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Diversity of coelomycetous fungi in human infections: A 10-y experience of two European reference centres

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ABSTRACT

The coelomycetous fungi are difficult to properly identify from their phenotypic characterization and their role as etiologic agents of human infections is not clear. We studied the species distribution of these fungi among clinical isolates that had been collected and stored over a ten-year period in two European reference laboratories (France and Spain). We identified phenotypically and molecularly 97 isolates by sequencing the D1-D2 fragment of the 28S nrRNA (LSU) gene and we provided the *in vitro* antifungal susceptibility pattern of seven antifungals against 46 isolates. Species of the orders *Pleosporales* and *Glomerellales* were present in both collections, and *Botryosphaeriales* and *Diaporthales* only in the French one. The most prevalent species were *Medicopsis romeroi*, *Neocucurbitaria keratinophila*, *Neocucurbitaria unguis-hominis* and *Paraconiothyrium cyclothyrioides*, which had been recovered primarily from superficial tissues. The *Didymellaceae* was the most common family represented, with 27 isolates distributed into five genera. Most of the isolates tested were susceptible to antifungals, and only the geometric mean (GM) and minimal inhibitory concentration (MIC) values of itraconazole and caspofungin had higher values. This study provides a good picture of the great diversity of coelomycetous fungi in the European clinical context, and the basis for future studies on this interesting but neglected group of fungi.

1. Introduction

Human infections by coelomycetous fungi are rare and poorly characterized due to the difficulty in identifying these fungi using only phenotypic tools. The coelomycetous fungi are characterized by the production of conidia (asexual spores) into fruiting bodies (= conidiomata). The class Coelomycetes today lack scientific validity due to the demonstrated polyphyletic character of this sort of fungi (Wijayawardene et al., 2016). They cause superficial or subcutaneous infections, mostly following a traumatic inoculation of contaminated plant material or soil particles during agricultural work in tropical and subtropical areas (Stchigel and Sutton, 2013). The most common coelomycetous fungi involved in these infections are the etiologic agents of black-grain eumycetoma, such as *Biatriospora mackinnonii*; *Falciformispora* spp., *Medicopsis romeroi*, and *Pseudochaetosphaeronema larense* (Ahmed et al., 2014). Other common coelomycetous fungi include *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum*, which typically cause onychomycosis, subcutaneous phaeohyphomycosis, and rarely eumycetoma (Stchigel and Sutton, 2013; de Hoog et al., 2000). In addition, many species of *Phoma* and *Pyrenochaeta* have been reported as occasional agents of localized and systemic infections in humans (Punithalingam, 1979; Ferrer et al., 2009). The taxonomy of several coelomycetous genera mentioned before have been revised recently but they still constitute a group of highly polyphyletic taxa

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that are usually difficult to identify phenotypically (de Gruyter et al., 2013; Chen et al., 2015).

In a recent study on coelomycetes conducted in the USA, Valenzuela-Lopez et al. (2017) identified 230 fungal strains by sequencing the D1-D2 domains of the 28S rRNA gene (LSU), from which 152 (66.1 %) strains belonged to the order *Pleosporales*, the rest being distributed in several orders of the phylum *Ascomycota*. Most of these strains were recovered from superficial tissue. *N. dimidiatum*, *Paraconiothyrium cyclothyrioides* and members of the family *Didymellaceae* were the most prevalent taxa. In addition, those authors demonstrated the usefulness of the LSU as a good molecular marker for a preliminary identification of coelomycetous fungi at genus level. However, the nucleotide sequences of more phylogenetically informative genes need analysing in order to identify the fungi at species level. Genes such as the RNA polymerase II subunit 2 (*rpb2*), translation elongation factor 1-alpha (*tef1*), beta-tubulin (*tub2*) and the ribosomal internal transcribed spacer region (ITS), combined in a multi-locus analysis, have all been recommended for this purpose (Valenzuela-Lopez et al., 2018a).

Until now, the coelomycetous fungi involved in invasive fungal infections (IFIs) are poorly known in Europe, probably due to the infrequency of these fungi and the complexity of their identification in the absence of characteristic fruiting bodies when grown on culture media used in the clinical lab. In a recent French study, eighteen proven cases of cutaneous and subcutaneous primary infections by coelomycetous fungi were reported and analysed in patients from tropical and subtropical regions (Guégan et al., 2016).

For a better knowledge of the diversity of coelomycetous fungi involved in human infections, we studied a large set of clinical isolates that had been identified in two mycology reference centres in France and Spain, and determined their *in vitro* antifungal susceptibility pattern.

2. Material and methods

2.1. Fungal isolates

We studied 97 isolates of coelomycetous fungi recovered from clinical specimens, 51 isolates (CNRMA) of which were provided by the French National Reference Centre for Invasive Mycoses and Antifungals (NRCMA) at the *Institut Pasteur*, Paris. The Spanish National Centre of Microbiology at the *Instituto de Salud Carlos III*, Madrid provided 46 isolates (CNM-CM). The isolates were collected between 2005 and 2015. Table 1 gives information about the country of isolation and the location of the infection in the body.

2.2. Morphological and physiological characterization

For morphology studies, the isolates were cultured following the recipes and descriptions by Valenzuela-Lopez et al. (2018b).

