INTRODUCTION

The genus *Chrysosporium* comprises a large number of ubiquitous anamorphic species, which are predominantly found in soil, marine and freshwater sediments, decaying wood, feathers, skin and hair of mammals, reptiles and birds (Rees 1967a, b, c, de Hoog et al. 2000, Hubalek 2000, Mandeel et al. 2009). *Chrysosporium* is usually characterised by whitish to pale colonies and conidia sessile or arising on short stalks from the fertile hyphae. The conidia are broader than the diameter of the hyphae, and they are usually subglobose, pyriform or claviform and are released rheotaxially (Sigler 1997, de Hoog et al. 2000). Due to the large number of species of *Chrysosporium* (approximately seventy), the poor morphological differentiation of its species and, in some cases, the absence of an associated sexual morph, they are not easy to identify and the distinction from similar genera such as *Geymecyes*, *Malbranchea*, or *Sporotrichum*, among others, is difficult (Vidal et al. 2000).

Based on the analysis of the sequences of the internal transcribed spacer region (ITS), Vidal et al. (2000) demonstrated that *Chrysosporium* is polyphyletic and the phylogenetic relationships of *Chrysosporium merdarium*, the type species of the genus, revealed that it belongs to the *Gymnosphaeaceae* (Onygenales). Those same authors also indicated that some morphological characters traditionally used in taxonomy such as the colour of the colony, the growth rate at different temperatures, conidiogenesis, and conidial morphology, are subject to homoplasy and, in some cases, are not useful to resolve the species boundaries.

Some *Chrysosporium* species develop telemorphs belonging to very diverse genera in the families *Arthrodermataceae* (Curt rh 1985), *Ascosphaeriaceae* (van Oorschot 1980), *Chaetomiaceae* (Vidal et al. 2000), *Gymnosphaeaceae* (van Oorschot 1980, Curt rh 1985), *Lasiosphaeraceae* (Mouchacca & Gams 1993, Ueda 1994), *Onygenaceae* (van Oorschot 1980, Curr rh 1985), *Monascaceae* (Pettersson et al. 2011) and *Myxotrichaceae* (Vidal et al. 2000).

Most of the *Chrysosporium* isolates found in the clinical laboratory are contaminants but some species occasionally infect humans. Most produce skin and nail lesions although some deep infections, mainly in immunocompromised patients, have also been reported (Sigler 1997, Sigler et al. 1998, Rolides et al. 1999, de Hoog et al. 2000, Stebbins et al. 2004, Abdel-Razik & Zaki 2008). In most of those reports, however the etiologic agent has been identified only at the genus level. One of the most relevant pathogenic species is *Nannizziopsis vriesii*, which has a *Chrysosporium* anamorph, causing severe and often fatal dermatomycosis in different species of reptiles (Paré et al. 1997, Nichols et al. 1999, Thomas et al. 2002, Bertelsen et al. 2005, Mitchell et al. 2006, Paré et al. 2006, Bowman et al. 2007, Paré & Jacobson 2007, Han et al. 2010, Hedley et al. 2010, Helliebuyck et al. 2010, Allender et al. 2011, Johnson et al. 2011). However, *N. vriesii* also produces infections in humans (Stebbins et al. 2004, Brandt et al. 2005, Steininger...
et al. 2005). It has been suggested that other *Chrysosporium* species, morphologically similar to *N. vriesii*, could also be involved in human and animal infections (Brandt et al. 2005, Abarca et al. 2008, 2009, 2010). The recent description of *C. guarroi*, which infects reptiles (Abarca et al. 2010) and is phylogenetically related to *N. vriesii*, suggests the existence of a complex of morphologically similar species.

Using phenotypic and molecular methods, we have studied a set of clinical *Chrysosporium* isolates from different reptiles and humans that are morphologically similar to *N. vriesii*, in order to better characterise these fungi and to determine their phylogenetic boundaries.

