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Cladosporium Species Recovered from Clinical Samples in the United States

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Cladosporium **species are ubiquitous, saprobic, dematiaceous fungi, only infrequently associated with human and animal opportunistic infections. We have studied a large set of *Cladosporium* isolates recovered from clinical samples in the United States to ascertain the predominant species there in light of recent taxonomic changes in this genus and to determine whether some could possibly be rare potential pathogens. A total of 92 isolates were identified using phenotypic and molecular methods, which included sequence analysis of the internal transcribed spacer (ITS) region and a fragment of the large subunit (LSU) of the nuclear ribosomal DNA (rDNA), as well as fragments of the translation elongation factor 1 alpha (*EF-1α*) and actin (*Act*) genes. The most frequent species was *Cladosporium halotolerans* (14.8%), followed by *C. tenuissimum* (10.2%), *C. subuliforme* (5.7%), and *C. pseudocladosporioides* (4.5%). However, 39.8% of the isolates did not correspond to any known species and were deemed to comprise at least 17 new lineages for *Cladosporium*. The most frequent anatomic site of isolation was the respiratory tract (54.5%), followed by superficial (28.4%) and deep tissues and fluids (14.7%). Species of the two recently described cladosporium-like genera *Toxicocladosporium* and *Penidiella* are reported for the first time from clinical samples. *In vitro* susceptibility testing of 92 isolates against nine antifungal drugs showed a variety of results but high activity overall for the azoles, echinocandins, and terbinafine.**

Cladosporium species are among the most common fungal inhabitants worldwide, being isolated from almost any environmental source and geographic location (1). The genus is characterized by the typical form of its conidiophores, which are erect, straight or geniculate, produce abundant branched acropetal chains of smooth to roughened dry conidia, and show a distinct darkened coronate hilum, i.e., conidial scar characterized by a thick rim surrounding a central convex dome (2, 3). The relatively small conidia are easily detached and disseminated by the wind, *Cladosporium* being one of the most frequently isolated airborne fungi (2, 4).

The most common *Cladosporium* species are primarily isolated from soil and plant material, where they are frequently encountered as saprobes or secondary invaders on follicular lesions, concomitant with other plant-pathogenic fungi (1, 5, 6). However, several species are important pathogens of plants and some are also able to affect animals, including humans (7–9). *Cladosporium* is usually associated with allergic rhinitis (10) or localized superficial or deep lesions (11–14) but, rarely, can cause disseminated infections (7, 15–17).

The genus *Cladosporium* has been shown to be both morphologically and phylogenetically heterogeneous (18). On the basis of molecular data, the true human-pathogenic species *C. bantiana*, *C. carrionii*, and *C. devriesii*, characterized by their thermotolerance and the absence of conidiophores with pigmented conidial scars, were transferred to *Cladophialophora* (1, 7, 18). More recently, *Cladosporium* underwent extensive revisions based on polyphasic approaches (1, 3, 19–21), which resulted in the delimitation of 169 species currently accepted in *Cladosporium sensu stricto* (*Cladosporiaceae*, *Capnodiales*). On the other hand, a great number of taxa were excluded from that genus, now being considered doubtful species or accommodated into several related new genera, such as *Hyalodendriella*, *Ochrocladosporium*, *Rachicladosporium*, *Rhizocladosporium*, *Toxicocladosporium*, and *Verrucocladosporium* (1, 3).

The diversity of *Cladosporium* species associated with human disease is currently reduced to four, i.e., *C. cladosporioides*, *C. herbarum*, *C. oxysporum*, and *C. sphaerospermum* (7). Most of these data, however, are based on a reduced number of clinical cases with the identification of the etiological agents not confirmed by reliable methods. Moreover, three of the clinically relevant species, *C. cladosporioides*, *C. herbarum*, and *C. sphaerospermum*, have been demonstrated to be species complexes (19–21) encompassing several morphologically sibling species that can only be distinguished by means of phylogenetic analyses (1, 7). The clinical significance of these phylogenetic species, however, has yet to be evaluated (22).

The objective of this work was to assess the diversity of *Cladosporium* species associated with human and animal disease by analyzing a large set of isolates from clinical specimens by means of phenotypic and DNA sequence data analyses. In addition, the *in vitro* susceptibility of these isolates was evaluated against nine clinically available antifungal drugs.

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TABLE 1 Clinical isolates, type or reference strains, and sequences included in this study

