Polyphasic identification of three new species in *Alternaria* section *Infectoriae* causing human cutaneous infection

Running title: Novel species causing cutaneous alternariosis

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ABSTRACT

Background: Cutaneous phaeohyphomycosis is an emerging disease in immunocompromised patients, being *Alternaria* one of the most common genera reported as a causative agent. Species identification is not carried out mainly due to the complexity of the genus. Analysis of the ITS barcode has become standard for fungal identification, but in *Alternaria* it is only able to discriminate among species-groups or sections.

Methods: We present three cases of cutaneous infection caused by *Alternaria* isolates morphologically identified as belonging to section *Infectoriae*. They have been morphologically characterized and phylogenetically delineated with five molecular markers (ITS, *ATPase*, *gapdh*, *rpb*2 and *tef*1).

Results: Mycotic infections have been diagnosed by repeated cultures and histopathological examination in two of the cases. The polyphasic approach has allowed to delineate three new species of *Alternaria* section *Infectoriae*, i.e. *A. anthropophila, A. atrobrunnea* and *A. guarroi*. *ATPase* has been the only locus able to discriminate most of the species (29 out of 31) currently sequenced in this section, including *A. infectoria* the commonest reported species causing alternariosis. Susceptibility test showed different antifungal patterns for the three species, although terbinafine was the most active *in vitro* drug against these fungi.

Conclusions: The *ATPase* gene is recommended as an alternative barcode locus to identify *Alternaria* clinical isolates in section *Infectoriae*. Our results reinforce the relevance of identification of *Alternaria* isolates at the species level and the necessity to carry out antifungal susceptibility testing to determine the most adequate drug for treatment.

Keywords: opportunistic infections, alternariosis, immunocompromised patients, antifungal susceptibility, *Alternaria infectoria*, *Pleosporaceae*, molecular identification, taxonomy.

1. **Introduction**

Alternaria is a cosmopolitan dematiaceous genus, whose species are mostly saprophytic, but also endophytic and phytopathogenic, generating important damages in diverse agronomic products[1,2]. In the last decades, it also emerges as an important human opportunistic pathogenic mold especially affecting immunocompromised patients and commonly causing cutaneous and subcutaneous infections, but cases of rhinosinusitis, oculomycosis, onychomycosis and invasive disease have been also reported[3-6]. The increasing incidence of alternariosis in immunosuppressed population is mainly due to transplants (bone marrow or solid organ transplant), to patients with cancer treatments or to primary or acquired immunodeficiency[3,4,7,8,9]. In absence of any standard guidance, multiple therapeutic options have been used for these infections, including thermotherapy[10], but itraconazole (ITC) has been the antifungal therapy most frequently used and, generally, with a satisfactory outcome[3,4,11]. However, posaconazole (PSC) is currently chosen as a better option since it usually has lower minimal inhibitory concentration (MIC) values, a better body tissues distribution and less drug interactions than itraconazole[4,12].

Although the most commonly reported species are *A. alternata*, *A. infectoria, A. tennuissima* and *A. chartarum*, it is of note that species identification is not performed in practically half of the cases of alternariosis reported[3,4]. This is probably due to the great number of species described in the genus and to the difficulties in the interpretation of the morphological features of its isolates, which often lead to incorrect identification[3]. In particular, the morphological identification of *A. infectoria* is problematic mainly due to the scarcity or lack of sporulation, producing white or nearly white colonies, mainly when grown on nutrient-rich media[13,14]. Therefore, alternative methods, such as DNA sequencing, are necessary for the correct identification of *Alternaria* species. In this sense, re-identification of *Alternaria* clinical isolates by sequence analysis highlights that *A. infectoria,* rather than *A. alternata,* is the most frequently identified species, at least as causative agent of cutaneous alternariosis[4,15,16].

Several molecular taxonomic studies have contributed to stablish the modern concept of *Alternaria*. The genus is divided into 27 monophyletic subgeneric groups, called sections, and comprises about 280 accepted species[17-21]. Although the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (rDNA) has been proposed as an universal barcode for fungal classification[22], it shows a low discriminatory power for distinguishing closely related *Alternaria* species due to the limited numbers of informative sites[2,17,19]. In fact, *Alternaria* identification based on the ITS barcode is limited to species complex or section, being therefore required a multilocus sequence analysis for the species delimitation of these fungi[2,17,21]. Unlike other genera of human opportunists such as *Aspergillus* and *Penicillium*, standard methodology for molecular identification of *Alternaria* species has not been yet stablished, even combinations of different genes are used depending on the *Alternaria* section studied[2,21,23]. In the case of the section *Infectoriae* the combination of ITS, RNA polymerase second largest subunit (*rpb*2), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), translation elongation factor 1-alpha (*tef*1) or plasma membrane ATPase (*ATPase*) have been used for species recognition[17,20,21,24]. Currently, this section comprises 36 species, of which only three have been described as opportunistic pathogenic species, i.e. *A. infectoria, A. caespitosa* and *A. triticina*[3,25].

Herein we present three cases with skin and soft tissue infection due to *Alternaria* in immunocompromised patients, who were admitted in 2017 at the Sant Joan Universitary Hospital in Reus, Spain. The isolates from the three cases showed the typical morphological features of those species of *Alternaria* section *Infectoriae* (i.e. short primary conidiophores with one or several conidiogenous loci, and small conidia with few longitudinal septa forming branched chains). Based on the multilocus sequence analysis of five genes and on morphological data, the three isolates have been recognized as new species in the genus, which extend the list of *Alternaria* species able to cause human infection. The in vitro susceptibility profile against eight antifungal drugs is provided for the three fungi.

Case reports

Case 1. A 46-year-old male, with pulmonary transplantation two years earlier and under immunosuppressive therapy (tacrolimus 2.5 mg/12 h and mycophenolate mofetil 500 mg/12 h), presented to us with a painless nodule on his right lower extremity. A punch biopsy on the nodule was carried out for histopathological study and cultured following the recommendations of the Spanish Society of Infectious Diseases and Clinical Microbiology[26]. The hematoxylin and eosin (H&E) stain (Fig. 1A) showed an important inflammatory granulomatous lesion with suppurative reaction. Grocott's methenamine silver (GMS) stain revealed abundant septate, hyphal elements in dermis and hypodermis (Fig. 1B). After 72 h of incubation at 25 and 37 ºC on Sabouraud dextrose agar (SAB) supplemented with gentamicin and chloramphenicol (BioMérieux S.A.), tissue cultures formed numerous colonies of a melanized filamentous fungus. Cultures were negative for bacteria. On SAB the sporulation was scarce, but morphological features of the few conidia produced allowed us to identify the fungus as *Alternaria* sp. The patient was treated with topical applications of voriconazole (VRC) for 20 days showing clinical improvement, with the subsequent exeresis of the nodule. At the 12-month follow-up visit, there was no relapse.

Case 2. A 73-year-old male, with a clinical history of prostate adenocarcinoma in stage IV and receiving radiotherapy treatment, was admitted to the hospital due to a clinical condition compatible with sepsis. Several weeks earlier, he had successfully been treated with cloxacillin (1g/6h i.v. for 10 days) due to positive cultures for *Staphylococcus aureus* from an infected lesion on the right index that advanced to cellulitis. Three days after admission, the patient presented multiple skin ulcers on both lower extremities at the knees level. Skin biopsies from each knee were obtained and the histopathological analysis with H&E and GMS stains revealed in both biopsies abundant septate hyphae in dermis and hypodermis with a suppurative granulomatous inflammation similar to that observed in the first case (Fig. 1). Cultures from the tissue biopsied on SAB at 25 and 37 ºC yielded colonies of a melanized filamentous fungus after 72 h of incubation, whose micromorphological features matched *Alternaria* sp.. Bacteriological cultures were negative. The treatment was initiated with liposomal amphotericin B (LAMB) 2 mg/kg/24h i.v. by 10 days, but it was replaced by VRC due to the side effects of the

first drug. The VRC was administered intravenously 6 mg/kg/12 h at the first day followed by 4 mg/kg/12 h until complete 7 days. The patient evolved favorably with healing of the skin lesions and was discharged from the hospital with no apparent systemic signs of infection. He died 12 months after the diagnosis of alternariosis due to unrelated causes, with no recurrence of the fungal lesions.

Case 3. A 71–year-old female was referred to the hospital due to a heart failure. She presented a multi-pathological clinical history, which consisted in arterial hypertension, diabetes mellitus, renal failure, with a chronic ischemic heart disease history, severe and diffuse three-vessel disease and dilated myocardiopathy of ischemic origin. On examination, the patient displayed numerous skin ulcerative lesions in her lower extremities. Exudate samples from different ulcers were cultured and colonies of a melanized filamentous fungus compatible with *Alternaria* were obtained on SAB after 72 h of incubation at 25 and 37 ºC. The ulcers improved with antibiotic treatment and nursing cures without specific treatment against *Alternaria*. One month later, the patient evolved with cardiogenic shock, multi-organ failure and *exitus*, but without a complete resolution of her cutaneous infection.