2.3. Molecular identification and phylogenetic analysis

Total genomic DNA was extracted following the protocols by Valenzuela-Lopez et al. (2017). Preliminary molecular identification of the isolates was made using LSU nucleotide sequences in $BLAST_N$ searches. Twenty-eight LSU sequences of type or reference strains deposited in the GenBank database by the Westerdijk Fungal Biodiversity Institute (CBS) and the Mae Fah Luang University (MFLUCC) culture collections were used for identification and phylogenetic purposes. DNA sequences generated in this study were deposited in GenBank (accession numbers are given in Table 1).

For the phylogenetic study, sequences were aligned using the ClustalW application (Thompson et al., 1994) of the MEGA 6.06 (Tamura et al., 2013) computer program, and manually adjusted using the same software platform. Phylogenetic reconstructions were made by maximum-likelihood (ML) and Bayesian inference (BI) with MEGA 6.06 and MrBayes 3.2.4 (Huelsenbeck and Ronquist, 2001), respectively. The best substitution model for the gene matrix (TN93 + G) was estimated using MEGA 6.06. For ML analyses, nearest-neighbour interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1000 ML bootstrapped pseudoreplicates. Bootstrap support (BS) of $\geq$70 was considered significant. For BI analyses, Markov chain Monte Carlo (MCMC) sampling was carried out with four million generations, with samples taken every 1000 generations. The 50 % majority rule consensus trees and posterior probability values (PP) were calculated after removing the first 25 % of the resulting trees for burn-in. A PP value of $\geq$0.95 was considered significant. Reference strains of *Colletotrichum gigasporum* (CBS 159.75), *Colletotrichum gloeosporioides* (CBS 122687) and *Colletotrichum hippeastri* (CBS 241.78) were used as outgroup.

2.4. Antifungal susceptibility testing

The *in vitro* susceptibility testing in both reference centres (n = 46 isolates) followed the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2008) procedure. The antifungals used were amphotericin B (Sigma–Aldrich Química, Madrid, Spain), itraconazole (Sigma–Aldrich Química, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), voriconazole (Pfizer S.A., Madrid, Spain), caspofungin (Merck & Co., Inc., Rahway, N.J.), micafungin (Astellas Pharma Inc, Tokyo, Japan) and terbinafine (Novartis, Basel, Switzerland). For the NCRMA, all antifungal drugs were obtained from ALSACHIM, Strasbourg, France.

The isolates were cultured on potato carrot agar (PCA; 20 g each of filtered potatoes and carrots, 20 g of agar, 1 L of distilled water) or oatmeal agar (OA; 30 g of filtered oat flakes, 15 g of agar–agar, 1 L tap water) for seven to 30 d at 25 °C and 30 °C to obtain sporulation. Conidia were then collected in sterile water containing 0.01 % (v/v) Tween 80 (Sigma–Aldrich, St. Louis, MO, USA), and the suspension was adjusted to 2–5 x 10^5 conidia/mL. The minimal effective concentration (MEC) was determined for each echinocandin and the minimal inhibitory concentration (MIC) for the other drugs (90 % inhibition for amphotericin B and 80 % for the azoles) after 24 h and 48 h of incubation at 35 °C. *Aspergillus flavus* ATCC 204304 and *Aspergillus fumigatus* ATCC 204305 were used as quality control strains in all tests carried out. Susceptibility profiles were determined for 46 isolates since non-sporulating isolates were excluded at the NRCMA.

3. Results

3.1. Locations of infections

The majority of the isolates were recovered from superficial tissue, mainly skin (44 %; 43/97), eyes (27 %; 26/97), nails/hairs (18 %; 17/97) and mouth/sinus (2 %; 2/97). A few were recovered from deeper sites: bones (4 %, 4/97), blood (2 %, 2/97), cerebrospinal fluid (n = 1), bone marrow (n = 1) and lung (n = 1) (Tables 1 and 2).

3.2. Phylogenetic analyses

The maximum-likelihood (ML) phylogenetic analysis of the LSU sequences (approximately 584 pb) demonstrated that the 97 isolates were distributed into four orders, but scattered into fourteen

Table 1
Taxonomical identification of the isolates studied, origin and GenBank accession numbers. New sequences generated are indicate in bold.