Species	Strain/isolate no.[a]	Origin (country)[b]	GenBank nucleotide accession no. for:			
			ITS	LSU	*EF-1α*	*Act*
Cercospora beticola	CBS 116456^T	*Beta vulgaris* (Italy)	NR121315	GU214404	AY840494	AY840458
Cercospora olivascens	CBS 253.67^T	Unknown	NR111773			
Cladosporium allicinum	CBS 121624^T	*Hordeum vulgare* (Belgium)	EF679350		EF679425	EF679502
	UTHSC DI-13-173	Human, lung (USA)	LN834353		LN834449	LN834537
	UTHSC DI-13-176	Human, skin (USA)	LN834354		LN834450	LN834538
	UTHSC DI-13-266	Canine, skin (USA)	LN834355		LN834451	LN834539
Cladosporium angustisporum	CBS 125983^T	*Alloxylon wickhamii* (Australia)	HM147995		HM148236	HM148482
	UTHSC DI-13-240	Human, toe nail (USA)	LN834356		LN834452	LN834540
Cladosporium asperulatum	CBS 126339	*Eucalyptus* leaf litter (India)	HM147997		HM148238	HM148484
	CBS 126340^T	*Protea susannae* (Portugal)	HM147998		HM148239	HM148485
	UTHSC DI-13-216	Feline, nasal (USA)	LN834357		LN834453	LN834541
Cladosporium cladosporioides	CBS 101367	Soil (Brasil)	HM148002		HM148243	HM148489
	CBS 112388^T	Indoor air (Germany)	HM148003		HM148244	HM148490
	UTHSC DI-13-204	Human, abdomen (USA)	LN834358		LN834454	LN834542
	UTHSC DI-13-209	Human, pleural (USA)	LN834359		LN834455	LN834543
	UTHSC DI-13-215	Human, sputum (USA)	LN834360		LN834456	LN834544
Cladosporium colocasiae	CBS 386.64^T	*Colocasia antiquorum* (Taiwan)	HM148067		HM148310	HM148555
	CBS 119542	*Colocasia esculanta* (Taiwan)	HM148066		HM148309	HM148554
Cladosporium cucumerinum	CBS 171.52^T	Fruit of *Cucumis sativus* (Netherlands)	HM148072		HM148316	HM148561
	CBS 173.54	Fruit of *Cucumis sativus* (Netherlands)	HM148074		HM148318	HM148563
Cladosporium flabelliforme	CBS 126345^T	*Melaleuca cajuputi* (Australia)	HM148092		HM148336	HM148581
	UTHSC DI-13-267	Human, sputum (USA)	LN834361		LN834457	LN834545
Cladosporium funiculosum	CBS 122128	Unknown	HM148093		HM148337	HM148582
	CBS 122129^T	Leaf of *Vigna umbellata* (Japan)	HM148094		HM148338	HM148583
	UTHSC DI-13-175	Human, BAL fluid (USA)	LN834362		LN834458	LN834546
	UTHSC DI-13-223	Human, BAL fluid (USA)	LN834363		LN834459	LN834547
	UTHSC DI-13-242	Human, nasal wash (USA)	LN834364		LN834460	LN834548
Cladosporium halotolerans	CBS 119416^T	Hypersaline water (Namibia)	DQ780364		JN906989	EF101397
	FMR 13493	Human, unknown (Spain)	LN834365		LN834461	LN834549
	UTHSC DI-13-164	Human, bone marrow (USA)	LN834366		LN834462	LN834550
	UTHSC DI-13-182	Marine mammal, dermis (USA)	LN834367		LN834463	LN834551
	UTHSC DI-13-183	Human, bronchus (USA)	LN834368		LN834464	LN834552
	UTHSC DI-13-206	Human, BAL fluid (USA)	LN834369		LN834465	LN834553
	UTHSC DI-13-213	Human, lymph node (USA)	LN834370		LN834466	LN834554
	UTHSC DI-13-221	Human, bone marrow (USA)	LN834371		LN834467	LN834555
	UTHSC DI-13-231	Catheter tip (USA)	LN834372		LN834468	LN834556
	UTHSC DI-13-249	Human, nasal (USA)	LN834373		LN834469	LN834557
	UTHSC DI-13-250	Human, scalp (USA)	LN834374		LN834470	LN834558
	UTHSC DI-13-252	Human, toe nail (USA)	LN834375		LN834471	LN834559
	UTHSC DI-13-259	Human, BAL fluid (USA)	LN834376		LN834472	LN834560
	UTHSC DI-13-263	Human, BAL fluid (USA)	LN834377		LN834473	LN834561
Cladosporium herbaroides	CBS 121626^T	Hypersaline water (Israel)	EF679357		EF679432	EF679509
Cladosporium herbarum	CBS 121621^T	*Hordeum vulgare* (Netherlands)	EF679363		EF679440	EF679516
	UTHSC DI-13-220	Human, BAL fluid (USA)	LN834378		LN834474	LN834562
Cladosporium iranicum	CBS 126346^T	Leaf of *Citrus sinensis* (Iran)	HM148110		HM148354	HM148599
Cladosporium iridis	CBS 138.40^T	Leaf of *Iris* sp. (Netherlands)	EF679370		EF679447	EF679523
Cladosporium macrocarpum	CBS 121623^T	*Spinacia oleracea* (USA)	EF679375		EF679453	EF679529
	UTHSC DI-13-191	Human, face (USA)	LN834379		LN834475	LN834563
Cladosporium oxysporum	CBS 125991	Soil (China)	HM148118		HM148362	HM148607
	CBS 126351	Indoor air (Venezuela)	HM148119		HM148363	HM148608
Cladosporium perangustum	CBS 125996^T	*Cussonia* sp. (South Africa)	HM148121		HM148365	HM148610
	UTHSC DI-13-208	Canine, BAL fluid (USA)	LN834380		LN834476	LN834564
Cladosporium pseudocladosporioides	CBS 117153	Leaf of *Paeonia* sp. (Germany)	HM148157		HM148401	HM148646
	CBS 125993^T	Outside air (Netherlands)	HM148158		HM148402	HM148647
	UTHSC DI-13-187	Turtle, unknown (USA)	LN834381		LN834477	LN834565
	UTHSC DI-13-232	Human, shoulder (USA)	LN834382		LN834478	LN834566
	UTHSC DI-13-233	Human, BAL fluid (USA)	LN834383		LN834479	LN834567
	UTHSC DI-13-261	Human, sputum (USA)	LN834384		LN834480	LN834568

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TABLE 1 (Continued)