The three patients presented anemia and thrombocytopenia at the time of diagnosis, only the first patient had acute renal failure and the last one had cholestasis. Clinical findings of the three patients at the admission are summarized in Table 1.

One fungal isolate from each case was send to the Mycology Unit of the University Rovira I Virgili (Spain) for species identification and antifungal susceptibility testing. The isolates were deposited as FMR 16235 (case 1), FMR 16556 (case 2) and FMR 16868 (case 3) and all them were morphologically identified as *Alternaria* spp. from section *Infectoriae*.

2. **Material and methods**

2.1. DNA extraction and sequencing

We selected five phylogenetic markers used in previous molecular studies on *Alternaria* identification[17,20,21]. These were the complete ITS1-5.8SrDNA-ITS2 fragment, and fragments of the genes *rpb*2, *gapdh*, *tef*1 and *ATPase.* The DNA was extracted from strains cultured on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) after 7 days of incubation at 25 °C in the dark, using the modified protocol of Müller et al.[27]. The amplification of the ITS region was carried out using the primer pairs ITS5/ITS4[28], *rpb*2 region using RPB2–5F2 and fRPB2–7cR[29], *ATPase* using ATPDF1 and ATPDR1[30], *gapdh* using gpd1 and gpd2[31], and the partial gene *tef*1 with the primers EF1-728F and EF1-986R[32]. The five regions were sequenced at Macrogen Europe (Macrogen Inc. Amsterdam, The Netherlands), using the same primer pairs and the consensus sequences were obtained in SeqMan software v. 7.0.0 (DNAStar Lasergene, Madison, WI, USA).

The novel sequences generated in the study were deposited in the NCBI´s GenBank nucleotide database (Table 2).

2.2. Phylogenetic analysis

The phylogenetic analyses included sequences of 28 ex-type strains corresponding to most of the species that comprise the section *Infectoriae,* the three case isolates, and the ex-type strains of *A. abundans* (CBS 534.83) and *A. breviramosa* (CBS 121331) of the section *Chalastospora* as outgroup (Table 2). Additionally, sequences of tree unidentified clinical isolates of *Alternaria* from our collection that showed to be genetically similar to some of the case isolates were also included in the analyses (Table 2).

The alignment of each locus was performed in Mega software (Molecular Evolutionary Genetics Analysis) v.6.0.[33], through Clustal W algorithm[34] and refined with MUSCLE[35] or manually if necessary. The combined analysis of the five phylogenetic markers was tested through Incongruence Length Difference (ILD) implemented in the Winclada program[36]. Phylogenetic analysis was performed using the maximum likelihood method (ML) under Mega software v.6.0. and Bayesian Inference (BI) approaches under MrBayes v. 3.2.6[37]. The best nucleotide substitution model determined using jModelTest[38] for the ML of the combined

analysis of the five phylogenetical markers, was General Time Reversible with Gamma distribution and Invariant sites (G+I). For the BI phylogenetic analysis, the best nucleotide substitution model was chosen using jModelTest[38]. For the ITS region and *rpb*2, we used Kimura 2-parameter with Invariant sites (K80+I), for *gapdh* Kimura 2-parameter with Gamma distribution (K80+G), for *tef*1 symmetrical model with Gamma distribution (SYM+G) and for *ATPase* General Time Reversible with Gamma distribution and Invariant sites (GTR+G+I). The parameters settings used were two simultaneous runs of 5.000.000 generations, four Markov chains, sampled every 1000 generations. The 50% majority-rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the samples. For ML analysis, ML bootstrap values (BML) ≥ 70% were considered significant, and for BI, PP values of ≥ 0.95.

2.3. Phenotypic study

Morphological characterization of the isolates was carried out on potato carrot agar (PCA; potato 20 g, carrot 20 g, agar 13 g, distilled water 1 L), oatmeal agar (OA; oatmeal 30 g, agar 13 g, distilled water 1 L) and PDA after 7 days of incubation at 25 °C in dark. Colour of the colonies in descriptions was based on Kornerup and Wanscher[39]. The measurements and description of the microscopic structures were made after 14 days at 25 °C in dark, from the specimen mounted in Shear's solution. The Zeiss Axio-Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera was used to obtain the photomicrographs.

2.4. Antifungal susceptibility

The in vitro antifungal susceptibility profile of the three clinical isolates was performed according to the CLSI M38-A2 method[40]. We evaluated antifungal activity of AMB (Sigma-Aldrich Quimica S.A., Madrid, Spain), VRC (Pfizer S.A., Madrid, Spain), PSC (Schering-Plough Research Institut, NJ, USA), ITC (Jansen Pharmaceuticals, Beerse, Belgium), caspofungin (CFG) (Merk & Co., Inc., Rahway, USA), anidulafungin (AFG) (Pfizer S.A., Madrid, Spain), micafungin (MFG) (Astellas Pharma, Madrid, Spain) and terbinafine (TBF) (Sigma Aldrich

Química S.A., Madrid, Spain). Microdilution plates were incubated at 35º C for 48h and read visually. The MIC was defined as the lowest drug concentration that produced 100% inhibition of visible fungal growth for AMB and azoles (ITC, PSC and VRC), and 80% for TBF. For the echinocandins (AFG, CFG and MFG) the minimum effective concentration (MEC) was determined microscopically as the lowest concentration of drug at where visible morphological changes in growth were observed (i.e. small, rounded, compact hyphal forms) compared to the growth in control. *Candida parapsilosis* ATCC 22019 was used as quality control strain for all tests that were performed in duplicate.

2.5. Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the research in this article related to micro-organisms.

3. **Results**

3.1. Phylogeny

The topology of the phylogenetic trees for single partitions were congruent and according the ILD test (P = 0.16) could be combined. The final concatenated sequence alignment, with 36 strains and the five loci, comprised 3014 bp (ITS 526 bp, *rpb*2 573 bp, *ATPase* 1182 bp, *gapdh* 483 bp and *tef*1 250 bp) with 453 variable sites (ITS 26 bp, *rpb*2 118 bp, *ATPase* 153 bp, *gapdh* 94 bp and *tef*1 62 bp) of which 275 were phylogenetically informative (ITS 18 bp, *rpb*2 79 bp, *ATPase* 83 bp, *gapdh* 60 bp and *tef*1 35 bp). The topology of the trees inferred by the two phylogenetic methods (ML and BI) were basically the same, with minor differences in statisticaly supported groupings. The ML phylogenetic tree with the bootstrap and posterior probability values showed that the isolates of our patients were nested into the section *Infectoriae* (Fig. 2). However, they appeared as lineages clearly distinct from the 28 ex-type strains of the species included in this study. Therefore, total of 31 lineages representing the different species in the section were recognized. Isolate FMR 16235 of case 1 was grouped with other isolates

(FMR 17278, FMR 17288 and FMR 17296) of clinical origin, forming all a highly supported terminal clade. Isolates FMR 16556 and FMR 16868 of cases 2 and 3, respectively, grouped in a basal clade together with *A. slovaca* and *A. quericola.* However, the phylogenetic relationship among them was uncertain because the internal branches showed low statistical support, and our isolates were located in independents branches, distant from the other accepted species. Based on the polyphasic approach, we propose and describe three new taxa in the section *Infectoriae* that were able to grow at 37 ºC and can be distinguished from related species by colony features and microscopic morphology of their conidia.

The individual phylogenetic analysis of each locus showed different abilities to discriminate among species in the section *Infectoriae*. ITS was able to differentiate less than 10% (3/31) of the species of the section included in the analysis, being only *A. broccoli-italicae, A. intercepta* and *A. metachromatica* identified with this genetic marker. *Gapdh* was able to differentiate 45% (14/31) of the species. *Tef*1 was able to separate 61% (19/31) of the species in the section. *Rpb*2 resolved 68% (21/31) of the species, being unable to distinguish *A. arbusti, A. cerasidanica, A. conjuncta, A. ethzedia, A. oregonensis, A. roseogrisea, A. triticimaculans, A. triticina, A. ventricosa* and *A. viburni*. The highest percentage of species resolution, 94% (29/31), was achieved with the *ATPase* dataset. The only species that could not be distinguished were *A. ethzedia* and *A. triticimaculans*.