Order	Species	Strain no.[a]	Origin	Country	GenBank accesion no.[b]
Botryosphaeriales	*Diplodia seriata*	CBS 112555^T	*Vitis vinifera* dead plant	Portugal	KF766327
		CNRMA 6.1007	bone	France	**LT965964**
	Lasiodiplodia sp.	CNRMA 15.383	eye	France (West Indies, Guadeloupe)	**LT965965**
	Lasiodiplodia theobromae	CBS 164.96^T	fruit along coral reef coast	Papua New Guinea	NG_042460
		CNRMA 10.1369	skin	France (West Indies, Martinique)	**LT965966**
		CNRMA 10.813	eye	France (West Indies, Martinique)	**LT965967**
		CNRMA 11.360	eye	France (West Indies, Martinique)	**LT965968**
		CNRMA 13.891	skin	France	**LT965969**
		CNRMA 14.708	eye	France (West Indies, Guadeloupe)	**LT965970**
	Neofusicoccum luteum	CBS 110299	*Vitis vinifera* cane	Portugal	AY928043
		CNRMA 12.597	eye	France	**LT965971**
Diaporthales	*Diaporthe sclerotioides*	CBS 296.67^T	*Cucumis sativus* root	The Netherlands	AF439628
	Diaporthe sp.	CBS 477	*Cucumis sativus*	USA	AF439631
		CNRMA 8.522	eye	France	**LT965972**
		CNRMA 9.205	eye	France (West Indies, Guadeloupe)	**LT965973**
		CNRMA 11.385	eye	France (West Indies, Martinique)	**LT965974**
		CNRMA 12.311	blood	France	**LT965975**
		CNRMA 13.515	skin	France	**LT965976**
		CNRMA 14.198	skin	France	**LT965977**
Glomerellales	*Colletotrichum gigasporum*	CBS 159.75	air and stored grains	India	DQ286206
		CNRMA 16.553	skin	France (West Indies, Guadeloupe)	**LT965978**
	Colletotrichum gloeosporioides	CBS 122687	*Leucospermum* sp. leaf litter	South Africa	EU552111
		CNRMA 15.504	eye	France (West Indies, Martinique)	**LT965979**
	Colletotrichum hippeastri	CBS 241.78	*Hippeastrum* sp.	The Netherlands	DQ286167
	Colletotrichum sp.	CNM-CM4760	corneal swab	Spain	**LT965980**
		CNM-CM 6116	conjuntival	Spain	**LT965981**
		CNM-CM 7345	humour acuosus	Spain	**LT965982**
Pleosporales	*Didymella gardeniae*	CBS 626.68^T	*Gardenia jasminoides* leaf	India	GQ387595
		CNM-CM 3697	nail	Spain	**LT965983**
		CNM-CM 3895	nail	Spain	**LT965984**
		CNM-CM 5036	scales	Spain	**LT965985**
		CNM-CM 5814	conjunctival exudate	Spain	**LT965986**
		CNM-CM 7499	conjunctival exudate	Spain	**LT965987**
		CNRMA 11.794	skin	France	**LT965988**
	Didymella glomerata	CBS 528.66	*Chrysanthemum* sp. cutting	The Netherlands	EU754184
		CNM-CM 3356	toenail	Spain	**LT965989**
		CNM-CM 3546	nail	Spain	**LT965990**
		CNM-CM 4675	nail	Spain	**LT965991**
		CNM-CM 7099	cutaneous exudate	Spain	**LT965992**
		CNRMA 9.1046	skin	France	**LT965993**
		CNRMA 10.867	skin	France	**LT965994**
		CNRMA 15.6	mouth/sinus	France	**LT965995**
	Epicoccum nigrum	CBS 173.73^T	*Dactylis glomerata* seed	USA	GU237975
		CNM-CM 5281	skin	Spain	**LT965996**
		CNM-CM 5724	vitreous humour	Spain	**LT965997**
	Epicoccum sorghinum	CBS 179.80	*Sorghum vulgare*	Puerto Rico	GU237978
		CNRMA 7.167	bone	France (New Caledonia)	**LT965998**
		CNRMA 10.947	skin	France (New Caledonia)	**LT965999**
		CNRMA 10.948	skin	France (New Caledonia)	**LT966000**
	Medicopsis romeroi	CBS 252.60^T	maduromycosis	Venezuela	EU754207
		CNM-CM 3387	knee ulcer	Spain	**LT966001**
		CNM-CM 7645	cutaneous exudate	Spain	**LT966002**
		CNRMA 4.200	eye	France	**LT966003**
		CNRMA 5.321	skin	France	**LT966005**
		CNRMA 7.1225	skin	France	**LT966007**
		CNRMA 8.1363	skin	France	**LT966008**
		CNRMA 11.680	skin	France	**LT966010**
		CNRMA 11.949	bone	France	**LT966011**
		CNRMA 14.407	skin	France	**LT966013**
		CNRMA 15.461	bone	France	**LT966014**
		CNRMA 15.7	skin	France	**LT966015**
	Neoascochyta desmazieri	CBS 297.69^T	*Lolium perenne*	Germany	KT389726
		CNM-CM 6201	nail	Spain	**LT966016**
	Neocucurbitaria cava	CBS 257.68^T	wheat-field soil	Germany	EU754199
		CNRMA 15.708	mouth/sinus	France	**LT966017**
	Neocucurbitaria keratinophila	CBS 121759^T	corneal scrapings	Spain	LT623215
		CNM-CM 5882	cutaneous exudate	Spain	**LT966018**
		CNM-CM 6401	fingernail	Spain	**LT966019**
		CNM-CM 6455	cutaneous exudate	Spain	**LT966020**
		CNM-CM 7013	cutaneous exudate	Spain	**LT966021**
		CNM-CM 7457	cutaneous exudate	Spain	**LT966022**
		CNM-CM 7731	cutaneous exudate	Spain	**LT966023**
		CNM-CM 8010	conjunctival exudate	Spain	**LT966024**

(*continued on next page*)

Table 1 (*continued*)