Species	Strain/isolate no.[a]	Origin (country)[b]	GenBank nucleotide accession no. for:			
			ITS	LSU	*EF-1α*	*Act*
Cladosporium ramotenellum	CBS 121628^T	Hypersaline water (Slovenia)	EF679384		EF679462	EF679538
	UTHSC DI-13-166	Human, nasal tissue (USA)	LN834385		LN834481	LN834569
	UTHSC DI-13-222	Animal, Nasal (USA)	LN834386		LN834482	LN834570
	UTHSC DI-13-224	Animal, Nasal (USA)	LN834387		LN834483	LN834571
Cladosporium sinuosum	CBS 121629^T	*Fuchsia excorticata* (New Zealand)	EF679386		EF679464	EF679540
Cladosporium sphaerospermum	CBS 193.54^T	Human, nail (Netherlands)	DQ780343		EU570261	EU570269
	UTHSC DI-13-184	Frog, abscess (USA)	LN834388		LN834484	LN834572
	UTHSC DI-13-229	Human, BAL fluid (USA)	LN834389		LN834485	LN834573
	UTHSC DI-13-237	Human, BAL fluid (USA)	LN834390		LN834486	LN834574
Cladosporium subinflatum	CBS 121630^T	Hypersaline water (Slovenia)	EF679389		EF679467	EF679543
	UTHSC DI-13-189	Human, toe nail (USA)	LN834391		LN834487	LN834575
Cladosporium subtilissimum	CBS 113754^T	Grape berry (USA)	EF679397		EF679475	EF679551
Cladosporium subuliforme	CBS 126500^T	*Chamaedorea metallica* (Thailand)	HM148196		HM148441	HM148686
	UTHSC DI-13-171	Human, CSF (USA)	LN834392		LN834488	LN834576
	UTHSC DI-13-180	Human, BAL fluid (USA)	LN834393		LN834489	LN834577
	UTHSC DI-13-214	Human, BAL fluid (USA)	LN834394		LN834490	LN834578
	UTHSC DI-13-254	Human, BAL fluid (USA)	LN834395		LN834491	LN834579
	UTHSC DI-13-255	Human, toe nail (USA)	LN834396		LN834492	LN834580
Cladosporium tenuissimum	CBS 125995^T	Fruits of *Lagerstroemia* sp. (USA)	HM148197		HM148442	HM148687
	UTHSC DI-13-174	Marine mammal, lung (USA)	LN834397		LN834493	LN834581
	UTHSC DI-13-177	Human, skin (USA)	LN834398		LN834494	LN834582
	UTHSC DI-13-188	Human, BAL fluid (USA)	LN834399		LN834495	LN834583
	UTHSC DI-13-205	Human, BAL fluid (USA)	LN834400		LN834496	LN834584
	UTHSC DI-13-236	Human, nasal (USA)	LN834401		LN834497	LN834585
	UTHSC DI-13-239	Human, sputum (USA)	LN834402		LN834498	LN834586
	UTHSC DI-13-253	Human, BAL fluid (USA)	LN834403		LN834499	LN834587
	UTHSC DI-13-258	Human, thorancentesis fluid (USA)	LN834404		LN834500	LN834588
	UTHSC DI-13-274	Human, toe (USA)	LN834405		LN834501	LN834589
Cladosporium sp.	UTHSC DI-13-165	Human, arm drainage (USA)	LN834406		LN834502	LN834590
	UTHSC DI-13-168	Human, BAL fluid (USA)	LN834407		LN834503	LN834591
	UTHSC DI-13-169	Human, BAL fluid (USA)	LN834408		LN834504	LN834592
	UTHSC DI-13-170	Human, toe nail (USA)	LN834409		LN834505	LN834593
	UTHSC DI-13-178	Animal, abscess (USA)	LN834410		LN834506	LN834594
	UTHSC DI-13-179	Human, hand (USA)	LN834411		LN834507	LN834595
	UTHSC DI-13-190	Human, CSF (USA)	LN834412		LN834508	LN834596
	UTHSC DI-13-207	Human, CSF (USA)	LN834413		LN834509	LN834597
	UTHSC DI-13-210	Human, skin (USA)	LN834414		LN834510	LN834598
	UTHSC DI-13-211	Human, BAL fluid (USA)	LN834415		LN834511	LN834599
	UTHSC DI-13-212	Human, ethmoid sinus (USA)	LN834416		LN834512	LN834600
	UTHSC DI-13-217	Human, nasal (USA)	LN834417		LN834513	LN834601
	UTHSC DI-13-218	Human, BAL fluid (USA)	LN834418		LN834514	LN834602
	UTHSC DI-13-219	Human, foot (USA)	LN834419		LN834515	LN834603
	UTHSC DI-13-225	Animal, BAL fluid (USA)	LN834420		LN834516	LN834604
	UTHSC DI-13-226	Human, BAL fluid (USA)	LN834421		LN834517	LN834605
	UTHSC DI-13-227	Human, sputum (USA)	LN834422		LN834518	LN834606
	UTHSC DI-13-228	Human, foot skin (USA)	LN834423		LN834519	LN834607
	UTHSC DI-13-234	Human, sputum (USA)	LN834424		LN834520	LN834608
	UTHSC DI-13-235	Human, BAL fluid (USA)	LN834425		LN834521	LN834609
	UTHSC DI-13-238	Human, leg (USA)	LN834426		LN834522	LN834610
	UTHSC DI-13-241	Human, foot (USA)	LN834427		LN834523	LN834611
	UTHSC DI-13-244	Human, BAL fluid (USA)	LN834428		LN834524	LN834612
	UTHSC DI-13-245	Human, toe (USA)	LN834429		LN834525	LN834613
	UTHSC DI-13-246	Human, BAL fluid (USA)	LN834430		LN834526	LN834614
	UTHSC DI-13-247	Human, BAL fluid (USA)	LN834431		LN834527	LN834615
	UTHSC DI-13-251	Human, BAL fluid (USA)	LN834432		LN834528	LN834616
	UTHSC DI-13-257	Human, sputum (USA)	LN834433		LN834529	LN834617
	UTHSC DI-13-262	Dolphin, bronchus (USA)	LN834434		LN834530	LN834618

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TABLE 1 (Continued)

Species	Strain/isolate no.[a]	Origin (country)[b]	GenBank nucleotide accession no. for:			
			ITS	LSU	*EF-1*α	*Act*
	UTHSC DI-13-265	Human, BAL fluid (USA)	LN834435		LN834531	LN834619
	UTHSC DI-13-268	Human, toe nail (USA)	LN834436		LN834532	LN834620
	UTHSC DI-13-269	Human, BAL fluid (USA)	LN834437		LN834533	LN834621
	UTHSC DI-13-270	Human, nail (USA)	LN834438		LN834534	LN834622
	UTHSC DI-13-271	Human, BAL fluid (USA)	LN834439		LN834535	LN834623
	UTHSC DI-13-273	Human, toe nails (USA)	LN834440		LN834536	LN834624
Cladosporium variabile	CBS 121636^T	*Spinacia oleracea* (USA)	EF679402		EF679480	EF679556
Penidiella sp.	UTHSC DI-13-256	Human, nail (USA)	LN834441	LN834445		
Toxicocladosporium banksiae	CBS 128215^T	Leaf of *Banksia emulata* (Australia)	HQ599598	HQ599599		
Toxicocladosporium chlamydosporum	CBS 124157^T	Leaf of *Eucalyptus camaldulensis* (Madagascar)	FJ790283	FJ790301		
Toxicocladosporium ficiniae	CBS 136406^T	Leaf of *Ficinia* sp. (South Africa)	KF777190	KF777241		
Toxicocladosporium irritans	CBS 185.58^T	Moldy paint (Suriname)	EU040243	EU040243		
	UTHSC DI-13-181	Human, blood (USA)	LN834442	LN834446		
	UTHSC DI-13-230	Human, finger nail (USA)	LN834443	LN834447		
Toxicocladosporium pini	CBS 138005^T	Needles of *Pinus* sp. (China)	KJ869160	KJ869217		
Toxicocladosporium posoqueriae	CBS 133583^T	Leaf of *Posoqueria latifolia* (Australia)	NR121555	KC005803		
Toxicocladosporium protearum	CBS 126499^T	Leaf of *Protea burchellii* (South Africa)	HQ599586	HQ599587		
Toxicocladosporium pseudoveloxum	CBS 128775^T	Leaf of *Phaenocoma prolifera* (South Africa)	JF499847	JF499867		
Toxicocladosporium rubrigenum	CBS 124158^T	Leaf of *Eucalyptus camaldulensis* (Madagascar)	FJ790287	FJ790305		
Toxicocladosporium sp.	UTHSC DI-13-172	Human, BAL fluid (USA)	LN834444	LN834448		
Toxicocladosporium strelitziae	CBS 132535^T	Leaf of *Strelitzia reginae* (South Africa)	NR111765	JX069858		
Toxicocladosporium veloxum	CBS 124159^T	Leaf of *Eucalyptus camaldunensis* (Madagascar)	FJ790288	FJ790306		

[a] CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; FMR, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, TX, USA; ^{T}, ex-type strain.
[b] BAL fluid, bronchoalveolar lavage fluid specimen; CSF, cerebrospinal fluid.

MATERIALS AND METHODS

Fungal isolates. A total of 92 isolates tentatively identified as *Cladosporium* spp. were included in this study (Table 1). All of the isolates were obtained from human and animal clinical specimens, mostly from the United States, received in the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio (UTHSC) from different parts of the country mainly for identification purposes.

Phenotypic identification. The isolates were morphologically characterized following the procedures outlined in Bensch et al. (1), Crous et al. (23), Schubert et al. (19), and Zalar et al. (20). Briefly, all of the isolates were grown on synthetic nutrient-poor agar (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 1 liter water) and potato dextrose agar (PDA) (Pronadisa, Spain) for 7 days at 25°C. Microscopic observations were made from cultures on SNA mounted in Shear's solution (23). Colony characteristics were recorded from cultures on SNA and PDA. For the estimation of cardinal growth temperatures, the isolates were grown on PDA agar for 14 days at temperatures ranging from 15°C to 35°C at intervals of 5°C, as well as at 32°C and 37°C.