### Table 1 Fungi included in this study

| Species | Origin | GenBank accession no. |
|---------|--------|-----------------------|
| *Nannizziopsis vriesii* | RKI 04-0104 Human, brain abscess, Nigerian man, Germany | HF547853 HF547877 HF547869 HF547878 |
| *Amauroascus niger* | UTHSC 04-2056 Pigongia viticeps, USA | HF547854 HF547879 HF547870 HF547880 |
| *Lecythophora hoffmannii* | UTHSC 06-1419 Pigongia viticeps, USA | HF547855 HF547881 HF547871 HF547882 |
| *Eremascus fertilis* | CCFVB CH12 Pigongia viticeps, Spain | HF547856 HF547883 EU883993* HF547884 |
| *Chrysosporium ophiodiicola* | UTHSC R 4 263 Physignathus sp. (water dragon), USA | HF547857 HF547885 HF547872 HF547886 |
| *Nannizziopsis pluriseptata* | UTHSC R 4 380 Snake, multifocal dermatitis, USA | HF547858 HF547887 HF547873 HF547888 |
| *Chrysosporium ophiodiicola* | UTHSC 10-1045 Skink lizard, USA | HF547859 HF547889 HF547874 HF547890 |
| *Nannizziopsis vriesii* | CBS 122913^1^ Snake, subcutaneous granuloma, USA | EU15820* HF547891 EU15819* HF547892 |
| *Nannizziopsis guarroi* | IMI 149964^1^ Ameiva sp., skin and lungs, USA | AJ176515* HF547893 AJ131687* HF547894 |
| *Chrysosporium ophiodiicola* | CBS 124450^1^ Iguana iguana, Spain | FJ863684* HF547860 |
| *Nannizziopsis vriesii* | CCFVB CH11 Iguana iguana, Spain | HF547861 |
| *Nannizziopsis vriesii* | CCFVB CH14 Iguana iguana, Spain | HF547862 |
| *Nannizziopsis vriesii* | CCFVB CH15 Iguana iguana, Spain | HF547863 |
| *Nannizziopsis vriesii* | UTHSC R 4 309 Snake, USA | HF547864 |
| *Nannizziopsis vriesii* | UTHSC 05-1370 Pigongia viticeps, USA | HF547865 |
| *Nannizziopsis vriesii* | UTHSC 06-3993 Agama agama, USA | HF547866 HF547897 HF547875 HF547898 |
| *Nannizziopsis vriesii* | UTHSC 07-3227 Pigongia viticeps, USA | HF547867 |
| *Nannizziopsis vriesii* | UTHSC R 4 317 Human, disseminated disease, Nigerian man, USA | HF547868 HF547899 HF547876 HF547900 |
| *Uncinocarpus reesii* | ATCC 34533 Feathers, Australia | AY176724* |
| *Amauroascus niger* | ATCC 22339 T Soil, USA | AY176706* |
| *Chrysosporium tropicum* | MUCU 100861 | Wolf dung, Solomon Islands AY176731* |
| *Aphanomucor meijetii* | ATCC 22144^7^ | HF547875* |
| *Chrysosporium keratinophilum* | CBS 392.76 | Soil, New Zealand AY176730* |
| *Arthrodema catenati* | OMM H1-10 | Human, Canada AY176736* |
| *Arthrodema olale* | UAMH 2338 | Human, skin scrapings and hair, Canada AY176735* |
| *Arthrodema ciferii* | ATCC 24447^7^ | Soil, USA EF143625* |
| *Ctenomyces serratus* | CBS 187.61^1^ | Soil, Australia AY176733* |
| *Chrysosporium vallaranae* | UAMH 6914 | Dung of Apojej lagopus, Chile AY176732* |
| *Gymnaascus littoralis* | CBS 454.73 | Conch shell, Canada FJ358272* |
| *Gymnaascus aurantius* | ATCC 22094^1^ | Soil, Russia AY176747* |
| *Gymnascus ruber* | CBS 352.90 | Soil, England AY176748* |
| *Ascospora subglobosa* | Voucher A.A. Wynns 5004(C) | Megachile rotundata, USA HQ540517* |
| *Ascospora apis* | CBS 252.32 | Apis melliflca, Denmark AYO04344* |
| *Parascocidioideae brasiliensis* | IMTSP 556 | Human, Brazil U181263* |
| *Ajellomyces dermatitidis* | ATCC 18187^7^ | Human, USA AY176704* |
| *Ajellomyces capsulatus* | UAMH 7114 | Soil, USA AF038353* |
| *Byssochlamys nivea* | CBS 100.11^7^ | Geastrum coronatum, Sweden AY176750* |
| *Eurotium herbariorum* | ATCC 1869 | Unpainted board, USA AY176751* |
| *Peyrococcus alliaceus* | ATCC 1689 | Soil, Australia AY176752* |
| *Araunomyces nodosotus* | CCF 3957 | Human, nail infection, Czech Republic HM205103* |
| *Araunomyces glareous* | CBS 116129 | Human, thumb nail, Canada FJ358273* |
| *Araunomyces minimus* | CBS 324.70 | Decayed wood, Canada FJ358274* |
| *Eremascus fertilis* | KVL 10-09 | Pollen, Denmark HQ540515* |
| *Lecythophora hoffmannii* | CBS 140.41 | Sewage water, England AB261976* |
| *Betisia allii* | KVL 10-08 | Pollen, Denmark H0540516* |

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* Ex-type strain.

* sequences retrieved from the GenBank database.