Results of the *in vitro* susceptibility testing were broadly variable among the three case isolates (Table 3). The drug that showed good activity for the isolates was TBF, with MICs ranging from 0.5 to 2 µg/mL. By contrast, AMB demonstrated poor activity for all them (2 to 8 µg/mL). The echinocandins (AFG, CFG and MFG) displayed good activity against FMR 16235 and FMR 16868, with values ranging between 0.03 to 1 µg/mL. Among these, MFG exhibited the lower MECs values (0.03 µg/mL for FMR 16868 and 0.125 µg/mL for FMR 16235). However, the three echinocandins showed poor activity against FMR 16556, with MECs ranging from 4 to 8 µg/mL. In general, the azoles showed low MICs values against FMR 16235 and FMR 16556 (between 0.125–2 µg/mL) with exception of ITC for FMR 16556

(MIC >16 µg/mL). Conversely, no azole demonstrated activity against FMR 16868 (MICs from 8 to >16 µg/mL).

3.2. Taxonomy

Alternaria anthropophila Iturrieta-González, Gené, Alastruey & Dania García, sp. nov. — MycoBank MB 829636, Fig. 3

Etymology. Name referred to the source of the isolates, human clinical specimens.

Colonies on PDA reaching 71 mm diam. after 7 d at 25 °C, white to greyish yellow (1A1/4B4), white at the periphery, flat, densely floccose, margin regular; reverse brown to greyish orange (6E4/5B4), white at the periphery. On PCA attaining 65 mm diam. after 7 d at 25 °C, olive-brown (4D4), flat, slightly floccose, margin fimbriate; reverse olive-brown (4D4). On OA reaching 61 mm diam. after 7 d at 25 °C, dull green (30E4), flat, slightly floccose, margin regular; reverse dull green (30E4).

Mycelium superficial and immersed, composed of septate, branched, 2–5 µm wide, smooth-walled to verruculose, hyaline to pale brown hyphae. Conidiophores solitary, arising directly from aerial hyphae, erect to slightly flexuous, occasionally geniculate at the apex, with up to 9 septa, unbranched or with up to two branches, 26–120 × 4–5(–7) µm, brown, smooth or verruculose, with 1–3 lateral or terminal conidiogenous loci. Conidia solitary or in chains of up to 9 conidia, commonly ellipsoidal, ovoid or obclavate, 11–63 × 6–11 µm, verruculose to verrucose, pale brown to brown, with some darkened middle transverse septa, (0–)2–4(–10) transverse septa, and 0–1 longitudinal or oblique septa per transverse segment; the primary conidia commonly produce secondary conidiophores that usually consist in a successive geniculate terminal extension, up to 150 µm long, bearing conidia solitary or in short chains. Sexual form not observed.

Cardinal temperatures for growth — Optimum 25 °C, maximum 37 °C, minimum 5 °C.

Specimens examined. Spain, Aragón, human skin lesion, May 2003, *Ana Alastruey* (FMR 17278 = CNM 2519); Catalonia, Reus, human subcutaneous nodule, Feb. 2017, *Isabel Pujol* (holotype CBS H-23916, culture ex-type CBS 145587 = FMR 16235); Cantabria, human pericardial liquid, Jul. 2009, *Ana Alastruey* (FMR 17296 = CNM 5813); Galicia, human subcutaneous nodule, Dec. 2005, *Ana Alastruey* (FMR 17288 = CNM 3823).

Note: *A. anthropophila* is included in a poorly supported clade together with the plant related species *A. californica, A. daucicaulis*, *A. hordeicola* and *A. hordeiaustralica*. *Alternaria californica* differs by producing unbranched and shorter conidiophores (30–100 µm) and conidia with up to 16 transverse septa[41]. The other three species (*A. daucicaulis*, *A. hordeicola* and *A. hordeiaustralica*) are characterized by the production of smooth or slightly punctate conidia in culture and by the production of a *Lewia*-like sexual morph on natural substrates[41,42].

Alternaria atrobrunnea Iturrieta-González, Pujol, Dania García & Gené, sp. nov. — MycoBank MB 829637, Fig. 4

Etymology. Name referred to the colour of the colony reverse on PDA

Colonies on PDA reaching 75 mm diam. after 7 d at 25 °C, grey to greyish yellow (4D1/4C8), flat, cottony, margin fimbriate; reverse yellowish brown to dark blond (5F4/5D4), yellowish grey at the periphery (4B2). On PCA attaining 83 mm diam. after 7 d at 25 °C, olive to dark green (3E3/29F7), flat, floccose, margin regular; reverse dark green (29F3). On OA reaching 85 mm diam. after 7 d at 25 °C, olive to dark green (3E3/29F4), flat, slightly floccose, margin regular; reverse dark green (29F8).

Mycelium superficial and immersed, composed of septate, branched, 2–6(–11) µm wide, subhyaline to pale brown, smooth to verruculose hyphae. Conidiophores solitary, arising directly from aerial hyphae, erect to slightly flexuous, with up to 8-septate, unbranched, 14–39 × 3–5 µm, brown, smooth or verruculose, with 1 terminal conidiogenous locius Conidia solitary or in simple short chains with up to 5

conidia, ovoid or obclavate, 7–44 × 5–12 µm, verrucose to tuberculate, brown, with some darkened middle transverse septa, 3–8 transverse septa, and 0–1(–2) longitudinal or oblique septa per transverse segment; some primary conidia produce secondary conidiophores as lateral or terminal extensions from the conidial body, bearing conidia in short chains. Sexual form not observed.

Cardinal temperatures for growth — Optimum 25 °C, maximum 37 °C, minimum 5 °C.

Specimen examined. Spain, Catalonia, Reus, exudate from an ulcerative skin lesion, Oct. 2017, *Isabel Pujol* (Holotype CBS H-23918, culture ex-type CBS 145589 = FMR 16868)

Note: *Alternaria atrobrunnea* is placed in a poorly supported basal clade of the phylogenetic tree (Fig. 2) together with *A. quercicola*, a species described from *Quercus brantii* (*Fagaceae*) in Iran[43]. However, *A. quercicola* differs from our new species in the conidial ornamentation, being rough-walled towards the base and smooth-walled towards the apex, and in length, (25–)31–51(–57) µm long[43].

Alternaria guarroi Iturrieta-González, Dania García, Pujol & Gené sp. nov. — MycoBank MB 829638, Fig. 5

Etymology. Name in honour of Josep Guarro for his extensive work on medical mycology.

Colonies on PDA reaching 80 mm diam. after 7 d at 25 °C, white, flat, cottony, margin regular; reverse pale yellow (4A3). On PCA attaining 82 mm diam. after 7 d at 25 °C, colorless, flat, with predominantly hyaline immersed mycelium, margin regular; reverse colorless. On OA reaching 83 mm diam. after 7 d at 25 °C, golden grey (4C2), flat, slightly floccose, margin regular; reverse olive-brown (4E4).

Mycelium superficial and immersed, composed of septate, branched, 2–7 µm wide, smooth to verruculose, subhyaline to pale brown hyphae. Conidiophores solitary, erect to slightly flexuous, occasionally geniculate at the apex with up to 6–septate,

mostly unbranched, 11–55 × 3–4 µm, brown, smooth or verruculose, with 1–3 lateral or terminal conidiogenous loci. Conidia solitary or in short unbranched chains of up to 5 conidia, ellipsoidal, obclavate or ovoid, 5–31 × 3–10(–12) µm, smooth to verruculose, brown, with some darkened middle transverse septa, 1–5(–9) transverse septa, and 0–1 longitudinal or oblique septa per transverse segment; these primary conidia produce secondary conidiophores that consist in a subapical extension from the conidial body, with a terminal conidiogenous locus bearing solitary or a short chain of conidia. Sexual form not observed.

Cardinal temperatures for growth — Optimum 25 °C, maximum 37 °C, minimum 5 °C.

Specimen examined. Spain, Catalonia, Reus, biopsy from ulcerative skin lesion, Apr. 2017, *Isabel Pujol* (Holotype CBS H-23917, culture ex-type CBS 145588 = FMR 16556)

Note: *Alternaria guarroi* and *A. slovaca* are in the same basal clade but with low statistical support. The latter species, which was also described from dermic human lesions in Bratislava (Czechoslovakia), differs from *A. guarroi* in producing solely chlamydospores and sporadically blastospores[17], which were originally described as brownish, aseptate, obovoid spores of 1–1.5 × 1 µm growing directly from mycelium cells and never germinating[44].

4. **Discussion**

Phaeohyphomycosis is an increasingly recognized infection, in which *Alternaria* is one of the most commonly reported agents, mainly associated with both superficial and deep local infection in patients with impaired immunity, especially due to solid organ transplantation[5,8,9,16,45-47]. Pulmonary transplantation, prostrate adenocarcinoma and a multiple pathological clinical history were predisposing factors to induce an immunocompromised condition in our patients to suffer cutaneous infection by this ubiquitous fungal genus. Respective mycotic infections were diagnosed by repeated cultures, in addition to histopathological

examination of the biopsy specimens with visible fungal elements in two of the cases.