DNA extraction, amplification, and sequencing. Total genomic DNA was extracted from mycelia obtained from colonies growing on PDA, using FastPrep (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. DNA was quantified using the NanoDrop 3000 (Thermo Scientific, Madrid, Spain).

The primers ITS5 and ITS4 (24) were used to amplify a region spanning internal transcribed spacer 1 (ITS1) and ITS2 and the 5.8S gene of the ribosomal DNA (rDNA); the primer pair LR0R/LR5 (25, 26) was used to amplify a fragment of the large subunit (LSU) gene of the rDNA; and the EF-728F/EF-986R and ACT-512F/ACT-783R primer pairs (27) were used for the translation elongation factor 1α gene (*EF-1*α) and the actin gene (*Act*), respectively.

Sequencing was performed in both directions using the same PCR primers at Macrogen Europe (Macrogen, Inc., Amsterdam, the Netherlands). Consensus sequences were obtained using SeqMan version 7.0.0 (DNAStar Lasergene, Madison, WI).

Molecular identification and phylogenetic analyses. An initial presumptive generic identification of the isolates was performed based on BLAST searches of ITS and LSU sequences in the GenBank (http://www.ncbi.nlm.nih.gov/) and CBS (http://www.cbs.knaw.nl/) databases. Multiple sequence alignments of each locus were performed in MEGA version 6 (28) using the ClustalW application (29), refined with MUSCLE (30), and manually adjusted if necessary. Phylogenetic reconstructions were made using maximum-likelihood (ML) and Bayesian Inference (BI) under MEGA version 6 and MrBayes version 3.1.2 (31), respectively. The best nucleotide substitution model (generalized time-reversible model with gamma distribution and a portion of invariable sites [GTR+G+I] for the three independent data sets) was estimated using MrModelTest version 2.3 (32) following the Akaike criterion. Phylogenetic analyses using ML were at first made individually for each locus and compared in order to assess for any incongruent results between nodes with high statistical support. As no incongruences were observed, the four loci were combined as follows: ITS, *EF-1*α, and *Act* for members of *Cladosporium sensu stricto* and ITS combined with LSU for members of other cladosporiumlike genera. For the ML analysis, nearest-neighbor interchange (NNI) was used as the heuristic method for tree inference. Support for the internal branches was assessed by a search of 1,000 bootstrapped sets of

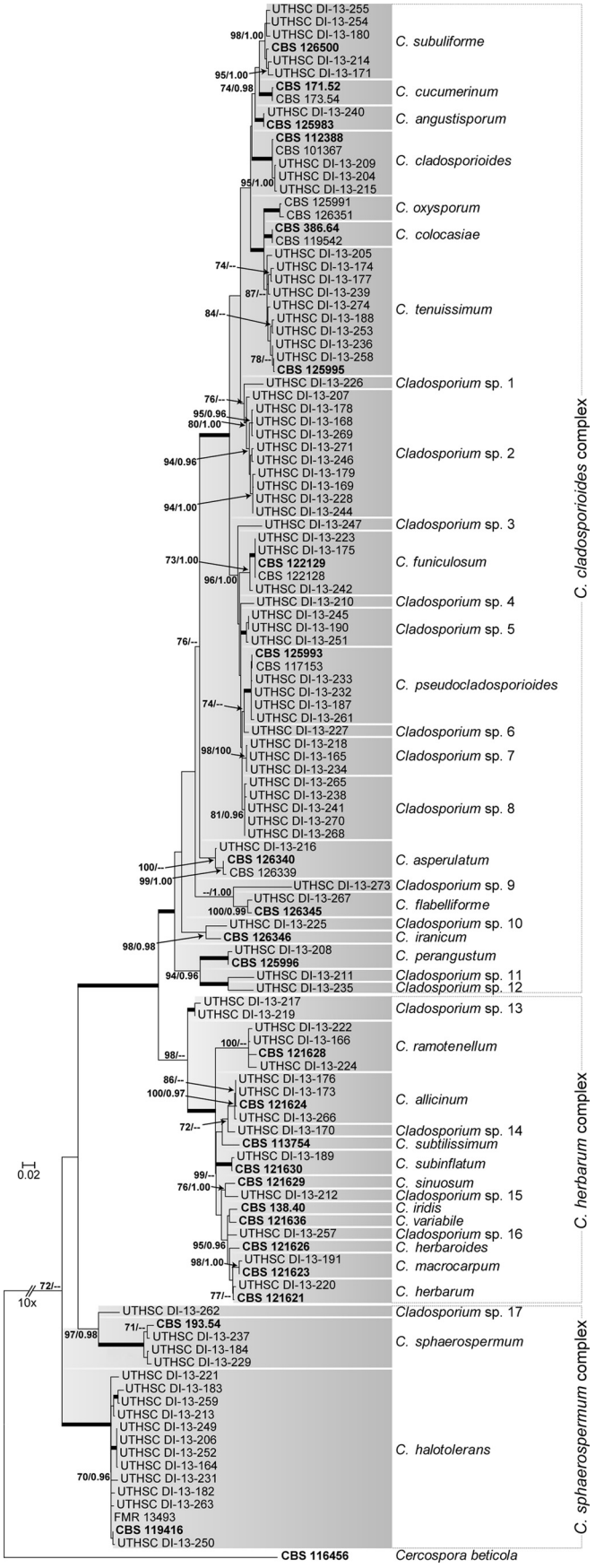

FIG 1 Maximum-likelihood (ML) tree inferred from combined ITS, *EF-1*α, and *Act* sequences of *Cladosporium* isolates. Branch lengths are proportional to phylogenetic distance. ML bootstrap support (BS) values of ≥70% and posterior probability (PP) values of ≥0.95 are shown above the branches. Thickened branches indicate BS of 100% and PP of 1.00. *Cercospora beticola* (CBS 116456) was used to root the tree. Type strains are indicated in bold font. CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; FMR, Facultat de Medicina, Reus, Spain; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, TX.

data. A bootstrap support value of ≥70 was considered significant. For BI analysis, two simultaneous runs of 10,000,000 generations were performed and samples were stored every 1,000 generations. The 50% majority-rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the samples. A PP value of ≥0.95 was considered significant.

The first combined phylogenetic analysis with ITS, *EF-1*α, and *Act* sequences of clinical isolates belonging to *Cladosporium sensu stricto* and all the available type and reference strains was carried out following the alignments of Bensch et al. (1; data not shown). Only sequences of those species closely related (>95% similarity) to the clinical isolates tested here were included in the final analysis.