1. ATCC: American Type Culture Collection, USA; CAFB: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCF: Culture Collection of Fungi, Department of Botany, Charles University in Prague, Czech Republic; CCFVB: Culture Collection of the Veterinary Mycology Group, Bellatera, Barcelona, Spain; IMI: International Mycological Institute Culture Collection, Surrey, United Kingdom; IMTSP: Instituto de Medicina Tropical de São Paulo Culture Collection, São Paulo, Brazil; KVL: Entomopathogenic Fungal Culture Collection at Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Denmark; MUCU: Mycotheque de l’Universite Catholique de Louvain, Louvain la Neuve, Belgium; OMH: Ontario Ministry of Health, Toronto, Ontario, Canada; RKI: Robert Koch Institute, Berlin, Germany; UAMH: Microfungus Collection and Herbarium, University of Alberta, Canada; UTHSC: Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, USA.

### MATERIALS AND METHODS

**Fungal isolates**

The clinical isolates and reference strains included in the study are detailed in Table 1. Only two strains of *N. vriesii* were included in this study. This is the type species of the genus, i.e., the type strain and a clinical strain from Germany. Of the other species of *Nannizziopsis (N. albicans, N. hispanica, N. mirabilis, N. patagonica and N. tropicalis)* only live cultures of *N. albicans* are available, but this species is phylogenetically related to *Amauroascus (Onygenaceae)* (Solé et al. 2002) and was not included in the study.
Molecular study

DNA was extracted according to Perdomo et al. (2011). Detailed protocols for the amplification of D1 and D2 domains of the 28S rDNA (D1-D2), the internal transcribed spacer region (ITS), and a fragment of actin (ACT) and β-tubulin (TUB) genes were described in Cano et al. (2004) (ITS), Voigt & Wöstemeyer (2000) (ACT), and Gilgado et al. (2005) (D1-D2 and TUB). PCR products were purified and sequenced at Macrogen Corp. Europe (Amsterdam Zuid-Oost, The Netherlands) with a 3730XL DNA analyzer (Applied Biosystems). The program SeqMan (Lasergene, Madison, Wisconsin) was used to obtain consensus sequences of each isolate. DNA sequences were aligned with the program ClustalX v. 1.8 (Thompson et al. 1997) with default parameters, followed by manual adjustments with a text editor. A D1-D2 and an ITS BLAST were carried out with the ex-type strains of \textit{N. vriesii} and \textit{C. guarroi} in order to select the closest species and to include it in the phylogenetic study. Sequences retrieved from GenBank and included in the phylogenetic analysis are listed in Table 1. Phylogenetic analysis of the D1-D2 encompassed representatives of the clinical isolates and reference strains of the families within the order \textit{Onygenales} (\textit{Ajellomyctecataceae, Arachnomyctecataceae, Arthrodertmataceae, Ascosphaeraceae, Gymnoascaceae} and \textit{Onygenaceae}) as well as some representatives of \textit{Eurotiales}. \textit{Eremascus fertilis} (HQ540515) and \textit{Lecythophora hoffmannii} (AB261976) were used as outgroups. The combined dataset (ITS, ACT and TUB), included representatives of the clinical isolates and the type strains of \textit{C. guarroi}, \textit{C. ophidiicoila} and \textit{N. vriesii}. The phylogenetic analyses were conducted using MEGA v. 5.05 (Tamura et al. 2011) with maximum likelihood (ML) algorithm, using Tamura 3-parameter substitution model with gamma distribution (D1-D2) and Tamura-Nei with gamma distribution (combined dataset). The robustness of branches was assessed by bootstrap analysis of 1 000 replicates. Bayesian analyses (BA) were carried out using MrBayes v. 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Bayesian analyses were performed by running 1 000 000 generations in four chains, saving the current tree every 100 generations. The last 18 000 trees were used to construct a 50 % majority-rule consensus tree and to determine the posterior probabilities of the branches. The sequences generated in this study and the alignments used in the phylogenetic analyses were deposited in GenBank (Table 1) and TreeBASE (accession URL: TB2:S13558), respectively.

Morphological studies

Colonial features were examined after 14 days of incubation on malt extract agar (MEA; Difco Laboratories, Detroit, MI, USA), oatmeal agar (OA; 30 g filtered oat flakes, 20 g agar, 1 L distilled water), potato dextrose agar (PDA; Pronadisa S.A., Spain), phytone yeast extract agar (PYE; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and Sabouraud dextrose agar (SDA; Pronadisa S.A., Spain). Colonial growth rates were determined at different temperatures (from 5 °C to 40 °C, in 5 °C intervals). To induce the formation of ascomata, the isolates were grown on OA and incubated at 25 °C and 30 °C for up to three months. Colour notations (in parenthesis) are from Konnerup & Wanscher (1978). Microscopic features were studied on PDA and PYE slide cultures incubated for 7–14 d at 30 °C, and mounted in lactic acid.