Order	Species	Strain no.[a]	Origin	Country	GenBank accesion no.[b]
		CNM-CM 8674	toenail	Spain	**LT966025**
	Neocucurbitaria unguis-hominis	CBS 112.79	airborn	Wales	GQ387622
		CNM-CM 7037	nail	Spain	**LT966026**
		CNM-CM 7089	cutaneous lession	Spain	**LT966027**
		CNM-CM 8717	urine	Spain	**LT966028**
		CNM-CM 8743	toenail	Spain	**LT966029**
		CNRMA 4.1112	eye	France	**LT966030**
		CNRMA 6.243	eye	France	**LT966031**
		CNRMA 16.153	eye	France	**LT966032**
		CNRMA 16.19	lung	France	**LT966033**
	Neocucurbitaria sp.	CNM-CM 6489	wound exudate	Spain	**LT966034**
		CNM-CM 7025	hair	Spain	**LT966035**
		CNM-CM 7132	toenail	Spain	**LT966036**
	Paraconiothyrium cyclothyrioides	CBS 972.95^T	soil	Papua New Guinea	JX496232
		CNM-CM 6313	conjunctival exudate	Spain	**LT966037**
		CNM-CM 6513	nail	Spain	**LT966038**
		CNM-CM 4767	abscess	Spain	**LT966039**
		CNRMA 11.383	skin	France (West Indies, Martinique)	**LT966041**
		CNRMA 11.855	skin	France	**LT966042**
		CNRMA 13.245	skin	France	**LT966043**
		CNRMA 16.374	skin	France (West Indies, Guadeloupe)	**LT966044**
		CNRMA 16.556	skin	France (West Indies, Guadeloupe)	**LT966045**
	Paraconiothyrium fuckelii	CBS 797.95	*Rubus* sp. dead stem	Denmark	JX496226
		CNRMA 3.240	eye	France	**LT966046**
		CNRMA 4.493	eye	France	**LT966047**
	Paraphaeosphaeria michotii	MFLUCC 13-0349	Poaceae dead leaves	Italy	KJ939282
		CNM-CM 6000	skin	Spain	**LT966048**
	Paraphoma fimeti	CBS 170.70^T	*Apium graveolens* seeds	The Netherlands	GQ387584
		CNM-CM 8075	wound exudate	Spain	**LT966049**
	Paraphoma sp.	CNRMA 9.467	skin	France	**LT966050**
		CNRMA 15.665	skin	France	**LT966051**
	Phaeosphaeriopsis obtusispora	CBS 246.64	*Aloe arborescens* dead leaf	Portugal	JX681119
	Phoma herbarum	CBS 615.75	*Rosa multiflora* dead stem	The Netherlands	EU754186
		CNM-CM 2132	right toe	Spain	**LT966052**
		CNM-CM 3526	bone marrow	Spain	**LT966053**
		CNM-CM 3597	blood culture	Spain	**LT966054**
		CNM-CM 8031	nail	Spain	**LT966055**
		CNRMA 9.1095	skin	France	**LT966056**
		CNRMA 11.1097	eye	France	**LT966057**
		CNRMA 12.1227	eye	France	**LT966058**
	pleosporelean fungus	CNRMA 11.1115	skin	France	**LT966059**
	Preussia sp.	CNM-CM 7343	nail	Spain	**LT966060**
	Preussia terricola	CBS 317.65^T	*Musa sapientum* rhizosphere	Honduras	GQ203725
	Preussia typharum	CBS 107.69	Dung of deer	Japan	GQ203726
		CNM-CM 7335	nail	Spain	**LT966061**
	Pseudophaeosphaeria rubi	MFLUCC 14-0259	*Rubus idaeus* dead branch	Italy	KX765299
	Tintelnotia destructans	CBS 127737^T	anterior eye chamber cornea	Germany	KY090664
		CNM-CM 7430	Unknown	Spain	**LT966062**
	Tintelnotia sp.	CNM-CM 7080	nail	Spain	**LT966063**
		CNM-CM 7981	cutaneous exudate	Spain	**LT966064**
	Xenodidymella saxea	CBS 419.92^T	Corroded mediterranean marble	Unknown	GU238141
		CNRMA 16.76	Cerebrospinal fluid	France	**LT966065**

[a] **CBS**: Strains from Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CNM-CM**: Isolates from the National Centre for Microbiology, Instituto Carlos III, Madrid, Spain; **CNRMA**: Isolates from the National Reference Center for Invasive Mycoses and Antifungals; Institut Pasteur, Paris, France; **MFLUCC**: Strains from Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. Type strains are indicated by a superscript^T.

[b] LSU, large subunit ribosomal DNA sequences. Sequences generated in this study are indicated in bold.

Table 2
Localization of infections due to coelomycetous fungi isolates.

Orders	no. of isolates obtained from:		
	Superficial infection	Deep infection	Total no. of isolates
Botryosphaeriales	7	1	8
Diaporthales	5	1	6
Glomerellales	5		5
Pleosporales	71	7	78
Total no. of isolates (%)	**88 (91)**	**9 (9)**	**97 (100)**

clades (Fig. 1). Most of the isolates (81 %; 78/97) belonged to the order *Pleosporales*, which were distributed into nine clades corresponding to 23 species of twelve genera, followed by those of the *Botryosphaeriales* (8 %; 8/97), the *Diaporthales* (6 %; 6/97) and the *Glomerellales* (5 %; 5/97).