Antifungal susceptibility. The antifungal susceptibility test was performed according to the CLSI M38-A2 standard (33) with slight modifications. The incubation temperature was set to 25°C, given the optimal growth requirements of *Cladosporium* and related taxa (1, 33). Nine antifungal agents were tested: amphotericin B (AMB), 5-fluorocytosine (5FC), itraconazole (ITC), posaconazole (PSC), voriconazole (VRC), terbinafine (TRB), anidulafungin (AFG), caspofungin (CFG), and micafungin (MFG). The minimal effective concentration (MEC), defined as the lowest drug concentration at which short, stubby, highly branched hyphae were observed, was determined at 24 h for the echinocandins, and the MIC was determined at 48 h for the remaining drugs. The MIC was defined as the lowest concentration exhibiting 100% inhibition of visible growth for AMB, ITC, PSC, and VRC or 50% and 80% reduction in growth for 5FC and TRB, respectively. *Paecilomyces variotii* ATCC MYA-3630 and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality control strains. Statistical analyses of the MIC/MEC data were performed using the Mann-Whitney test in Prism version 6.0 (GraphPad Software, San Diego, CA).

Nucleotide sequence accession numbers. DNA sequences determined in this study were deposited in GenBank under accession numbers LN834353 to LN834448 (rDNA), LN834449 to LN834536 (*EF-1*α), and LN834537 to LN834624 (*Act*) (Table 1).

RESULTS

Analysis of ITS and LSU sequences showed that 88 isolates (96%) belonged to *Cladosporium sensu stricto*, three isolates (3%) to the genus *Toxicocladosporium*, and one isolate (1%) to the genus *Penidiella*.

The phylogenetic analysis of *Cladosporium sensu stricto* included 121 taxa and 1,002 bp (447 bp for ITS, 337 bp for *EF-1*α, and 218 bp for *Act*), of which 485 bp were constant (347 bp for ITS, 60 bp for *EF-1*α, and 78 bp for *Act*), 496 were variable (97 bp for ITS, 260 bp for *EF-1*α, and 139 bp for *Act*) and 328 were parsimony informative (24 bp for ITS, 197 bp for *EF-1*α, and 107 bp for *Act*) (Fig. 1). The majority of isolates (57, 65%) nested into the *C. cladosporioides* complex: 28 belonged to nine species (i.e., *C. angustisporum*, *C. asperulatum*, *C. cladosporioides*, *C. flabelliforme*, *C. funiculosum*, *C. perangustum*, *C. pseudocladosporioides*, *C. subuliforme*, and *C. tenuissimum*), while 29 isolates clustered into 12 terminal subclades genetically distant from any currently known species of the genus. A total of 14 isolates were related to the *C.*

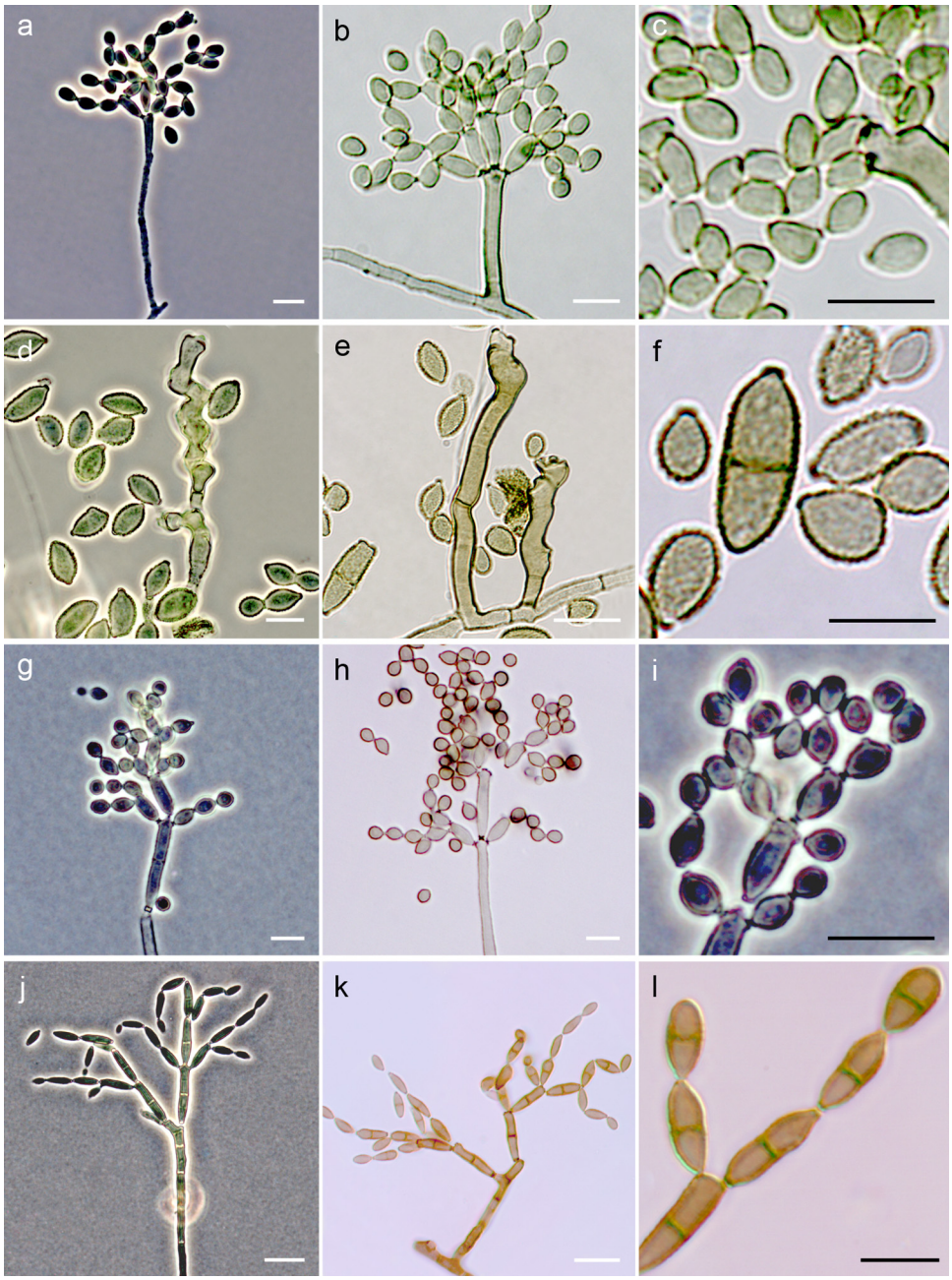

FIG 2 Conidiophores and conidia of fungi belonging to the *C. cladosporioides* complex (a to c), *C. herbarum* complex (d to f), *C. sphaerospermum* complex (g to i), and *Toxicocladosporium* spp. (j to l). White bars, 10 μm; black bars, 5 μm.

herbarum complex (16%), mostly corresponding to five species (i.e., *C. allicinum*, *C. herbarum*, *C. macrocarpum*, *C. ramotenellum*, and *C. subinflatum*), while five isolates clustered into four new lineages in the genus. Seventeen isolates were nested within the *C. sphaerospermum* complex (19%) and mostly belonged to two species (i.e., *C. halotolerans* and *C. sphaerospermum*), while a single isolate represented a new lineage.