Physiological studies

Production of urease was determined in Christensen's urea broth after incubation at 30 °C for 7 d. Lipase activity was tested by growing on Tween 80 opacity test medium (TOTM) according to Silfkin (2000), incubating the Petri dishes at 30 °C for 14 d. Growth on dermatophyte test medium (DTM) and colonial changes from yellow (acidic) to red (basic) were recorded after incubating the Petri dishes at room temperature (20 °C to 30 °C) for 14 d. Hydrolysis of milk solids was detected on bromocresol purple-milk solids-glucose agar (BCP-MSG) Petri dishes, according Kane et al. (1997), after incubation at 30 °C for 14 d. Cycloheximide tolerance was evaluated by growing isolates on SDA supplemented with 0.2 % of cycloheximide (Sigma, USA) at 30 °C for 14 d. Tolerance to NaCl was evaluated by growth of isolates on SDA, amended with 3 % and 5 % w/v NaCl, after incubation for 14 d at 30 °C. Hemolysis was evaluated by culturing isolates on blood agar (BioMérieux, France) for 14 d at 30 °C.

RESULTS

Molecular analysis

With the primers used, we were able to amplify and sequence 485–502 bp (D1-D2), 428–507 bp (ITS), 628–645 bp (ACT) and 402–419 bp (TUB). None of the isolates showed an ITS sequence identity higher than 95 % with the species of \textit{Chrysosporium} represented in GenBank, with the exception of eight reptilian isolates that showed 100 % identity with the type strain of \textit{C. guarroi} and one isolate from a human systemic infection in the USA (UTHSC R-4317) that showed a 97.8 % identity also with this species. Maximum likelihood (ML) and Bayesian analyses of D1-D2 dataset produced phylogenetic trees with similar topologies. Fig. 1 shows the D1-D2 ML tree including the bootstrap support (bs) and the posterior probabilities (pp). Three main clades could be distinguished within the ingroup corresponding to the orders \textit{Eurotiales} (97 % bs/1 pp), \textit{Arachnomyctecataceae} (97 % bs/1 pp) and \textit{Onygenales} (72 % bs/1 pp), respectively. The \textit{Onygenales} encompassed five well-supported clades, corresponding to the families \textit{Ascosphaeraceae} (99/1), \textit{Gymnoascaceae} (99/1), \textit{Arthrodertmataceae} (96/1) and \textit{Onygenaceae} (90/1), and the fifth one (99/1) that embraced the type strains of \textit{C. guarroi} and \textit{N. vriesii}, and most of our clinical isolates, and a poorly-supported group (66/1) that included some members of the family \textit{Ajellomyctecataceae}. This analysis did not resolve the taxonomic position of the type strain of \textit{C. ophidiicoila} and of the clinical isolate UTHSC R-4380 (provisionally named \textit{Chrysosporium} sp. 4), which consisted of two different branches between the \textit{Onygenaceae} and the \textit{Nannizziopis} group.

In the combined ITS-ACT-TUB ML tree (Fig. 2) several terminal well-supported branches representing undescribed species were shown. These were \textit{Chrysosporium} sp. 1, that included the isolates UTHSC 04-2056 and UTHSC 06-1419, \textit{Chrysosporium} sp. 2 (isolate CCFVB CH12), \textit{Chrysosporium} sp. 3 (isolate UTHSC R-4263) and \textit{Chrysosporium} sp. 5 (isolate UTHSC 10-1045). Additionally, three strains of \textit{C. guarroi} also formed a terminal clade (84 % bs) that included the type strain of this species, one reptilian isolate and a human clinical strain (UTHSC R-4317), which was slightly separated from the other two. The ex-type strain of \textit{N. vriesii}, and a human clinical strain, morphologically identified as \textit{N. vriesii} (RKI 04-0104), were separated from the rest of fungi included in the tree but also separated between them. The ex-type strain of \textit{C. ophidiicoila} (CBS 122913) and the isolate UTHSC R-4380 (\textit{Chrysosporium} sp. 4) were placed away from the others and in fact acted as outliers.

Morphological study

The isolates included in this study were characterised by the production of thin- and smooth-walled, small, mostly sessile and 1-celled conidia. The only species able to produce chlamydo spores was \textit{Chrysosporium} sp. 1 (Fig. 3f, g). Single intercalary conidia were formed by all the isolates, with those...
Fig. 1 Maximum-likelihood (ML) tree based on Tamura three-parameter corrected nucleotide distances among the D1 and D2 domains of the 28S rRNA gene sequences of taxa included in Table 1. Numbers on the branches are bootstrap ML values above 55 %, followed by Bayesian posterior probabilities (Bpp) above 0.6. Branch lengths are proportional to distance. Sequences not generated in this study and obtained from the GenBank database are indicated in parentheses. Ex-type strains of the different species are indicated with T. New species proposed in this study are indicated in bold. N. = Nannizziopsis. C. = Chrysosporium.

of Chrysosporium sp. 3 being the longest (Fig. 5e). All the species, with the exception of Chrysosporium sp. 4, produced arthroconidia in chains, usually terminal (Fig. 3e, 6f, g). In all the isolates the sessile and terminal conidia were similar in size, although some conidia of Chrysosporium sp. 4 (Fig. 7e) and Chrysosporium sp. 5 (Fig. 6h) were considerably longer (above 10 µm). Chrysosporium sp. 5 is the only species that produced up to 5-celled conidia (Fig. 6h). On PYE at 25 °C Chrysosporium guarroi showed the slowest growth rate (17–22 mm in 14 d), whereas Chrysosporium sp. 4 was the fastest (40–46 mm in 14 d).