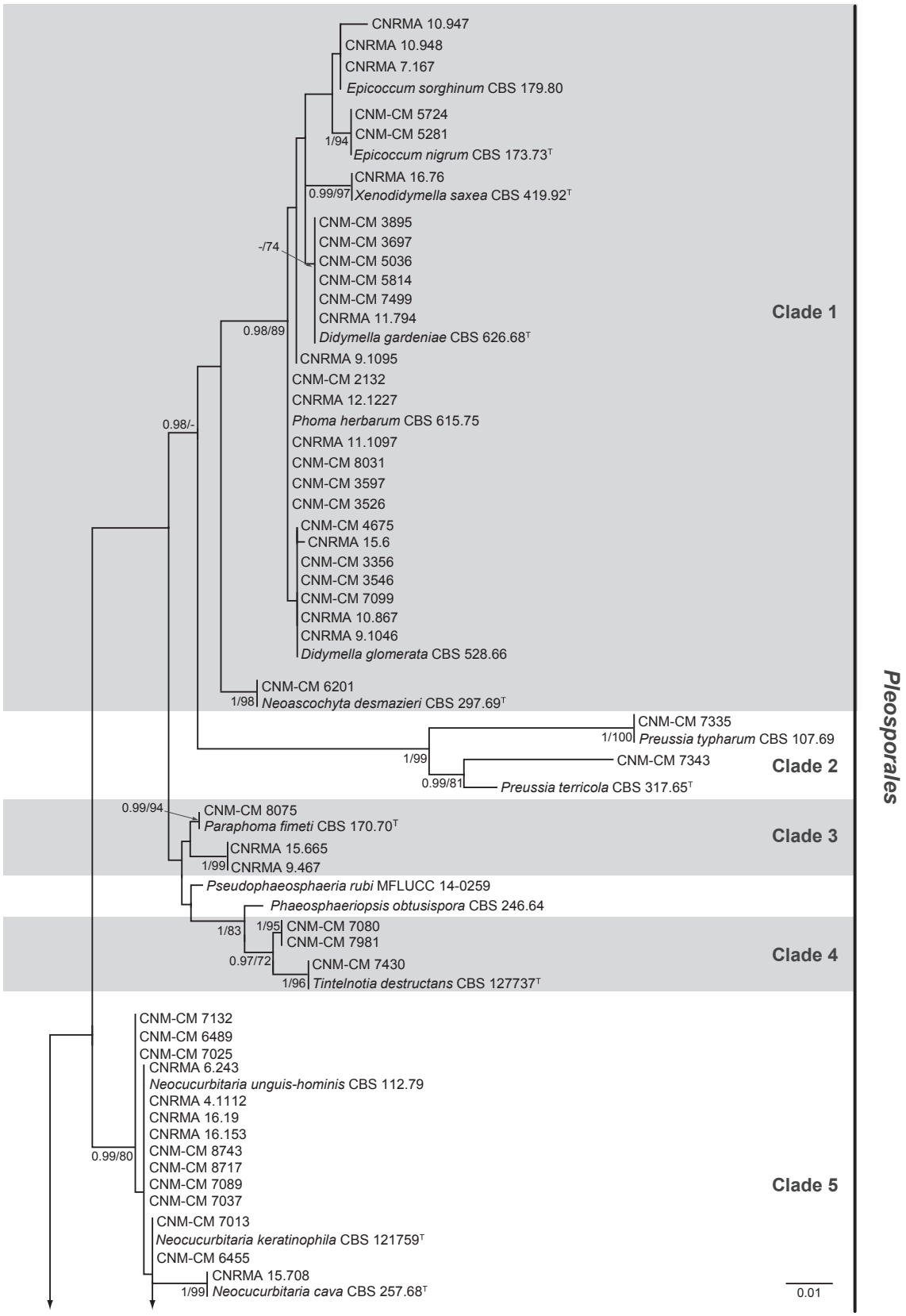

Fig. 1. Maximum likelihood tree obtained from the D1-D2 of LSU (584 bp) sequences of the 125 strains, where 28 belong to type or reference strains. The branch lengths are proportional to phylogenetic distance. Bayesian posterior probability scores ≥0.95 and Bootstrap support values ≥ 70 % are indicated on the nodes. Some branches were shortened to fit them to the page, these are indicated by two diagonal lines with the number of times a branch was shortened. The species of the genus *Colletotrichum* were used to root the tree. Superscript ^{T} indicated the type strains.

