	AFG	CFG	MFG	TBF
O. mirabilis (11)	GM	28.36	7.00	18.23	11.09	3.93	7.90	0.22	0.03
	MIC range	8–32	1–32	0.5–32	2–32	0.015–32	1–32	0.06–0.5	0.015–0.125
	MIC_{90}	32	32	32	32	4	4	0.25	0.02
O. cordanae (1)	GM	16	1	2	4	0.03	2	0.125	0.015
O. olivacea (1)	GM	32	1	1	4	0.03	1	0.125	0.015
O. ramosa (3)	GM	10.66	1	11.08	2.1	0.051	1	0.16	0.015
	MIC range	8–16	1	0.25–32	0.5–4	0.015–0.125	1	0.125–0.25	0.015
Overall (16)	GM	24.5	5.13	14.80	8.53	2.72	5.67	0.2	0.02
	MIC range	8–32	1–32	0.25–32	0.5–32	0.02–32	1–32	0.06–0.50	0.02–0.13
	MIC_{90}	32	2	32	16	4	8	0.25	0.02

[a] GM, geometric mean.
[b] MEC, minimal effective concentration; AMB, amphotericin B; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; TBF, terbinafine.

respectively, olive (3F5) to olive brown (4E4 to F4), slightly raised, velvety. Vegetative hyphae septate, pale brown, smooth and thin walled, 1.5 to 2 μm wide. Conidiophores differentiated arising directly from vegetative hyphae, erect, straight, simple, clavate or cylindrical with beaked apex, 15 to 20 μm by 1.5 to 2 μm, pale brown, thin and smooth walled, bearing one or more denticles in the apical region; denticles cylindrical, subhyaline to pale brown, up to 0.8 μm long. Conidia abundant on OA and PCA, absent on PDA and MEA 2%, mostly two celled, trilobate, T or Y shaped, (7)8 to 10(12) μm long, lower cell 1.5 to 2.5 μm wide, upper cell 3 to 6 μm wide, pale brown, rough and thin walled, released by rhexolytic secession. Chlamydospores abundant on PCA and OA, absent on MEA 2%, growing directly on vegetative hyphae, lateral, sessile, solitary, unicellular, globose or subglobose, 3 to 3.5 μm by 3 to 3.5 μm, brown, smooth and thick walled. Sexual morph not observed. Cardinal temperatures for growth: optimum 20 to 25°C, maximum 35°C, minimum 15°C.

Specimens examined: USA, California, from human nail, 2012, D. A. Sutton (CBS H-21780 holotype; cultures ex-type CBS 137173 = FMR 12514 = UTHSC 12-1082). Pennsylvania, from human skin, 2003, D. A. Sutton (CBS 137171 = FMR 12512 = UTHSC 03-3677). Utah, from bronchoalveolar lavage fluid, 2004, D. A. Sutton (CBS 137172 = FMR 12513 = UTHSC 04-2729).

DISCUSSION

In this study, we determined the distributions of *Verruconis* and *Ochroconis* species in a set of clinical specimens from human and animal origin from the United States, based on molecular and phenotypic analyses. *V. gallopava* was the most common species, found mainly on respiratory samples (BAL fluid and sputum), followed by deep-tissue samples (brain and others). These findings agree with those of previous studies, in which *V. gallopava* frequently involved the lung, producing either cavitary or noncavitary lung lesions. This fungus also shows special neurotropism in warm-blooded animals (21, 22). Although *V. gallopava* is commonly known as producing brain abscesses and encephalitis in birds and other animals, several reports document it as an etiologic agent in human disease. Most of these reports occur in immunocompromised patients, with organ transplantation being the most common underlying condition (13, 21, 22, 48). A few cases in immunocompetent patients with pulmonary manifestations have also been described. In these cases, *V. gallopava* was isolated from BAL fluid and lobectomy samples (49–51).

Some of our *V. gallopava* isolates showed atypical morphologies, such as yellowish white or moist colonies, slow growth at 25°C, and ellipsoidal conidia with an apiculate base. However, the molecular analysis demonstrated that they in fact belong to this species. The morphological variability in *V. gallopava* has also been reported in other studies (13, 28). Yarita et al. (13) analyzed four isolates from hot springs in Japan, which showed differences in the shape and size of the conidia, being slender or thicker and shorter than those of the type species; however, their D1/D2 regions were 99.7% identical with the those of the type strain of *V. gallopava*. The isolates studied by Dixon and Salkin (28) also produced a similar variation in conidial size.

V. gallopava has been described as a thermotolerant species (22, 32, 52), and all the isolates studied here grew well at 42°C. This explains its ability to survive in warm environments, like thermal soils or hot springs, and to infect warm-blooded animals, poultry, and other birds, in addition to humans. The *in vitro* thermotolerance is a useful physiological feature for identifying this species (13, 20).

In the present study, we report for the first time an albino isolate of *V. gallopava* (UTHSC 07-623), which was recovered from a canine intervertebral disc. This is an unusual phenomenon that occurs in a few other fungi, such as *Neoscytalidium hyalinum*, *Aspergillus flavus*, *Ophiostoma floccosum*, *Ophiostoma piceae*, and *Ophiostoma pluriannulatum* (53–55), which in general are involved almost exclusively in skin and nail disease (56, 57). The melanin plays an important role in fungal pathogenesis, and its absence often generates less virulent isolates than with pigmented ones (47, 58–60). Although we could not demonstrate that this isolate was the etiological agent of the infection, the presence of albino isolates recovered from deep tissues might suggest that this fungus has an additional mechanism of pathogenicity, apart from that of melanin. However, additional isolates should be studied to demonstrate this hypothesis.