Although several species have been associated as causal agent of alternariosis, *A. infectoria* is the most frequent species identified in recent studies[4,16,48,49]. However, in most cases its identification is limited to morphological features of the fungus growing in culture and confirmed by the analysis of the ITS barcode. Even, in some cases, only ITS analysis is carried out directly from paraffin-embedded tissue samples without culturing[16,50]. Nevertheless, as stated before, the so called barcode for fungi has very limited resolution to distinguish closely related species in *Alternaria*, usually being only able to distinguish among *Alternaria* species-groups or sections[17,19]. Therefore, the incidence of *A. infectoria* as causative agent of alternariosis is probably overestimated in the above mentioned studies, since the analysis of ITS sequences only is able to confirm the relationship of the case isolate to *Alternaria* section *Infectoriae*. In the present study, sequence analysis of five loci (ITS, *ATPase, gapdh, tef1* and *rpb*2) has allowed to distinguish most species currently accepted in section *Infectoriae*, and to delineate three novel taxa in this group of *Alternaria*, i.e. *A. anthropophila*, *A. atrobrunnea* and *A. guarroi* (Fig. 2). Of note is that the former one has been phylogenetically strongly supported with sequences of other clinical isolates that could not be identified previously due to their limited sporulation in culture. However, the taxonomic structure of the section remains obscure, since the genetic markers used have not been able to resolve the phylogenetic relationships among many of the species included in the analysis. Anyway, we agree with Lawrence et al.[19,30] in that the *ATPase* is a very suitable genetic markers for molecular identification of *Alternaria* species and this could be used in clinical laboratories for identifying these fungi. In fact, it has been able to discriminate practically all the species in *Alternaria* section *Infectoriae* included in this study (Fig. S1 in the supplementary material). Identification at the species level in such section is relevant since, as the new species described here, it includes fungi able to growth at the body temperature and, therefore, with potential to cause animal and human infections. However, only the correct identification of *Alternaria* isolates

can lead to determine the epidemiology of its species or to establish proper treatment to resolve infections caused by this complex group of fungi.

Given that no optimal treatment has been defined for *Alternaria* infections, several therapeutic options are used, depending on the status of the patient concerned and the extent of disease. The most commonly antifungal therapy includes ITC, VRC, and PSC[52], being the former widely used in cutaneous alternariosis and usually combined with surgical excision of involved tissue and reduction of the immunosuppression in cases of transplant recipients[3,4,5,7,16,52]. In our case, in the first patient the infection was resolved with surgical excision of the nodule and topical VRC; the second patient with multiple skin lesions was initially treated with i.v. LAMB but due to side effects this was switched to VRC; while multiple lesions in the third patient were improved with nursing cures without specific antifungal treatment but follow up was not possible because she was exitus (Table 1). It of note however that, due to hepatic impairment as side effect reported for VRC[50,53] or the significant drug-drug interaction when using ITC[11], currently PSC has shown to be a good option to treat cutaneous alternariosis[4, 50,54]. This correlates with results of *in vitro* antifungal testing in several studies, which show that, among azoles, PSC is the most potent drug against *Alternaria* species[7,55,56]. In the present study, isolates of *A. anthropophila* and *A. guarroi* of the first two cases, respectively (Table 3), showed low MICs to this drug. Although it was not the azol of election for treatment of our patients but VRC, which showed moderate activity, they cured with the treatment. Conversely, no azole was active against *A. atrobrunnea* isolated from the third case. This variable antifungal profile was also observed in the case of equinocandins, for which *A. anthropophila* and *A. atrobrunnea* exhibited low MEC values (0.03 to 1 µg/mL) respect to the high values (4 to 8 µg/mL) in *A. guarroi* (Table 3). Different antifungal patterns have also been found in this class of drugs in other studies when susceptibility data is compared among *Alternaria* species[55,56]. Therefore, these results reinforce the relevance of identification of *Alternaria* isolates at the species level and the necessity to carry out antifungal susceptibility testing to determine the most successful drug for alternariosis treatment. However, only the analysis of susceptibility patterns of

more isolates of well-delineated species will allow us to elucidate whether a correct identification can predict the best drug for treatment.

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Fig. 1. Histological section of the skin biopsy from case 1. Hematoxylin and eosin (H&E) staining showing an inflammatory granulomatous lesion (A); Grocott's Methenamine Silver (GMS) staining revealing septate hyphae (B). (original magnifications: H&E x100, GMS x400).

Fig. 2. Maximum Likelihood (ML) tree constructed with ITS, *ATPase*, *gapdh*, *rpb*2, and *tef*1 sequences from 34 strains representatives of the section *Infectoriae*. The phylogenetic tree was rooted with *Alternaria abundans* and *A. breviramosa*. Bootstrap support values for ML greater than 70% and Bayesian posterior probabilities greater than 0.95 are given near nodes. Names of species newly described here are indicated in bold. Branch lengths are proportional to distance. ^{T} Ex-type strain.

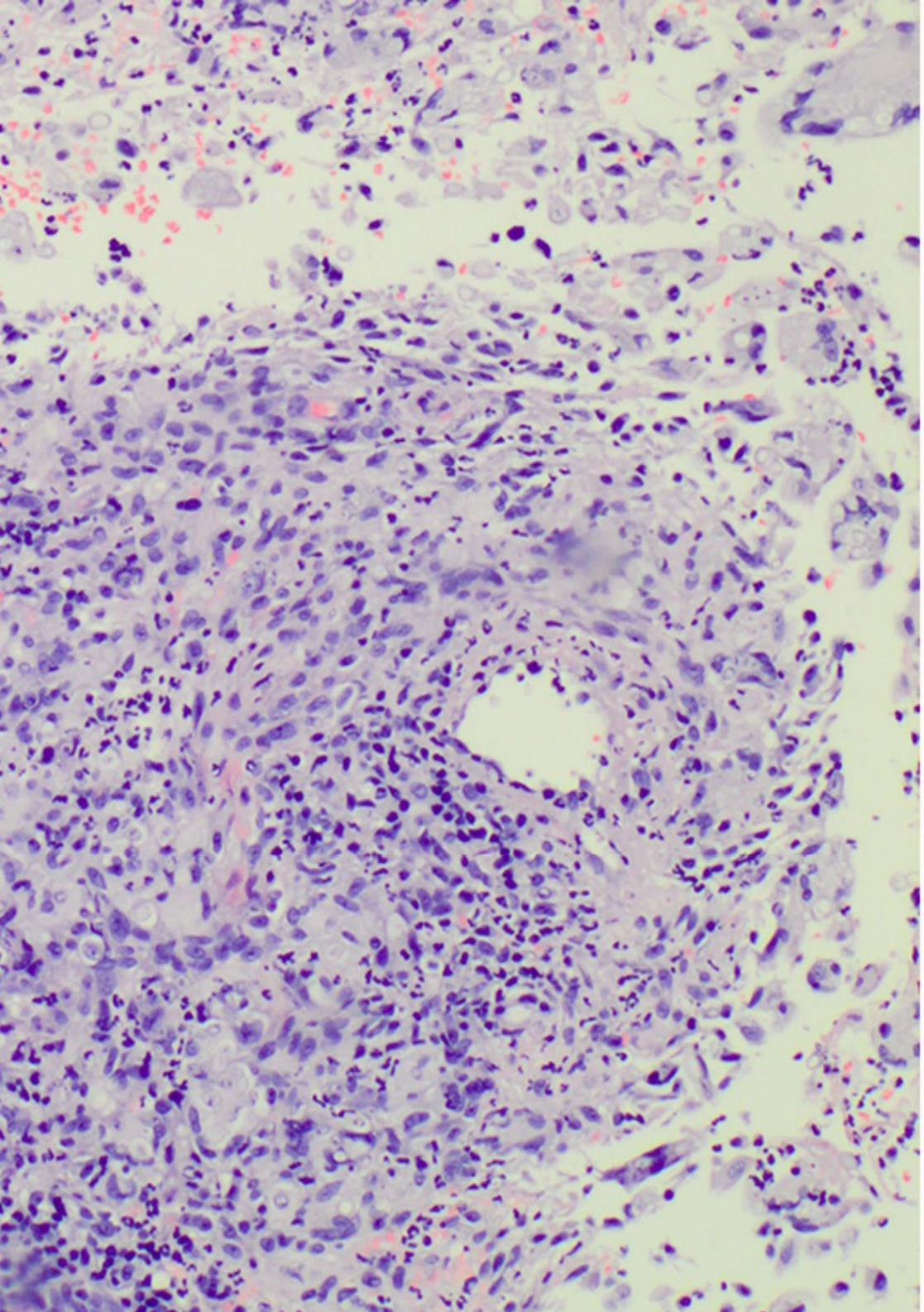
Fig. 3. *Alternaria anthropophila* sp. nov., FMR 16235. A-C. Colonies on (A) PDA, (B) PCA, (C) OA, at 25 ºC after 7d. D–G. Conidiophores and conidia at 25 ºC from PCA after 14 days (D) and from OA after 30 days (E–I). Scale bars E=20 µm; D, F–I=10 µm.