Physiological characterisation

The urease test was strain dependent, however the majority of the isolates were positive. All the fungi tested grew on DTM changing the colour of the medium from yellow to red (data not shown). The results of the other physiological tests are summarised in Table 2. With the exception of C. ophiidiocola, all...
Key physiological features of fungi included in this study.

| Fungi                      | BCP-MS-G agar | Hemolysis | Lipase | Cycloheximide tolerance | Growth on PYE at 15 °C | Growth on PYE at 40 °C |
|----------------------------|---------------|-----------|--------|-------------------------|------------------------|------------------------|
| Nannizziopsis vriesii IMI 149994T |               | +         | +      | –                       | +                      | +                      |
| Nannizziopsis guarroi UTHSC R-4309 |               | –         | –      | +                       | +                      | +                      |
| Nannizziopsis guarroi UTHSC R-4262 |               | –         | +      | –                       | +                      | –                      |
| Nannizziopsis guarroi UTHSC R-4317 (+) |               | +         | +      | +                       | +                      | –                      |
| Nannizziopsis chlamydospora UTHSC 04-2056 |               | +         | +      | +                       | +                      | +                      |
| Nannizziopsis arthrosporioides UTHSC R-4263 |               | +         | +      | +                       | +                      | +                      |
| Chrysosporium ophiodiicola |               | +         | +      | +                       | +                      | +                      |
| Chrysosporium sp. 1 |               | +         | +      | +                       | +                      | +                      |
| Nannizziopsis draconii CCFVB CH12 |               | +         | +      | +                       | +                      | +                      |
| Nannizziopsis pluriseptata UTHSC 10-1045 |               | +         | +      | +                       | +                      | +                      |

- = absence; + = positive; (+) = scarce positive. Growth on SDA plus 5% NaCl: – = absence; + = positive; (+) = scarce positive growth.

* Reactions on BCP-MS-G agar: – = absence; + = positive; (+) = scarce positive. Growth on SDA plus 5% NaCl: – = absence; + = positive; (+) = scarce positive growth.

Nannizziopsis chlamydospora Stchigel, D.A. Sutton, Cano & Guarro, sp. nov. (Chrysosporium sp. 1) — MycoBank MB801986; Fig. 3a–i

Etymology. From Greek chlamydo-, cloak, and from Latin -spora, spore.

Colonies on PYE at 30 °C attaining a diameter of 41–48 mm after 14 d, yellowish white (M. 4A2), elevated at the centre and radially folded, compact, with an irregular margin; reverse yellowish white (M. 4A2). Hyphae hyaline, septate, smooth-walled, 1–3(–5) µm wide. Conidia unicellular, mostly sessile, on short protrusions or on side branches, less frequently terminal, hyaline, thin- and smooth-walled, pyriform, claviform, or cylindrical, 3–9 × 1.5–2 µm; intercalary conidia, cylindrical to doliiform, 6–10 × 1.5–2 µm; arthroconidia catenate, cylindrical to doliiform, 4–10 × 2–4 µm. Chlamydospores globose, broadly ellipsoidal or irregular, smooth- and thick-walled, 5–15(–20) µm diam. Sexual morph not observed. Felt (skunk-like) odour produced on all the culture media tested.

Minimum and maximum temperature of growth — 5 °C and 40 °C, respectively. Colonies reaching a diameter of 33–39 mm on PDA, 37–41 mm on SDA, 35–37 mm on MEA and 25–32 mm on OA after 14 d at 25 °C.

Specimens examined. USA, ex Pogona vitticeps ex-type CBS 133985, UTHSC 04-2056, FMR 10835; ex Pogona vitticeps dermal lesion, holotype CBS-H80115; cultures ex-type CBS 133985, UTHSC 04-2056, FMR 10835; ex Pogona vitticeps dermal lesion, UTHSC 06-1419.

Nannizziopsis draconii J. Cabañas, Abarca, Stchigel, Cano & Guarro, sp. nov. (Chrysosporium sp. 2) — MycoBank MB801987; Fig. 4a–h

Etymology. From Latin draco, dragon, referring to the source (a lizard) from where the fungus was isolated.