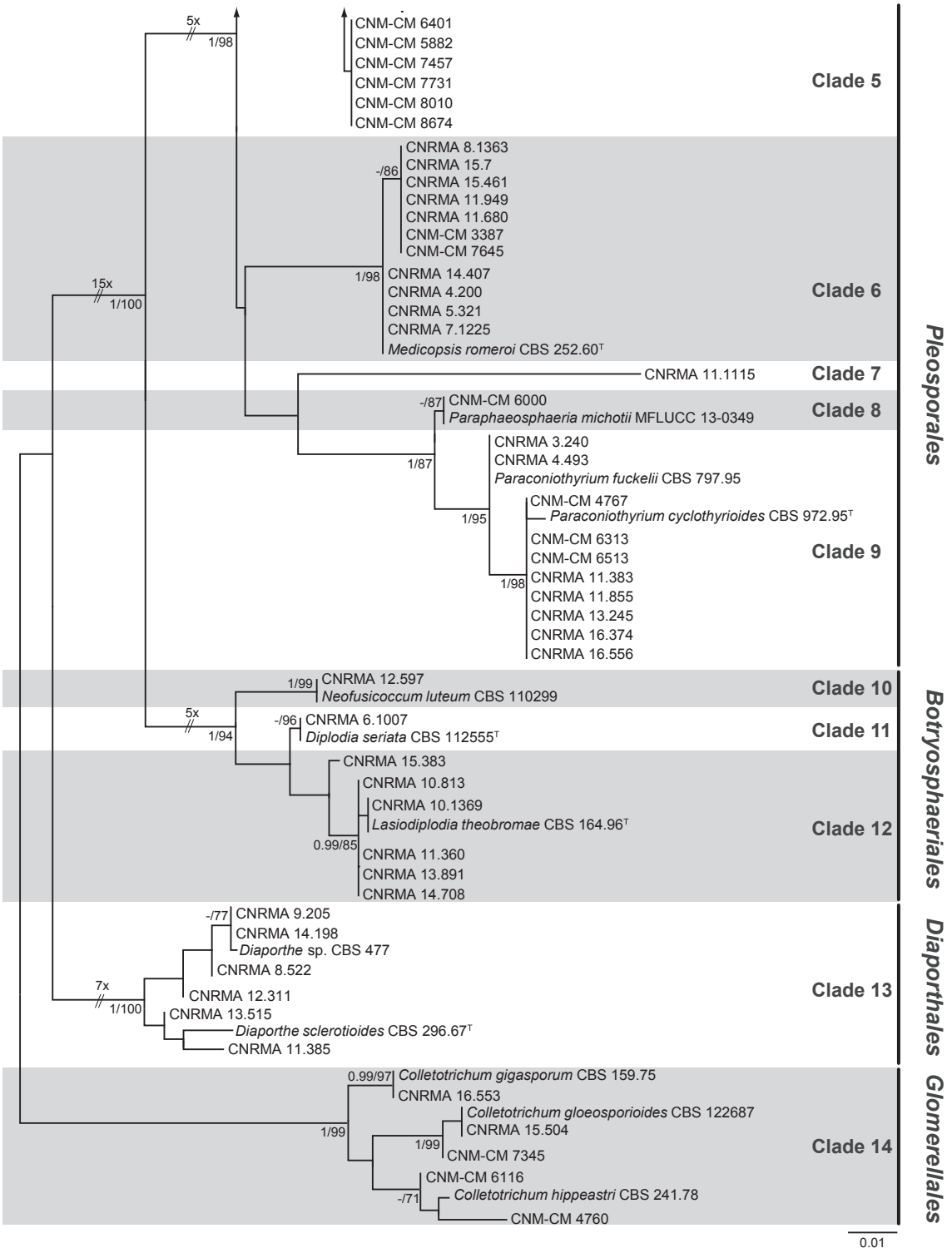

Fig. 1. (*continued*).

The most common species identified was *M. romeroi* (11 %; 11/97), followed by *P. cyclothyrioides*, *Neocucurbitaria keratinophila* and *Neocucurbitaria unguis-hominis* (8 % each; 8/97). These species were mostly isolated from cutaneous lesions (Table 2).

Clade 1 of the *Pleosporales* corresponded to the family *Didymellaceae*, which included 27 isolates distributed into five genera, morphologically characterized by their production of pycnidial conidiomata and hyaline, aseptate conidia. The five genera were *Didymella*, *Epicoccum*, *Neoascochyta*, *Phoma* and *Xenodidymella*. *Didymella* was represented by 13 isolates, six of them clustering with the type strain of *Didymella gardeniae* (CBS 626.68), and the other seven clustered with a reference strain of *Didymella glomerata* (CBS 528.66). The genus *Epicoccum* grouped five of the isolates, three of them clustering with a reference strain of *Epicoccum*

sorghinum (CBS 179.80) and the other two with the type strain of the type species of the genus, *Epicoccum nigrum* (CBS 173.73). The genus *Phoma* was represented by seven clinical isolates and a reference strain of *Phoma herbarum* (CBS 615.75). Two additional isolates included in this clade (CNRMA 16.76 and CNM-CM 6201) grouped with the type strains of *Xenodidymella saxea* (CBS 419.92) and *Neoascochyta desmazieri* (CBS 297.69), respectively.

Clade 2 had two species of *Preussia*: CNM-CM 7335 grouped with a reference strain of *Preussia typharum* (CBS 107.69), while CNM-CM 7343 represented an unknown species forming a sister clade with the type strain of *Preussia terricola* (CBS 317.65).

Clade 3 grouped three isolates of *Paraphoma*, one of them (CNM-CM 8075) clustered with the type strain of *Paraphoma fimeti* (CBS 170.70), and the remaining two (CNRMA 15.665 and CNRMA 9.467) representing unidentified phoma-like species.

Clade 4 had two sister clades of the genus *Tintelnotia*, which produced pycnidia and hyaline, aseptate conidia. The isolate CNM-CM 7430 was identified as *Tintelnotia destructans*. However, the other two isolates (CNM-CM 7080 and CNM-CM 7981) did not cluster with any known species of the genus and might represent new species.