Distinct morphological features of isolates in the *C. cladosporioides* complex included the formation of mostly unbranched, cylindrical conidiophores, bearing ovoidal to ellipsoidal intercalary and terminal conidia, smooth or rarely showing fine ornamentation (Fig. 2a to c); the maximum temperatures for growth were 32°C for *C. cladosporioides*, *C. flabelliforme*, *C. perangustum*, and *C. pseudocladosporioides* and 35°C for *C. angustisporum*, *C. funiculosum*, *C. subuliforme*, and *C. tenuissimum*. Isolates of the *C. herbarum* complex exhibited mostly nodulose conidiophores, bearing distinctly ornamented globose to subglobose terminal conidia (Fig. 2d to f); none of the isolates of this complex were able to grow at temperatures above 32°C, and *C. allicinum* exhibited a maximum growth temperature of 30°C. Isolates of the *C. sphaerospermum* complex formed cylindrical and branched conidiophores, bearing globose to subglobose conidia, smooth or

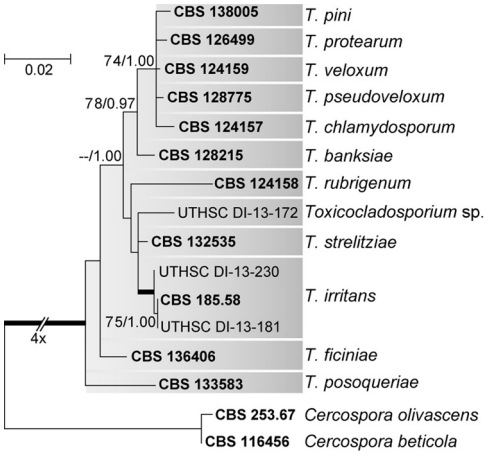

FIG 3 Maximum-likelihood (ML) tree inferred from combined ITS and LSU sequences of *Toxicocladosporium* isolates. Branch lengths are proportional to phylogenetic distance. ML bootstrap support (BS) values of ≥70% and posterior probability (PP) values of ≥0.95 are shown above the branches. Thickened branches indicate BS of 100% and PP of 1.00. *Cercospora beticola* (CBS 116456) and *Cercospora olivascens* (CBS 253.67) were used to root the tree. Type strains are indicated in bold font. CBS, CBS-KNAW Fungal Biodiversity Centre, the Netherlands; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, TX.

finely ornamented (Fig. 2g to i); the maximum temperatures for growth were 32°C for *C. sphaerospermum* and 35°C for *C. halotolerans*. None of the clinical isolates formed sexual morphs in culture.

Overall, the most commonly identified species was *C. halotolerans* (14.8%), followed by *C. tenuissimum* (10.2%) and *C. subuliforme* (5.7%). However, 39.8% of isolates did not match with any known taxa and represent at least 17 putative new *Cladosporium* species (Fig. 1). The most common anatomical site of isolation was the respiratory tract (54.5%), mainly from bronchoalveolar lavage (BAL) fluid and nasal specimens, followed by superficial sites (28.4%); these percentages were similar for all of the species and species complexes identified.

Phylogenetic analysis of the *Toxicocladosporium* isolates included 15 taxa and 984 bp (530 bp for LSU and 454 bp for ITS), of which 826 bp were constant (464 bp for LSU and 362 bp for ITS), 155 were variable (66 bp for LSU and 89 bp for ITS), and 129 were parsimony informative (56 bp for LSU and 73 bp for ITS) (Fig. 3). Two clinical isolates belonged to *Toxicocladosporium irritans*, while the isolate UTHSC DI-13-172 formed an independent lineage, genetically related to *Toxicocladosporium strelitziae* but showing distinctive morphological features and probably corresponding to a new species. The main morphological characteristics of members of *Toxicocladosporium* were the presence of nonnodulose conidiophores with dark and thickened cell walls and septa, producing conidia without the typical coronate scars of *Cladosporium* (Fig. 2j to l), and a maximum temperature for growth of 35°C.

According to the LSU sequence analysis, a single isolate (UTHSC DI-13-256), originally identified as *C. sphaerospermum*, was related to but distant (<98.2% sequence similarity) from members of the genus *Penidiella* (i.e., *Penidiella aggregata* and *Penidiella drakensbergensis*; sequence accession numbers JF499862 and KC005792, respectively) (data not shown). However, its final identification was not possible given the scarcity of DNA sequences of the latter species for comparison. This isolate was characterized by restricted growth (3 to 4 mm at 25°C for 7 days) and the production of solitary penicillate conidiophores, composed of chains of ramoconidia with slightly pigmented and thickened conidiogenous scars.

The results of the antifungal susceptibility testing are summarized in Table 2. The overall results for *Cladosporium* species showed a geometric mean (GM) MIC and MIC_{90} for AMB of 0.64 μg/ml and 2 μg/ml, respectively. Among the azoles, ITC and PSC were the most active, with both drugs having a GM MIC of 0.43 μg/ml and respective MIC_{90}s of 0.5 μg/ml and 1 μg/ml, while VRC showed a GM MIC and MIC_{90} of 1.68 μg/ml and 4 μg/ml, respectively. Flucytosine showed variable activity and had a GM MIC and MIC_{90} of 1.37 μg/ml and 4 μg/ml, respectively. TRB exhibited the most potent activity, with a GM MIC and MIC_{90} of 0.09 μg/ml and 1 μg/ml, respectively. With the exception of CFG, the echinocandins exhibited strong *in vitro* activity, with GM MIC values of 0.19 μg/ml and 0.12 μg/ml for AFG and MFG. All of the *Cladosporium* species tested showed similar susceptibility patterns except for *C. sphaerospermum*, where the three isolates tested exhibited higher MIC and MEC values, especially for the azoles, AMB, AFG, and MFG ($P < 0.001$). Comparison of antifungal susceptibility by species complex (Table 2) showed that AMB exhibited more potent activity against members of the *C. herbarum* complex, with GM MIC and MIC_{90} values of 0.18 μg/ml and 1 μg/ml ($P < 0.002$), while members of the *C. sphaerospermum* complex exhibited higher GM MIC and MIC_{90} values for AMB, PSC, ITC, and CSP ($P < 0.003$). *Toxicocladosporium* and *Penidiella* isolates exhibited similar susceptibility patterns, with mostly low GM MIC and MIC_{90} values against all antifungals tested but without statistically significant differences.