Colonies on PYE at 30 °C attaining a diameter of 32–38 mm after 14 d, yellowish white (M. 4A2), felted, slightly elevated at centre, with regular margin; reverse yellowish white (M. 4A3). Hyphae hyaline, septate, smooth-walled, 1–3(–5) µm wide. Conidia unicellular, mostly sessile, also produced on short protrusions or on side branches,
Fig. 2 Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, actin and β-tubulin). Bootstrap support values above 70 % are indicated at the nodes.

Fig. 3 Nannizziopsis chlamydospora UTHSC 04-2056 (= Chrysosporium sp. 1). a. Colony on blood agar; b. colonies on BCP-MS-G (reverse); c. colony on TOTM; d. conidiophores bearing sessile and intercalary conidia (black arrow), and conidia on side branches; e. long chains of lateral and terminal arthroconidia; f. chlamydospores in chains; g. a solitary chlamydospore and thick-walled hyphae; h, i. conidia. — Scale bars: d–g = 10 µm; h, i = 5 µm (d–h, differential interference contrast; i, phase contrast).
or terminal, hyaline, thin- and smooth-walled, claviform or cylin-
drical, 4–7 × 1.5–2(–2.5) μm; intercalary conidia scarce, cylin-
drical, 4–9 × 1.5–2 μm; arthroconidia catenate, mostly cylin-
drical or doliiform, scarcely produced, 5–9 × 1.5–2.5 μm. 
Chlamydospores absent. Sexual morph not observed. Fetid 
(skunk-like) odour produced on all the culture media tested. 
Minimum and maximum temperature of growth — 15 °C and 
35 °C, respectively. Colonies reaching a diameter of 32–35 mm 
on PDA, 34–37 mm on SDA, 35–43 mm on MEA and 32–40 
mm on OA after 14 d at 25 °C.

Specimen examined. Spain, ex Pogona vitticeps, holotype CBS H-21116, 
cultures ex-type CBS 133987, CCFVB CH12, FMR 10859.

**Nannizziopsis arthrosporioides** Stchigel, D.A. Sutton, Cano & Guarro, sp. nov. (Chrysosporium sp. 3) — MycoBank 
MB801988; Fig. 5a–f

Etymology. From the Greek arthron-, articulation, and from Latin -spora, 
spore.

Colonies on PYE at 30 °C attaining a diameter of 34–37 mm 
after 14 d, yellowish white (M. 1A2), zonate, felted, slightly 
cottony at centre, with lobate margins; reverse yellowish white 
(M. 4A2). Hyphae hyaline, septate, smooth-walled, 1–4 μm wide, 
straight or twisted. Conidia 1(–2)-celled, mostly sessile, also 
produced on short protrusions or terminal, hyaline, thin- 
and smooth-walled, subglobose, pyriform, obovate, or claviform to 
cylindrical, 2.5–7 × 1.5–3 μm; intercalary conidia present, 
similar to the arthroconidia in shape and size; arthroconidia 
arranged in short terminal and intercalary chains, doliiform to 
cylindrical or irregularly-shaped, 5–15 × 1.5–4 μm. Chlamydos-
spores absent. Sexual morph not observed. Fetid (skunk-like) 
odour present on all the culture media tested.

Minimum and maximum temperature of growth — 15 °C and 
30 °C, respectively. Colonies reaching a diam of 34–38 mm 
on PDA, 28–32 mm on SDA, 42–45 mm on MEA, and 30–33 
mm on OA, after 14 d at 25 °C.

Specimen examined. USA, ex Physignathus sp., holotype CBS H-21117, 
cultures ex-type CBS 133988, UTHSC R-4263, FMR 10842.
**Nannizziopsis pluriseptata** Stchigel, D.A. Sutton, Cano & Guarro, sp. nov. (*Chrysosporium* sp. 5) — MycoBank MB801989; Fig. 6a–h

*Etymology.* From the Latin *pluri-*—many, and *septum,* septum.

*Colonies* on PYE at 30 °C attaining a diameter of 38–40 mm after 14 d, white to orange white (M. 5A2), zonate, felted, slightly cottony at the centre, with regular margins; reverse orange white (M. 5A2). *Hyphae* hyaline, septate, smooth-walled, 1–5 µm wide, straight. *Conidia* 1(–5)-celled, mostly sessile, also produced on short protrusions or on side branches, or terminal, hyaline, thin- and smooth-walled, pyriform, obovate, claviform to cylindrical, 2.5–8(–15) × 1.5–2.5 µm; intercalary conidia occasionally present, cylindrical to doliiform or irregularly shaped, 2.5–5 × 2–2.5 µm; arthroconidia, disposed in lateral or terminal short chains, cylindrical to doliiform, 4–7 × 2.5–3.5 µm, usually bearing sessile conidia. *Chlamydospores* and *sexual morph* absent. Fetid (skunk-like) odour present on all culture media tested.