Clade 5 had 20 isolates of *Neocucurbitaria*. *N. keratinophila* and *N. unguis-hominis* were the most common species, both with eight isolates each. *Neocucurbitaria cava*, with a single isolate (CNRMA 15.708), was also included in this clade. Three Spanish isolates, CNM-CM 6489, CNM-CM 7025 and CNM-CM 7132 were identified as *Neocucurbitaria* sp. due to being phylogenetically different from the other isolates and, again, might be a new species of the genus. *Neocucurbitaria* spp. produces pycnidia, ornamented or not, with bristle-like setose structures, and hyaline, aseptate conidia.

Clade 6 had eleven isolates of *M. romeroi* (syn. *Pyrenochaeta romeroi)*, which produces pycnidia and hyaline, aseptate conidia.

Clade 7 is represented by a single isolate (CNRMA 11.1115), phylogenetically distinct from the known pleosporalean fungi, possibly representing a novel taxon.

Clades 8 and 9 belonged to the family *Didymosphaeriaceae*. Clade 8 included a single isolate (CNM-CM 6000) phylogenetically related to a reference strain of *Paraphaeosphaeria michotii* (MFLUCC 13-0349). Clade 9 grouped ten isolates, two related to a reference strain of *Paraconiothyrium fuckelii* (CBS 797.95) and eight with the type strain of *P. cyclothyrioides* (CBS 972.95). Members of the *Didymosphaeriaceae* form pycnidia and pale brown, 0-1 septate conidia.

The order *Botryosphaeriales* are present in Clades 10 to 12. Clade 10 had only one isolate (CNRMA 12.597) which clustered with a reference strain of *Neofusicoccum luteum* (CBS 110299); Clade 11 also had a single isolate (CNRMA 6.1007) that clustered with the type strain of *Diplodia seriata* (CBS 112555), and Clade 12 grouped six isolates, five of them clustering with the type strain of *L. theobromae*, and CNRMA 15.383 identified as *Lasiodiplodia* sp. These fungi produce stromatic conidiomata and aseptate, hyaline to brown, thick-walled conidia.

Clade 13 included the type strain of *Diaporthe sclerotioides* (CBS 296.67) and six isolates corresponding to unidentified species of the genus *Diaporthe* (*Diaporthales*), none of them able to be morphologically distinguished since they produce pycnidia and small hyaline conidia.

Clade 14, corresponding to the *Glomerellales*, was used as outgroup. Five isolates nested in the *Colletotrichum* clade, two clustering with reference strains of *C. gigasporum* (CBS 159.75) and *C. gloeosporioides* (CBS 122687), respectively; and the other three, could not be identified. All the isolates showed the typical morphology of *Colletotrichum*, i.e., acervuli, conidia variable in shape, flattened with thickened tip branches (appressoria).

3.3. Antifungal susceptibility testing

The MIC was determined for 46 of the isolates included here (16 from Spain and 30 from France) (Table 3, Table S1). Globally, the geometric mean (GM) and MIC_{50} values of itraconazole and caspofungin were the highest (Table 3). The MIC of amphotericin B (0.06–1 mg/L) was generally low among the *Pleosporales* with the exception of one isolate of *M. romeroi* and one of *D. gardeniae*, with MICs of 8 and 32 mg/L, respectively. The azole MICs ranged between 0.03 and 1 mg/L for isolates belonging to the genera *Paraconiothyrium*, *Paraphoma*, *Tintelnotia* and *Neocucurbitaria*, with the exception of two isolates of *N. unguis-hominis*, which showed higher values (16 mg/L). The terbinafine MIC was low except for *Diaporthe* spp. and a few isolates of *Colletotrichum* spp. and *M. romeroi*.

4. Discussion

The present study is the largest on this taxonomically complex group of fungi from clinical origin, with almost a hundred isolates morphologically and molecularly characterized from two southern European countries (France and Spain). Most of these coelomycetous fungi belonged to the order *Pleosporales* and were most commonly recovered from superficial infections. Similar results were observed in a previous work that focused on coelomycetous fungi collected at a North American reference centre (Valenzuela-Lopez et al., 2017). However, the diversity of the fungi identified in that study was higher, i.e. eleven orders were represented against four here.

In the present study, *M. romeroi* was the most frequently isolated species whereas the most common taxon in the American study was *N. dimidiatum*. Interestingly, while *M. romeroi* is usually reported as an etiologic agent of black grain eumycetoma (Ahmed et al., 2014; van de Sande, 2013), our isolates were mainly recovered from eye and non-mycetoma subcutaneous infections.

The second most frequently isolated species were *P. cyclothyrioides*, *N. unguis-hominis* and *N. keratinophila*. *P. cyclothyrioides* is an emerging pathogen (Colombier et al., 2015; Guégan et al., 2016; Valenzuela-Lopez et al., 2017) and was represented by eight isolates recovered from skin or superficial locations and mainly from tropical regions. *N. unguis-hominis*, initially described as an agent of human onychomycosis (Punithalingam and English, 1975), was equally distributed across both centres (n = 8 isolates). Regarding *N. keratinophyla*, this species was reported for the first time from a corneal infection in Spain (Ferrer et al., 2009). Interestingly, as well as being the first case reported for this species, all the isolates of *N. keratinophyla* were recovered in Spain from superficial tissue.