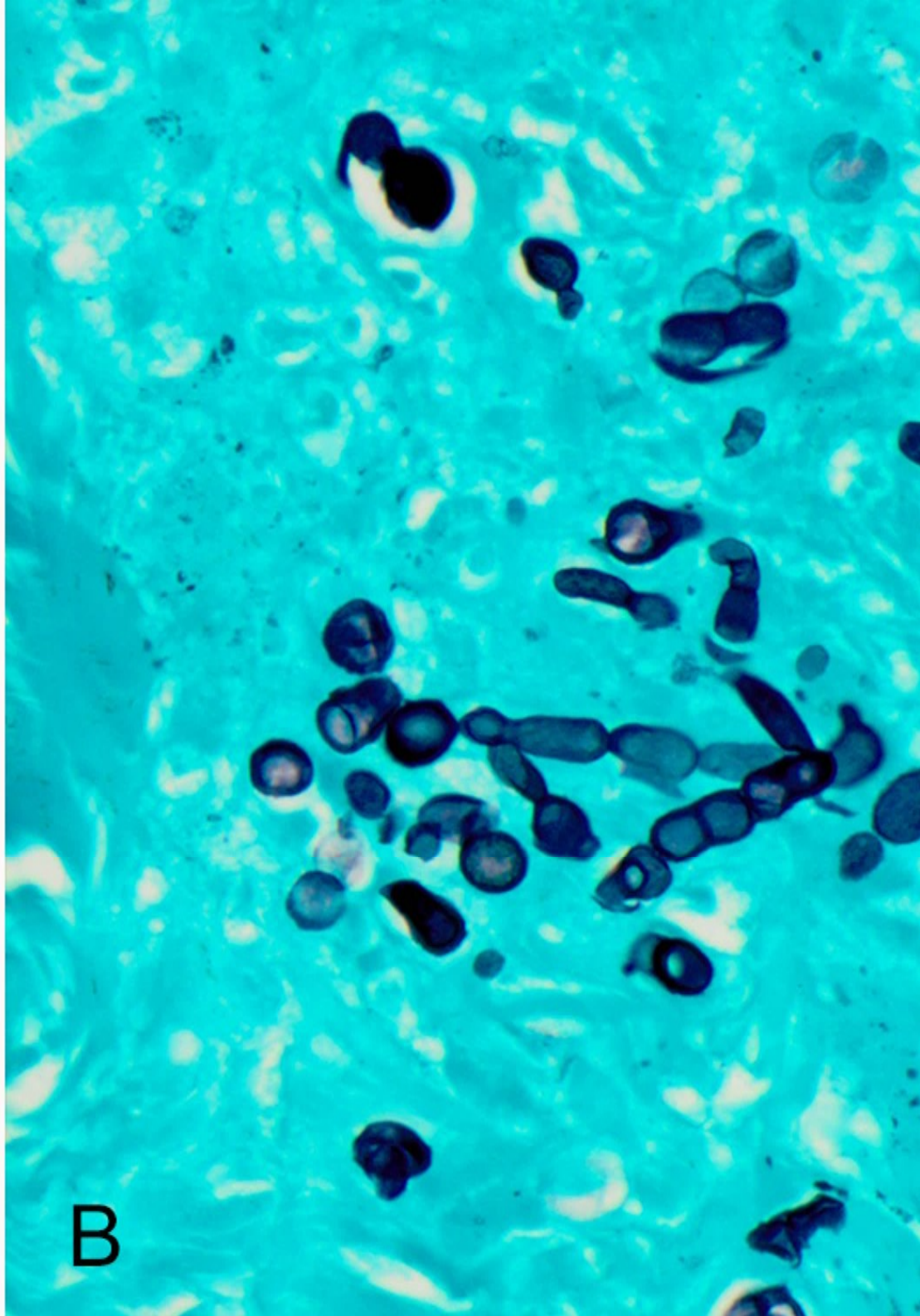
Fig. 4. *Alternaria atrobrunnea* sp. nov., FMR 16868. A-C. Colonies on (A) PDA, (B) PCA, (C) OA, at 25 ºC after 7d. D-G. Conidiophores and conidia from PCA after 14 days. Scale bars D–G=10 µm.

Fig. 5. *Alternaria guarroi* sp. nov., FMR 16556. A-C. Colonies on (A) PDA, (B) PCA, (C) OA, at 25 ºC after 7d. D-G. Conidiophores and conidia from PCA after 14 days. Scale bars D–G= 10 µm.

Supplementary material

Fig. S1. Maximum Likelihood (ML) tree constructed with *ATPase* sequences from 34 strains representatives of the section *Infectoriae*. The phylogenetic tree was rooted with *Alternaria abundans* and *A. breviramosa*. Bootstrap support values for ML greater than 70% and Bayesian posterior probabilities greater than 0.95 are given near nodes. Names of species newly described here are indicated in bold. Branch lengths are proportional to distance. ^{T} Ex-type strain.