DISCUSSION

Members of *Cladosporium* are relatively easy to identify to genus and species complex based on their typical conidiogenous structures. However, morphological identification of *Cladosporium* species is difficult given the high morphological similarity between closely related species. In light of our results, it is strongly recommended that phenotypic identifications be confirmed with DNA sequencing. Several authors have demonstrated the usefulness of the *EF-1*α and *Act* loci to allow a good species delimitation in *Cladosporium* (1, 19, 21). This is especially important for members of the *C. cladosporioides* complex, which demonstrated the greatest species diversity, the highest number of species associated with clinical samples, and also, the greatest number of undescribed species. Moreover, we found that *C. cladosporioides*, the species most frequently cited as being clinically relevant, was poorly represented in our set of isolates, while *C. asperulatum*, *C. funiculosum*, *C. flabelliforme*, *C. pseudocladosporioides*, *C. subuliforme*, and *C. tenuissimum* are described for the first time from clinical samples. Similarly, in the *C. sphaerospermum* complex, most of the isolates morphologically identified as *C. sphaerospermum* were genetically reidentified as belonging to the phenotypically similar species *C. halotolerans*, which according to our data, emerged as the most common species from clinical origins. The latter species has never been associated with human infection; however, some isolates had been reported from human or animal clinical samples (1). In the case of the *C. herbarum* complex, 13 of the 14 isolates morphologically identified as *C. herbarum*, also

TABLE 2 Results of *in vitro* antifungal susceptibility testing of the 92 clinical isolates included in the study

Genus	Species (no. of isolates tested)	MIC/MEC parameter[a]	Result (μg/ml) for[b]: AMB	5FC	VRC	PSC	ITC	TBF	CFG[c]	AFG[c]	MFG[c]
Cladosporium	*C. cladosporioides* complex (57)	Range	0.06–2	0.06–>32	0.25–16	<0.03–1	<0.03–2	<0.03–4	0.125–8	0.03–0.5	0.03–0.5
		GM	0.73	1.20	1.65	0.40	0.34	0.12	2.78	0.19	0.11
		MIC_{90}	1	4	4	0.5	0.5	1	8	0.5	0.25
	C. tenuissimum (9)	Range	0.5–1	1–>16	1–4	0.25–0.5	0.25–0.5	0.06–1	4–8	0.125–0.5	0.125–0.25
		GM	0.93	2.72	1.85	0.37	0.29	0.18	4.67	0.37	0.15
		MIC_{90}	1.00	4.00	2.00	0.50	0.50	0.25	8.00	0.50	0.25
	C. subuliforme (5)	Range	1–2	0.25–2	0.25–2	0.25–0.5	0.25	0.06–1	4–8	0.06–0.5	0.06–0.25
		GM	1.15	1.00	0.66	0.29	0.25	0.28	5.28	0.16	0.12
		MIC_{90}	1.00	2.00	1.00	0.25	0.25	1.00	8.00	0.25	0.13
	C. pseudocladosporioides (4)	Range	0.5–1	0.5–1	2–4	0.25–0.5	0.5–1	0.03–2	0.25–8	0.03–0.25	0.03–0.125
		GM	0.59	0.71	2.38	0.42	0.59	0.21	2.38	0.15	0.07
		MIC_{90}									
	C. cladosporioides (3)	Range	0.5–1	1–2	0.5–16	0.25–1	0.25–0.5	0.5–2	1–4	0.125–0.25	0.125
		GM	0.79	1.26	1.59	0.40	0.31	1.00	2.00	0.16	0.13
		MIC_{90}									
	C. funiculosum (3)	Range	0.06–1	0.5–1	0.5–2	0.25–0.5	0.125–0.25	0.03–0.06	4	0.06–0.25	0.06–0.125
		GM	0.31	0.63	1.00	0.31	0.20	0.04	4.00	0.12	0.08
		MIC_{90}									
	C. angustisporum (1)	Range	1.00	1.00	4.00	0.50	0.50	2.00	4.00	0.13	0.06
		GM									
		MIC_{90}									
	C. asperulatum (1)	Range	1	0.25	2	0.25	0.5	0.03	8	0.25	0.125
		GM									
		MIC_{90}									
	C. flabelliforme (1)	Range	2	2	2	0.5	0.25	0.03	4	0.25	0.125
		GM									
		MIC_{90}									
	C. perangustum (1)	Range	0.5	4	4	0.5	0.5	0.03	4	0.125	0.06
		GM									
		MIC_{90}									
	Cladosporium sp. (29)	Range	0.125–2	0.06–>16	0.25–8	<0.03–1	<0.03–2	<0.03–4	0.125–8	0.03–0.5	0.03–0.5
		GM	0.67	1.10	1.73	0.43	0.36	0.09	2.00	0.18	0.10
		MIC_{90}	1.00	4.00	4.00	0.50	0.50	1.00	8.00	0.50	0.25
	C. herbarum complex (14)	Range	<0.03–2	0.5–>16	0.5–8	0.06–0.5	0.06–1	<0.03–0.125	0.125–8	0.06–1	0.06–0.5
		GM	0.18	2.97	1.81	0.37	0.35	0.05	0.67	0.23	0.15
		MIC_{90}	1	8	4	0.5	0.5	0.125	2	0.5	0.5
	C. allicinum (3)	Range	<0.03–0.125	2–4	2–4	0.5	0.25–0.5	0.03–0.06	1.00	0.25–0.5	0.125–0.25
		GM	0.05	2.52	3.17	0.50	0.40	0.05	1.00	0.31	0.20
		MIC_{90}									
	C. ramotenellum (3)	Range	1–2	4–>16	1–2	0.5	0.5–1	<0.03–0.125	0.5–8	0.25–1	0.125–0.5
		GM	1.26	4.00	1.59	0.50	0.63	0.05	2.00	0.40	0.20
		MIC_{90}									
	C. herbarum (1)	Range	0.06	8	2	0.5	0.5	0.03	0.5	0.125	0.125
		GM									
		MIC_{90}									
	C. macrocarpum (1)	Range	0.5	2	1	0.25	0.5	0.125	1	0.125	0.5
		GM									
		MIC_{90}									
	C. subinflatum (1)	Range	0.5	0.5	4	0.5	0.5	0.03	0.5	0.5	0.25
		GM									
		MIC_{90}									
	Cladosporium sp. (5)	Range	0.06–0.5	2–4	0.5–8	0.06–0.5	0.06–0.5	<0.03–0.125	0.125–0.5	0.06–0.5	0.06–0.125
		GM	0.11	2.30	1.32	0.25	0.19	0.05	0.29	0.14	0.08
		MIC_{90}	1.00	4.00	4.00	0.50	0.50	1.00	8.00	0.50	0.25
	C. sphaerospermum complex (17)	Range	0.125–2	0.06–4	0.5–16	0.06–4	0.25–>16	<0.03–1	0.06–4	<0.03–1	0.06–1
		GM	1.13	1.13	1.70	0.64	1.13	0.06	1.27	0.15	0.13
		MIC_{90}	2	2	8	2	32	0.5	4	0.25	0.125
	C. halotolerans (13)	Range	0.125–2	0.06–4	0.5–2	0.06–1	0.25–2	<0.03–1	0.06–4	<0.03–0.25	0.06–0.125
		GM	1.00	0.90	1.11	0.47	0.56	0.08	1.00	0.11	0.10
		MIC_{90}	2.00	2.00	2.00	1.00	2.00	1.00	4.00	0.25	0.13
	C. sphaerospermum (3)	Range	1–2	2–4	8–16	2–4	>16	<0.03–0.125	2–4	0.25–1	0.125–1
		GM	1.59	2.52	10.08	2.52	>16	0.03	3.17	0.50	0.40
		MIC_{90}									
	Cladosporium sp. (1)	Range	2	2	2	0.5	0.5	<0.03	2	0.25	0.125
		GM									
		MIC_{90}									
	Overall (88)	Range	<0.03–2	0.06–>16	0.25–16	<0.03–4	<0.03–>16	<0.03–4	0.06–8	<0.03–1	0.03–1
		GM	0.64	1.37	1.68	0.43	0.43	0.09	1.91	0.19	0.12
		MIC_{90}	2.00	4.00	4.00	0.50	1.00	1.00	8.00	0.50	0.25
Toxicocladosporium	*T. irritans* (2)	Range	0.5–1	0.25–2	0.25	0.25–1	0.5	<0.03–0.06	0.125–2	0.06–0.25	0.06–0.5
		GM	0.71	0.71	0.25	0.50	0.50	0.03	0.50	0.12	0.17
		MIC_{90}									
	Toxicocladosporium sp. (1)	Range	0.5	0.125	0.25	0.125	0.125	<0.03	1	0.125	0.06
		GM									
		MIC_{90}									