Table 3
Overall *in vitro* antifungal activity against the 46 coelomycetous isolates as determined by EUCAST[a] methodology.

Antifungal agent	MIC/MEC values (mg/L)[b]				
	range	median	GM	MIC_{50}	MIC_{90}
Amphotericin B	0.03–16	0.5	0.41	0.25	1
Itraconazole	0.014–16	2	1.72	0.5	16
Voriconazole	0.03–16	0.5	0.70	0.6	4
Posaconazole	0.014–16	0.5	0.58	0.25	8
Caspofungin	0.125–16	2	2.17	1	8
Micafungin	0.015–16	0.5	0.53	0.125	8
Terbinafin	0.014–16	0.25	0.39	0.25	2

[a] EUCAST, European Committee on Antimicrobial Susceptibility Testing procedure.
[b] MIC, minimum inhibitory concentration; MEC, minimal effective concentration; MIC_{50} and MIC_{90}, MIC encompassing 50 and 90 % of isolates tested, respectively.

Other coelomycetous fungi we identified in the present work were *D. glomerata* and *Phoma herbarum*. Although *Phoma* spp. are commonly reported as a coelomycete involved in human infections (Punithalingam, 1979; Bennett et al., 2018), recent extensive changes in taxonomy and nomenclature have spread all but one of the species into different genera of the *Didymellaceae*, *Phoma herbarum* conserved as the type species of the genus (Chen et al., 2015; Valenzuela-Lopez et al., 2018b). Interestingly, *D. gardeniae* was commonly found in our study (five isolates from Spain and one from France).

Recently, Ahmed et al. (2017) proposed *T. destructans*, a new phoma-like fungus belonging to the *Phaeosphaeriaceae* able to cause eye and nail infections. They reported the successful use of terbinafine against a case of keratitis by this species. Two of the Spanish isolates recovered from superficial specimens (one cutaneous exudate and one nail sample) were molecularly related to the above-mentioned species but phylogenetically different and might represent a new taxon.

L. theobromae (order *Botryosphaeriales*) is the only species of this genus involved in human opportunistic infections (Saha et al., 2012). Valenzuela-Lopez et al. (2017) found a higher species diversity in the North American study than we report here, since five of the French isolates were identified as *L. theobromae*. The other three isolates of the *Botryosphaeriales* we found were related, one to a different species of *Lasiodiplodia* and the other two to other genera, specifically *Neofusicoccum* and *Diplodia*.

Four species of the genus *Diaporthe* (formerly *Phomopsis*; order *Diaporthales*), i.e. *D. bougainvilleicola*, *D. longicolla*, *Diaporthe phaseolorum* and *D. phoenicicola*, are considered opportunistic pathogens that cause mycoses that range from superficial to deep infections (Cariello et al., 2013; Gajjar et al., 2011; Garcia-Reyne et al., 2011; Iriart et al., 2011). Six isolates from France were phylogenetically placed into the latter genus. However, our results are only preliminary since only one phylogenetic marker was analysed. This fact was pointed out by other authors in this genus, which is a polyphyletic fungus (Gomes et al., 2013).

We also report the finding of five clinical isolates of *Colletotrichum*. Two of the isolates corresponded to *C. gigasporum* (formerly *Colletotrichum crassipes*) and *C. gloeosporioides*, taxa that have previously been reported as agents of keratitis, endophthalmitis and phaeohyphomicotic cyst; the other three isolates could not be identified at species level. This genus encompasses numerous plant pathogens that are found worldwide, although mainly in tropical and subtropical regions (Cannon et al., 2012). The taxonomy of *Colletotrichum* is complicated and the genus is organized in species-complexes (Cannon et al., 2012; Liu et al., 2014). Species such as *Colletotrichum coccodes*, *C. crassipes*, *Colletotrichum dematium*, *C. gloeosporioides*, *Colletotrichum graminicola* and *Colletotrichum truncatum* cause superficial and deep infections (endophthalmitis, keratitis, subcutaneous cyst or more rarely arthritis) (Cano et al., 2004). Further studies, including different phylogenetic markers, are needed to delimit the different species and clarify their pathogenic role.

The antifungal susceptibility of coelomycetous fungi involved in human infections is poorly known, mainly because they do not easily sporulate. In spite of the limited number of isolates tested here, amphotericin B seemed the most active drug *in vitro* together with terbinafine, in agreement with Valenzuela-Lopez et al. (2017). Until more *in vitro* data is available, the antifungal treatment of the infection by this sort of fungus remains purely empirical. In a recent study, Guégan et al. (2016) recommended extensive surgical resection of affected tissues as a first-line treatment for solitary subcutaneous lesions by coelomycetous fungi, followed by an antifungal therapy (posaconazole or voriconazole) in the case of relapse or amphotericin B in refractory cases.

Since our study is based on isolates from the two reference centres, we cannot comment on the incidence of infections due to coelomycetes nor compare their epidemiology between France and Spain. However, we still provide a good picture of the great diversity of coelomycetous fungi in the clinical context, and the basis for future studies on this interesting but neglected group of fungi.

Conflicts of interest

No conflict of interest declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.02.001.

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