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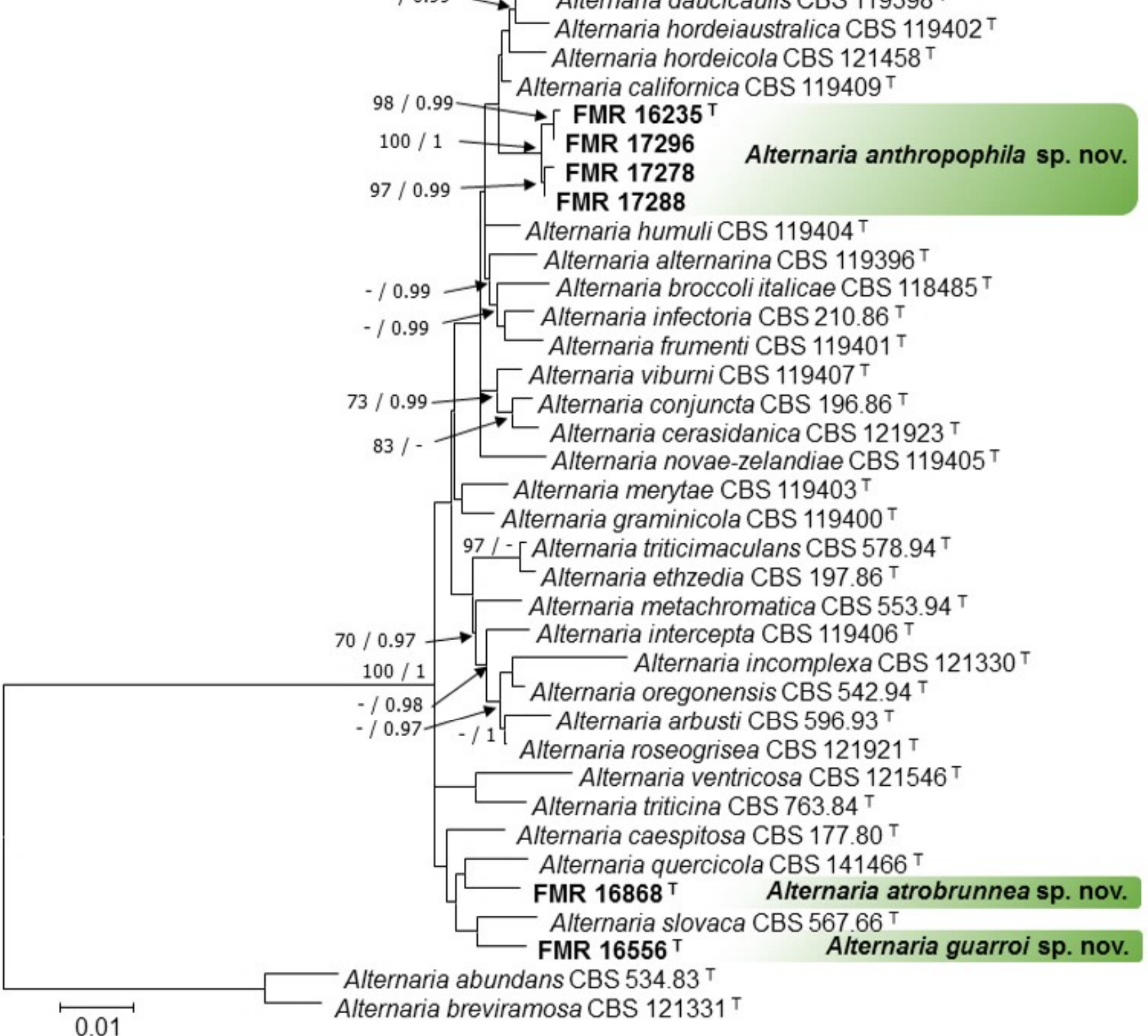
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FMR 17278
97 / 0.99
FMR 17288
Alternaria anthropophila sp. nov.
Alternaria humuli CBS 119404 T
Alternaria alternarina CBS 119396 T
- / 0.99
Alternaria broccoli italicae CBS 118485 T
- / 0.99
Alternaria infectoria CBS 210.86 T
Alternaria frumenti CBS 119401 T
Alternaria viburni CBS 119407 T
73 / 0.99
Alternaria conjuncta CBS 196.86 T
83 / -
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Alternaria merytae CBS 119403 T
Alternaria graminicola CBS 119400 T
97 / -
Alternaria triticimaculans CBS 578.94 T
Alternaria ethzedia CBS 197.86 T
Alternaria metachromatica CBS 553.94 T
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Alternaria intercepta CBS 119406 T
Alternaria incomplexa CBS 121330 T
100 / 1
Alternaria oregonensis CBS 542.94 T
- / 0.98
Alternaria arbusti CBS 596.93 T
- / 0.97
- / 1
Alternaria roseogrisea CBS 121921 T
Alternaria ventricosa CBS 121546 T