O. mirabilis was the most common species recovered from superficial lesions, but it was also isolated from BAL fluid samples. This species is a waterborne fungus usually isolated from moist places in bathrooms and rarely from soil and plant material. However, several isolates of this species have also been recovered from clinical samples, producing mild cutaneous infections (skin, fingers, and toenails) in humans and fishes (32). It has been suggested that the bathroom-associated fungi can penetrate the skin and the nails during showering, when these barriers are weakened (6, 61).

The species *O. constricta* and *O. humicola*, occasionally reported from superficial infections in humans (20, 21, 61), were not represented in our set of isolates. However, most of the isolates identified here as *O. mirabilis* were received as either *O. constricta* or *O. humicola*. Only subtle morphological features allow the distinction between these three species: *O. constricta* has poorly differentiated conidiophores and markedly verruculose conidia, with a conspicuous constriction at the septum, *O. mirabilis* has differentiated cylindrical conidiophores (up to 20 μm) and conidia slightly constricted at the septum, and *O. humicola* has rapid growth, longer conidiophores than the other two species, and cylindrical conidia with a smooth or slightly rugose wall.

We obtained a single isolate of *O. cordanae* from the tissue of a left thigh, which was previously identified as *O. humicola*. *O. cordanae* can be differentiated by its more slowly growing colonies, shorter conidiophores, and smaller conidia, which are mostly ellipsoidal. *O. cordanae* was recently described by Samerpitak et al. (32) as a cosmopolitan species commonly inhabiting living leaves, sometimes found on decaying vegetal material, and less frequently from ant nests, but it has never been obtained from clinical samples. Therefore, this is the first report of this species in the clinical setting. Because only one isolate was obtained from a superficial tissue sample and because of its mesophilic abilities, the human-pathogenic role of this species is still doubtful.

Based on our multilocus sequence analysis and detailed phenotypic study, three new species are proposed here, i.e., *O. icarus* from environmental sources and *O. ramosa* and *O. olivacea* from clinical specimens. Both *O. icarus* and *O. ramosa* are morphologically similar and phylogenetically related to *O. minima* (Fig. 2). However, they can be easily differentiated by their maximum temperatures for growth (37°C for *O. minima*, 35°C for *O. ramosa*, and 33°C for *O. icarus*). Additionally, in *O. minima*, the conidia are longer than those of the other two species (up to 13.5 μm), in

O. ramosa, the conidia have a rugose wall, and in *O. icarus*, the lower cell of the conidia is narrower than that of *O. minima* (1.5- to 2.5-μm wide for *O. icarus* versus up to 4.5 μm for *O. minima*).

In the phylogenetic analyses, *O. olivacea* was placed close to *O. verrucosa* (Fig. 2). Both species produce verrucose conidia, but in *O. verrucosa*, they are cylindrical or fusiform, mostly four celled, and sometimes arranged in acropetal, branched, or unbranched chains (32). In contrast, in *O. olivacea*, the conidia are cylindrical or ellipsoidal, mostly two celled, and not arranged in chains. Morphologically, *O. olivacea* is similar to *Ochroconis gamsii* and *O. humicola* in its production of one-celled conidia and erected long cylindrical conidiophores. However, in *O. gamsii*, the conidia are broadly fusiform and unilaterally flattened, and the conidiophores are darker, while in *O. humicola*, the conidia are broadly cylindrical and finely echinulate and the conidiophores are longer (up to 300 μm) (2, 62). *Ochroconis macrozamiae*, a recently described species on *Macrozamia* leaf litter, also resembles *O. olivacea*, but, in addition to having broadly fusiform conidia, it is phylogenetically close to *O. gamsii* (63).

Few *in vitro* antifungal susceptibility studies are available for *Ochroconis* species, as members of this genus are rarely involved in human disease. The most relevant species in the clinical setting, *O. gallopava*, was recently transferred to *Verruconis*. Recently, Seyedmousavi et al. (64) evaluated the antifungal susceptibilities of numerous strains of *Verruconis* and *Ochroconis* spp. from clinical and environmental origins against eight antifungal drugs, using the broth microdilution test. In that study, the isolates of *V. gallopava* showed low MICs for AFG, PSC, ITC, AMB, CFG, and VRC, while 5-flucytosine and fluconazole showed no activity. In contrast, only AFG, PSC, and CFG showed good *in vitro* activity against *O. mirabilis*, the most frequently isolated *Ochroconis* species from clinical sources in that study (64). These results are in disagreement with our data, for which these drugs demonstrated high MICs. TBF and MFG, which were not tested by Seyedmousavi et al. (64), were the only drugs with some activity against *O. mirabilis*. These differences in the data between those of Seyedmousavi et al. (64) and our study might be explained by the different origins of the *Ochroconis* isolates tested (Europe versus the United States) but also by differences in the procedure, i.e., the incubation temperature (25°C versus 30°C) and the methodology or criteria used to read the endpoint.

There are few clinical cases reported in the literature regarding *Ochroconis* infections. Mancini and McGinnis (65) described a case of pulmonary abscess by *O. constricta* in a heart transplant recipient. The patient was successfully treated with systemic AMB, resulting in the resolution of clinical symptoms and a cavitary lesion. Recently, Ge et al. (23) reported a human case of subcutaneous phaeohyphomycosis in an immunocompetent patient due to *Ochroconis tshawytschae*. Several short courses of treatment with ITC or TBF were begun, but no cure was obtained. A subcutaneous infection by *O. humicola* in a cat was reported by VanSteenhouse et al. (66). The fungus was recovered from a granulomatous lesion, and although no antifungal test was performed, the cat was successfully treated with ketoconazole.

Despite the fact that *V. gallopava* is the species most frequently implicated in the clinical setting, the repeated isolation of several species of *Ochroconis* in the clinical setting never before reported suggests their potential pathogenic role and deserves further research.

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