Minimum and maximum temperature of growth — 20 °C and 40 °C, respectively. Colonies reaching a diam of 20–36 mm on PDA, 33–35 mm on SDA, 35–37 mm on MEA and 23–25 mm on OA after 14 d at 25 °C.

*Specimen examined.* USA, ex skin of a skink (*Eumeces inexpectatus* Taylor), holotype CBS H-21118, cultures ex-type CBS 133989, UTHSC 10-1045, FMR 12084.

**Chrysosporium longisporum** Stchigel, D.A. Sutton, Cano & Guarro, sp. nov. (*Chrysosporium* sp. 4) — MycoBank MB801990; Fig. 7a–e

*Etymology.* From the Latin *longo-*—long, and *spora,* spore.

*Colonies* on PYE at 25 °C attaining a diameter of 40–46 mm after 14 d, white to pale orange (M. 6A3), zonate, felted, slightly cottony at centre, with regular margins; reverse pale orange (M. 5A2). *Hyphae* hyaline, septate, smooth-walled, 1–5 µm wide, straight. *Conidia* 1(–2)-celled, mostly sessile, or produced on short protrusions or on side branches or terminal, hyaline thin- and smooth-walled, pyriform, obovate, claviform to cylindrical, 3–13 × 2–3.5 µm; intercalary conidia present, cylindrical to doliiform, 3–6 × 2–3 µm, usually bearing sessile conidia; arthroconidia in chains absent. *Chlamydospores* and *sexual morph* absent. Fetid (skunk-like) odour present on all the culture media tested.

Minimum and maximum temperature of growth — 5 °C and 25 °C, respectively. Colonies reaching a diam of 40–44 mm on PDA, 40–46 mm on SDA, 45–50 mm on MEA and 25–50 mm on OA after 14 d at 25 °C.

*Specimen examined.* USA, ex dermic lesion of a tentacled snake (*Erepeton tentaculatum* Lacépède), holotype CBS H-21139, cultures ex-type CBS 133990, UTHSC R-4380, FMR 10617.
Fig. 6  *Nannizziopsis plurisepctata* UTHSC 10-1045 (= *Chrysosporium* sp. 5). a. Colonies on blood agar (the arrow shows the b-hemolysis halus); b. colony on BCP-MS-G (surface and reverse); c. colony on TOTM; d, e. fertile hyphae bearing mostly sessile conidia (arrow showing a intercalary conidium); f, g. arthroconidia; h. sessile conidia (observe the presence of up to 5-celled propagules). — Scale bars = 10 µm (d, f, differential interference contrast; e, g, h, phase contrast).
Nannizziopsis guarroi (J.Cabañas & Abarca) J.Cabañas, Abarca, Guarro, Stchigel & Cano, comb. nov. — MycoBank MB801991; Fig. 8a–g

Basionym. *Chrysosporium guarroi* J.Cabañas & Abarca, Med. Mycol. 48: 370. 2010.

Notes — All the clinical isolates, with the exception of *C. longisporum*, in the D1-D2 tree (Fig. 1) formed a well-supported clade (100 % bs/1 pp) within the *Onygenales*, and were phylogenetically separated from the other families of the order. All these species are phenotypically similar and share the ability to cause dermal lesions in reptiles. These characteristics support the proposal of a new family.

Nannizziopsiaceae Guarro, Stchigel, D.A. Sutton & Cano, fam. nov. — MycoBank MB802007

Type genus. *Nannizziopsis* (Apinis) Currah, Mycotaxon 24: 164. 1985.

*Ascomycota, Pezizomycotina, Eurotiomycetes, Eurotiomycetidae, Onygenales. Ascomata* (when present) discrete, spheri-
cal, whitish, with a peridium composed of a network of loosely interwoven, verrucose, hyaline hyphae which are constricted at the septa. *Asc*: spherical, 8-spored, soon evanescent. *Ascospores* spherical, hyaline, thick- and smooth-walled under light microscope, spiny to reticulate under scanning electron microscope. *Chrysosporium* anamorph consisting of sessile conidia, rarely intercalary, solitary, hyaline, smooth- and thin-walled, pyriform, obovate, obovoid, clavate or cylindrical,
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1-celled, rarely 2–5-celled, usually with broad basal scars; arthroconidia 1-celled, intercalary or terminally disposed, in chains. Fetid (skunk-like) odour is present in all the members of this family.