Alternaria triticina CBS 763.84 T
Alternaria caespitosa CBS 177.80 T
Alternaria quercicola CBS 141466 T
FMR 16868 T
Alternaria atrobrunnea sp. nov.
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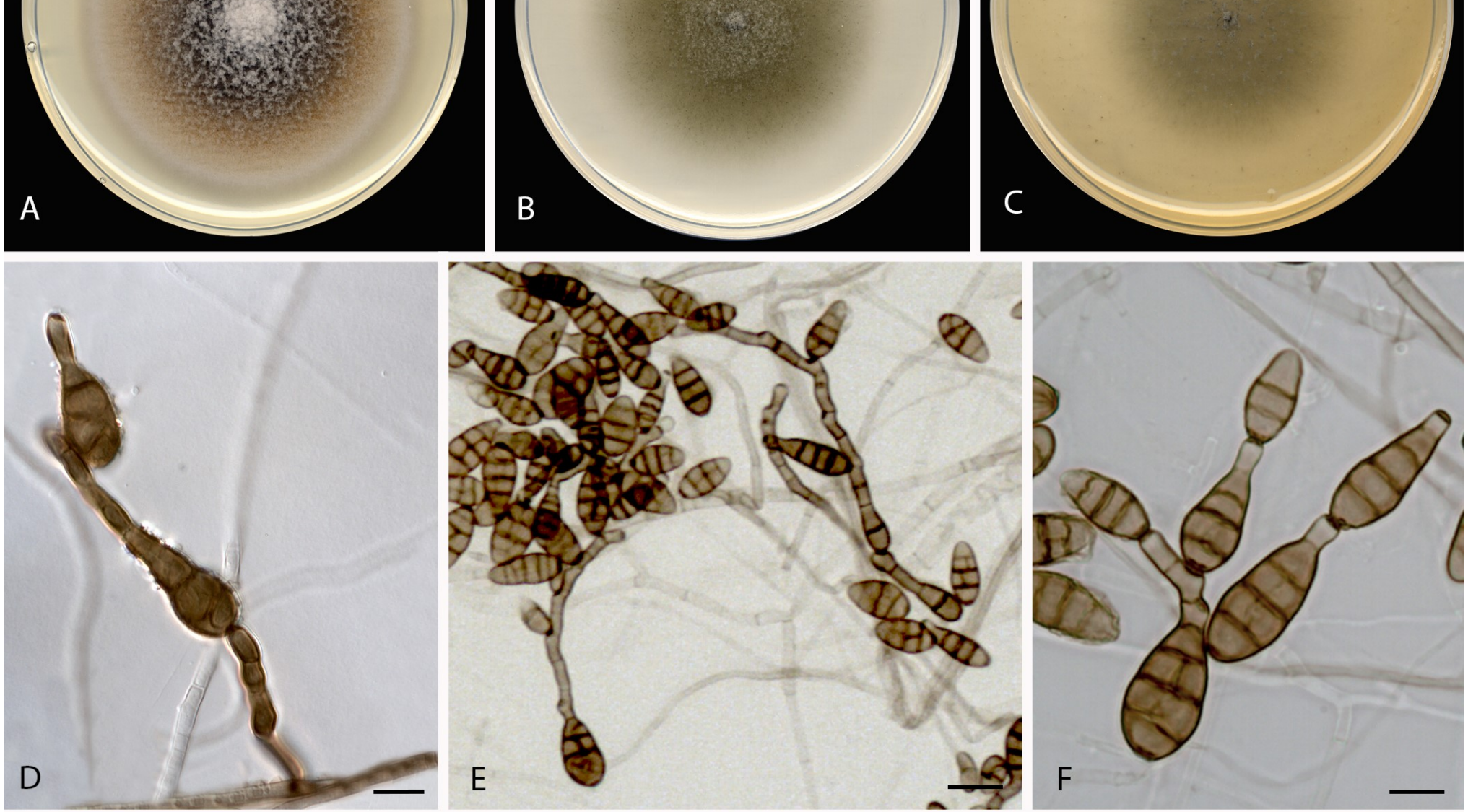
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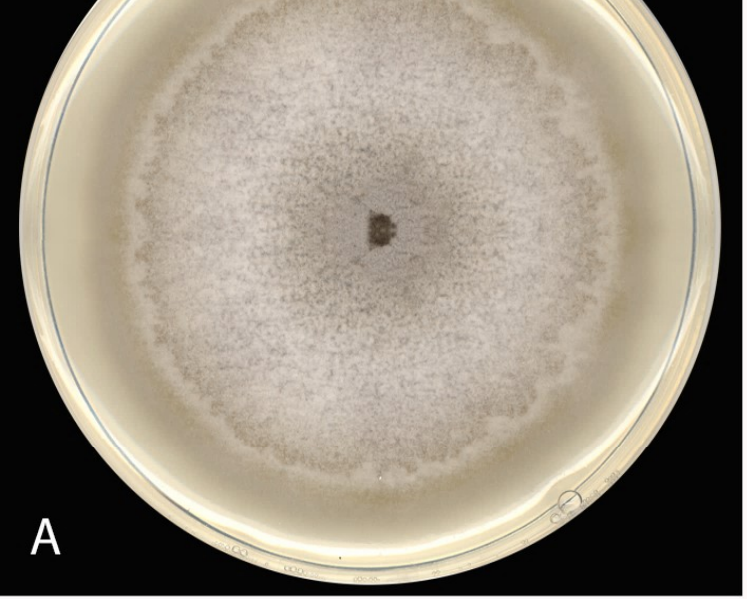
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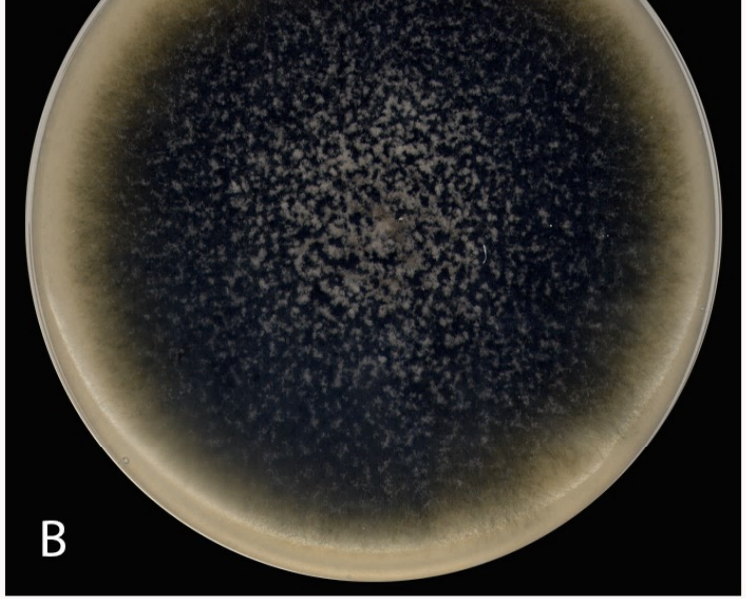
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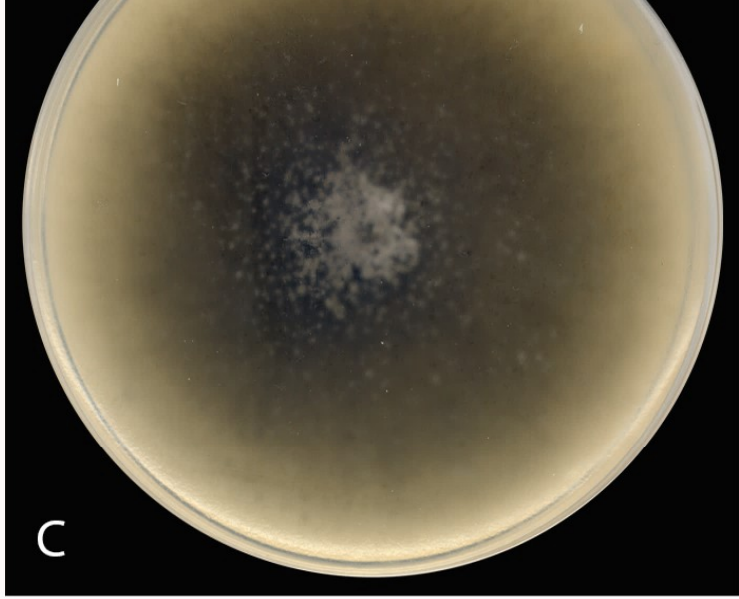
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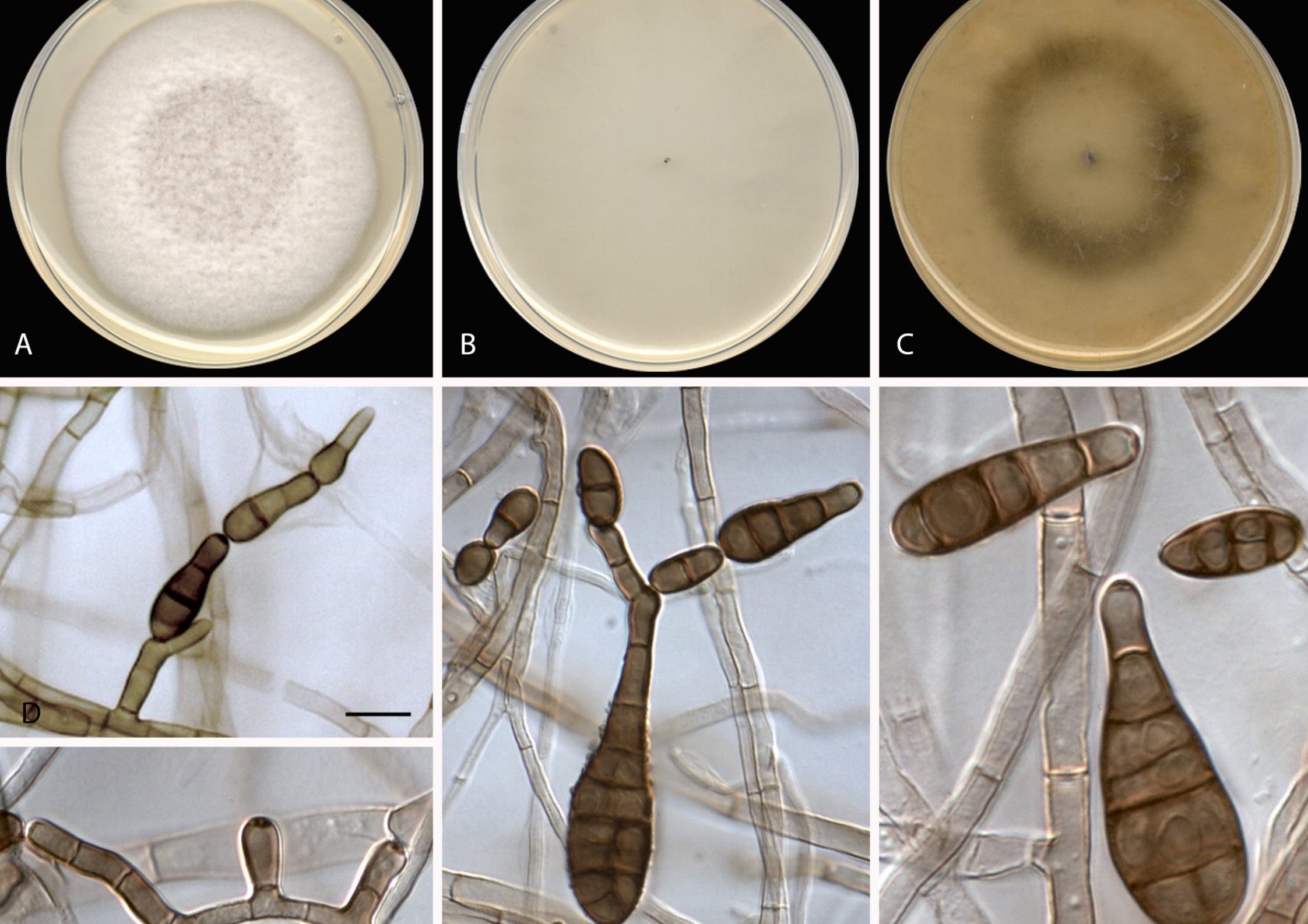
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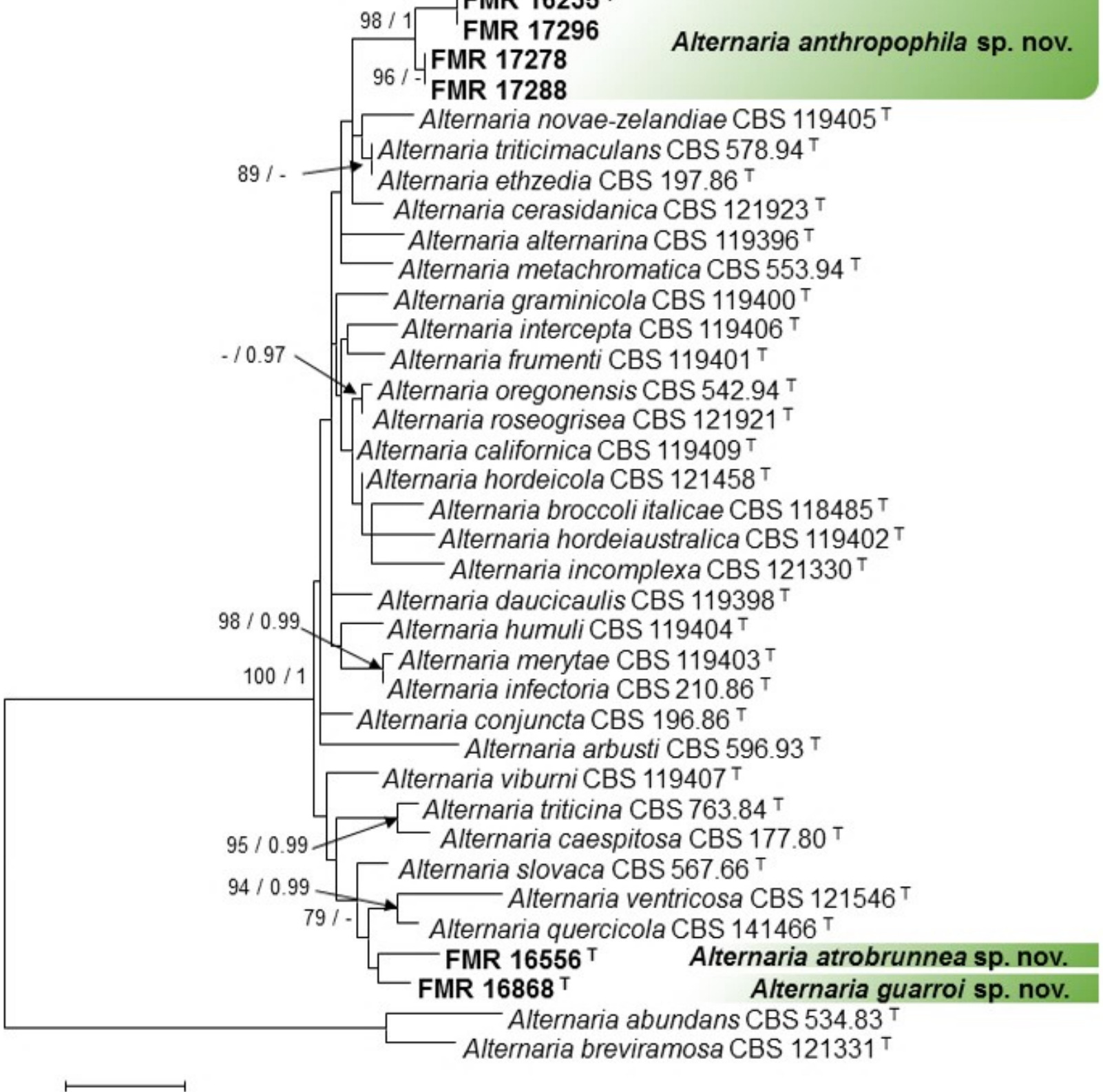
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Alternaria ventricosa CBS 121546 T
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Alternaria quercicola CBS 141466 T
FMR 16556 T
Alternaria atrobrunnea sp. nov.
FMR 16868 T
Alternaria guarroi sp. nov.
Alternaria abundans CBS 534.83 T
Alternaria breviramosa CBS 121331 T