(Continued on following page)

TABLE 2 (Continued)

Genus	Species (no. of isolates tested)	MIC/MEC parameter[a]	Result (μg/ml) for[b]: AMB	5FC	VRC	PSC	ITC	TBF	CFG[c]	AFG[c]	MFG[c]
	Overall (3)	Range	0.5–1	0.125–2	0.25	0.125–1	0.125–0.5	<0.03–0.06	0.125–2	0.06–0.25	0.06–0.5
		GM	0.63	0.40	0.25	0.31	0.31	0.02	0.63	0.12	0.12
		MIC_{90}									
Penidiella	*Penidiella* sp. (1)	Range	2	0.06	0.125	0.06	>0.03	>0.03	0.25	0.25	0.25
		GM									
		MIC_{90}									

[a] GMs and MIC_{90}s are shown only for species with ≥5 isolates. GM, geometric mean.
[b] AMB, amphotericin B; 5FC, flucytosine; VRC, voriconazole; PSC, posaconazole; ITC, itraconazole; CFG, caspofungin; AFG, anidulafungin; MFG, micafungin; TRB, terbinafine.
[c] These columns include MEC data.

considered a clinically relevant species, were found to belong to other species of this complex (i.e., *C. allicinum*, *C. macrocarpum*, and *C. ramotenellum*). While *C. macrocarpum* has been identified as the causative agent of human infections (17), *C. allicinum* and *C. ramotenellum* have never been reported before in the clinical setting, although some isolates have been recorded as obtained from human samples (1). However, due to the lack of clinical histories and histopathological findings, it was impossible for us to confirm a pathogenic role of the species reported here for the first time from clinical specimens.

It is remarkable that our phylogenetic analysis was unable to provide species-level identification of a high number of *Cladosporium* isolates (39.8%) that were originally considered to belong to several common morphospecies. Instead, those unidentified isolates were grouped into 5 terminal clades and 12 monotypic lineages, representing a large variety of phylogenetic species. It is probable that many of these clades and monotypic lineages represent new species; however, further studies combining phenotypic and molecular data would be necessary to confirm these findings. We report also for the first time the isolation of *Toxicocladosporium* and *Penidiella* species from clinical specimens. Isolates of these recently proposed genera were only known from leaves of several plants and from environmental sources (3, 34). According to our data, the vast majority of isolates were obtained from respiratory specimens, including BAL fluid, nasal, and sputum samples. This is not rare, because *Cladosporium* is preponderant in the airborne mycobiota (35), being considered one of the most important respiratory allergenic fungi, after *Alternaria* (10, 34, 36).

Reports of invasive infections by *Cladosporium* are extremely rare. Bentz and Sautter (37) reported a mixed disseminated infection by *Aspergillus fumigatus* and *C. cladosporioides* in an immunocompromised patient. *Cladosporium cladosporioides* and *C. macrocarpum* have been reported from two clinical cases involving the central nervous system (15, 17), while *C. sphaerospermum* was isolated from an intrabronchial infection (38). However, in none of these cases was the etiology of the infection supported by histopathological studies. The isolation of *Cladosporium* species from deep tissues seems improbable considering the inability of these organisms to grow at temperatures exceeding 35°C, and thermotolerance being one of the most important virulence factors for invasive or disseminated infections (39). In our study, less than half of the isolates exhibited very limited growth at 35°C, while none was able to grow at 37°C. However, surprisingly, several of our isolates were obtained from deep tissue samples, including bone marrow, cerebrospinal fluid (CSF), and lung and lymphatic tissue samples, among others. Isolation of these fungi from invasive infections may have been due to environmental contamination of the samples; however, occasionally isolates that fail to grow in culture at 37°C have been reported to cause invasive disease in immunocompromised individuals (40).

There is a paucity of information regarding antifungal susceptibility patterns for *Cladosporium* species. Most data are from a few reported clinical cases (7, 13, 15, 41). Our study provides the first *in vitro* data for a large set of clinical isolates, including several species obtained from diverse anatomical sites and not previously reported from clinical samples. Case reports have shown a favorable outcome using azole-based therapies. ITC has shown efficacy in the treatment of superficial infections caused by *C. cladosporioides*, *C. sphaerospermum*, and *C. oxysporum* (8, 14, 37, 41–44), while VRC was effective against *C. macrocarpum* in a brain abscess (17). This agrees with our *in vitro* data, which demonstrated that the azoles, particularly ITC and PSC, have good activity against *Cladosporium* species, although VRC displayed variable activity. AMB has been shown to be ineffective against *C. cladosporioides* (41) and *C. sphaerospermum* (38) in cases of skin and intrabronchial infections, respectively. Our results, however, suggest that this drug might be effective, especially against members of the *C. herbarum* complex. Kantarcioğlu and Yücel (45) reported potent *in vitro* activity of TRB against a set of unidentified *Cladosporium* species. Our data confirmed the results of that study, with TRB showing significant activity against all of the species tested. Echinocandin activity against *Cladosporium* species has not been previously evaluated; however, we observed that both AFG and MFG exhibited notable *in vitro* activity against all of our isolates, indicating that they could represent an important alternative for the treatment of infections by these fungi pending further confirmatory studies.

In conclusion, our study has significantly expanded the diversity of *Cladosporium* species seen in clinical specimens as a result of the molecular characterization of these isolates. We were unable, however, to document these organisms as etiologic agents in human or animal disease due to the lack of clinical information and/or histopathological findings. It is also important to note that most reported cases of *Cladosporium* infections lack molecular confirmation, and in those cases where they have been so characterized, the strains are not available. Given that many journals require the public availability of DNA sequence data, we recommend that clinical strains be deposited in international culture collections, thereby making them available for reidentification and further study.

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