Table 1. Clinical findings, treatment and outcome of the three reported cases.

Case	Age/ gender	Clinical history	Clinical presentation	Hematological conditions				Primary therapy	Outcome
				Hemoglobin (g/dL)	Leucocytes ($x10^3$/uL)	Platelets ($x10^3$/uL)	Creatinine (mg/dL)		
1	46/M	Pulmonary transplant (2 years prior), under immunosuppressive treatment	Solitary, nodule, right leg	12.7	6.27	120	1.46	VRC + surgery	Cure, no relapse
2	73/M	Prostate adenocarcinome stage IV, under radiotherapy treatment	Multiple skin ulcers on knees	9.8	5.5	109	0.40	LAMB, replaced with VRC	Cure, no relapse (dead other reasons)
3	71/F	Hypertension, diabetes mellitus, renal failure, chronic ischemic heart disease, polytreatment	Multiple ulcerative lesions in legs	10.8	5.8	83	0.74	Antibiotic, nursing cures	Improved (dead other reasons)

Table 2. *Alternaria* species included in the phylogenetic study and their respective origin and GenBank accession number.

Species	Strains[†]	Substrate	Locality	GenBank accession numbers[‡]				
				ITS	*gapdh*	*ATPase*	*tef*1	*rpb*2
Alternaria abundans	CBS 534.83 (T)	*Fragaria* sp.	New Zealand	JN383485	KC584154	JQ671802	KC584707	KC584448
A. alternarina	CBS 119396 (T)	*Avena sativa*	USA/Wisconsin	JQ693648	JQ646289	JQ671817	**LR134367**	JQ905199
A. anthropophila	FMR 16235 (T)	Human subcutaneous nodule	Spain/Catalonia	**LR537444**	**LR537034**	**LR537052**	**LR537046**	**LR537040**
	FMR 17278	Human skin biopsy	Spain/Aragón	**LR537030**	**LR537035**	**LR537054**	**LR537048**	**LR537042**
	FMR 17288	Human subcutaneous nodule	Spain/Galicia	**LR537032**	**LR537038**	**LR537055**	**LR537049**	**LR537043**
	FMR 17296	Pericardial liquid	Spain/Cantabria	**LR537445**	**LR537036**	**LR537053**	**LR537047**	**LR537041**
A. arbusti	CBS 596.93 (T)	*Pyrus pyrifolia*	USA/California	JQ693644	JQ646365	JQ671940	FJ214902	**LR134184**
A. armoraciae	CBS 118702 (T)	*Armoracia rusticana*	New Zealand	KC584182	KC584099	**LR134098**	KC584638	KC584379
A. atrobrunnea	FMR 16868 (T)	Human ulcerative skin lesion	Spain/Catalonia	**LR537033**	**LR537039**	**LR537057**	**LR537051**	**LR537044**
A. broccoli-italicae	CBS 118485 (T)	*Brassica oleracea* var.*italica*	Australia	KM821536	KM821538	KY412557	**LR134262**	**LR134194**
A. caespitosa	CBS 177.80 (T)	Human skin lesion	Spain	KC584250	KC584178	**LR134114**	KC584752	KC584492
A. californica	CBS 119409 (T)	*Triticum aestivum*	USA/California	JQ693645	JQ646285	JQ671813	**LR134245**	**LR134181**
A. cerasidanica	CBS 121923 (T)	fruit of *Prunus avium*	Denmark/near Skaelskor	**LR135744**	**LR135747**	**LR135748**	**LR135745**	**LR135746**
A. conjuncta	CBS 196.86 (T)	*Pastinaca sativa*	Switzerland	FJ266475	AY562401	JQ671824	KC584649	KC584390
A. daucicaulis	CBS 119398 (T)	*Daucus carota*	Canada/Ontario	JQ693653	JQ646294	JQ671822	**LR134241**	**LR134177**
A. ethzedia	CBS 197.86 (T)	*Brassica napus*	Switzerland	AY278833	AY278795	JQ671805	KC584657	KC584398
A. frumenti	CBS 119401 (T)	Undetermined Poaceae	New Zealand	JQ693654	JQ646295	JQ671823	**LR134370**	**LR134172**
A. graminicola	CBS 119400 (T)	Undetermined Poaceae	United Kingdom	JQ693650	JQ646291	JQ671819	**LR134249**	**LR134180**
A. guarroi	FMR 16556 (T)	Human ulcerative skin lesion	Spain/Catalonia	**LR537031**	**LR537037**	**LR537056**	**LR537050**	**LR537045**
A. hordeiaustralica	CBS 119402 (T)	*Hordeum vulgare*	South Australia	JQ693641	JQ646283	JQ671811	**LR134243**	**LR134179**
A. hordeicola	CBS 121458 (T)	*Hordeum vulgare*	Southwest Norway	JQ693642	JQ646284	JQ671812	**LR134371**	**LR134175**
A. humuli	CBS 119404 (T)	*Humulus lupulus*	France/Alsace	JQ693652	JQ646293	JQ671821	**LR134199**	**LR134174**
A. incomplexa	CBS 121330 (T)	Canal mud	USA/Idaho	JQ693658	JQ646287	JQ671815	**LR134250**	**LR134185**
A. infectoria	CBS 210.86 (T)	*Triticum aestivum*	United Kingdom	AF347034	AY278793	JQ671804	KC584662	KC584404
A. intercepta	CBS 119406 (T)	*Viburnum* sp.	USA/Chicago	JQ693656	JQ646297	JQ671826	FJ214927	**LR134170**
A. merytae	CBS 119403 (T)	*Meryta sinclairii*	New Zealand	JQ693651	JQ646292	JQ671820	**LR134198**	**LR134119**
A. metachromatica	CBS 553.94 (T)	*Triticum aestivum*	South Australia	JQ693660	AY562404	JQ671809	FJ214931	JQ905189
A. novae-zelandiae	CBS 119405 (T)	*Daucus carota*	New Zealand	JQ693655	JQ646296	JQ671825	**LR134197**	**LR134120**
A. oregonensis	CBS 542.94 (T)	*Triticum aestivum*	USA/Oregon	FJ266478	FJ266491	JQ671827	KC584674	KC584416
A. quercicola	CBS 141466 (T)	*Quercus brantii*	Iran/Fars province	KX228295	KX228362	**LR134115**	**LR134259**	**LR134188**
A. roseogrisea	CBS 121921 (T)	*Helianthus annuus*	USA/North Dakota	**LR134102**	**LR134103**	**LR134104**	**LR134260**	**LR134192**
A. slovaca	CBS 567.66 (T)	Human, lesion of ear	Czechoslovakia	KC584226	KC584150	**LR134368**	KC584702	KC584444
A. triticimaculans	CBS 578.94 (T)	*Triticum aestivum*	Argentina/La Plata	JQ693657	JQ646280	JQ671806	FJ214930	**LR134183**
A. triticina	CBS 763.84 (T)	*Triticum aestivum*	India/New Delhi	AY278834	JQ646281	JQ671808	FJ214942	**LR134186**
A. ventricosa	CBS 121546 (T)	*Pyrus bretschneideri*	USA/Washington	JQ693649	JQ646290	JQ671818	KY352501	**LR134134**
A. viburni	CBS 119407 (T)	*Viburnum* sp.	USA/Chicago	JQ693647	JQ646288	JQ671816	**LR134200**	**LR134166**

[†]CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; FMR: Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain. T: Ex-type strain

[‡]ITS: Internal Transcribed Spacers, *gapdh*: glyceraldehyde 3-phosphate dehydrogenase, *ATPase*: plasma membrane ATPase, *tef1*: translation elongation factor 1-alpha, *rpb2*: RNA polymerase second largest subunit. Newly generated sequences in this study and new species are indicated in bold.

Table 3. Results of in vitro antifungal susceptibility testing for the three case isolates of *Alternaria* section *Infectoriae*

	A. anthropophila (Case 1-FMR 16235)		*A. guarroi* (Case 2-FMR 16556)		*A. atrobrunnea* (Case 3-FMR 16868)	
Results (µg/mL) for[§]	MIC	MEC	MIC	MEC	MIC	MEC
AMB	2	-	2	-	8	-
ITC	0.125	-	>16	-	16	-
PSC	0.125	-	0.125	-	8	-
VRC	1	-	2	-	>16	-
AFG	-	0.5	-	8	-	0.06
CFG	-	0.5	-	4	-	1
MFG	-	0.125	-	4	-	0.03
TBF	0.5	-	1		2	-

[§]AMB, amphotericin B; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; AFG, anidulafungin; CFG, caspofungin; MFG, micafungin; TBF, terbinafine.