DISCUSSION

The order Onygenales comprises six families: Ajellomycetaceae, Arachnomycetaceae, Arthodermataceae, Ascosphaeraceae, Gymnascaceae and Onygenaceae (Lumbsch & Huhndorf 2010). Until now, these families, with the exception of the Onygenaceae, which is clearly polyphyletic (Sugiyama et al. 1999, Herr et al. 2001, Sugiyama & Mikawa 2001, Gibas et al. 2002, Untereiner et al. 2002, 2004), were well delimited. In our D1-D2 phylogenetic tree (Fig. 1), the members of the Arachnomycetaceae were located outside the Onygenales. The data supports the revalidation of the order Arachnomycetales proposed by Gibas et al. (2002). On the other hand, two species

Fig. 8 Nannizziopsis guarroii CBS 124553. a. Colonies on blood agar; b. colonies on BCP-MS-G; c. colony on TOTM; d, e. fertile hyphae bearing sessile conidia; f, g. sessile conidia. — Scale bars = 10 µm (d, f, differential interference contrast; e, g, phase contrast).
of Ascospheara (Ascospheareales), i.e., A. apis and A. subgloboasa, were included within the Onygenales, but the third member of this family used in our phylogenetic study, Bettsia alvei, was located out the Onygenales, Arachnomycetales and Eurotiales, and in fact acted as outgroup in the tree. This agrees with Wynns et al. (2012), in which that species together with Eremascus fertilis (Eremascaceae, Coryneliales) formed a group separated from the clade made up by the species of Ascospheara. Our analysis demonstrated that several fungi phylogenetically related and morphologically similar to the Chrysosporium anamorph of Nannizziopsis vriesii (Fig. 9) constitute a new lineage within the Onygenales, clearly differentiated and phylogenetically distant from the members of the other families of the order. This lineage is considered a new family. This family includes the genus Nannizziosypsis, with the type species N. vriesii, C. guarroi, which is here included in the
genus *Nannizziopsis*, and four of the five new species described here. *Chrysosporium guarroi* was recently described by Abarca et al. (2010) based on several isolates that caused different cases of dermatomycosis in pet green iguanas in Spain (Fig. 8). During the course of our study we also identified four isolates of that species from snakes, iguanas and bearded dragons, and one from a human specimen. The human isolate differed from those infecting reptiles in some molecular and physiological features; i.e., 20 bp/1514 bp in the combined dataset analysis and it showed positive growth on SDA plus 3 % NaCl and milk solid hydrolysis. The conidia of *C. guarroi* are similar to those of *Chrysosporium* anamorph of *N. vriesii*, but in the latter they are usually sessile, while those in *C. guarroi* are mostly borne at the ends of narrow stalks. Other recently described species morphologically similar to *Nannizziopsis* spp. is *C. ophiiodiicola* (Fig. 10), which was isolated from a mycotic granuloma of a black rat snake. This species is distinguished by its narrow and cylindrical conidia, mostly on long stalks, and because it was neither able to split urea nor produce hemolysis. In our phylogenetic tree, the taxonomic position of this species was unresolved.

The genus *Nannizziopsis* was reviewed, although only on the basis of morphological criteria, by Guarro et al. (1991), and they accepted the species *N. albicans*, *N. hispanica* and *N. vriesii*. *Nannizziopsis mirabilis* (Uchiyama et al. 1995), *N. tropicalis* (Cano et al. 1997) and *N. patagonica* (Udagawa & Uchiyama 1999) were later described on the same criteria. With the exception of *N. hispanica*, all species of *Nannizziopsis* produce a chrysosporium-like anamorph. Unfortunately, with the exception of *N. albicans*, living cultures of these species are not available. More recently, in different phylogenetic studies, *N. albicans* was placed very far from the type strain of *N. vriesii*, being later accommodated in the genus *Amauroascus* (Vidal et al. 2000, Solé et al. 2002). These data are congruent with the ornamentation of the ascospores, which is considered a useful criterion in the taxonomy of the *Onygenales*. Although, the sexual morph of *N. vriesii* is rarely produced in culture, its ascospores have been described as echinulate (Apinis 1970, Guarro et al. 1991), while those of *Amauroascus* spp. are clearly reticulate, as are those of *N. albicans*. As we mentioned above, we could not include in our molecular analysis, apart from *N. vriesii*, the type strains of the other previously described species of *Nannizziopsis*; however, on the basis of the charac-
teristics reported in their descriptions, we can infer that probably they would be better accommodated in Amuroascus. The new species described here were phenotypically very similar. Nannizziopsis chlamydospora can be distinguished from the other species of the genus because it produces chlamydospores and grows at 5 °C. Nannizziopsis dracanoi can be differentiated from the other species by the combination of several features, i.e. the ability to grow on BCP-MS-G agar alkalining the medium, tolerance to 0.2 % cycloheximide, and the inability to grow on SDA with 3 % NaCl. Nannizziopsis arthrosporioides produces abundant long arthroconidia. Nannizziopsis pluriseptata produces from 1- to 5-celled sessile conidia, alkalizes the BCP-MS-G agar and grows on SDA supplemented with 5 % NaCl. These pluriseptate conidia have some resemblance to those of the dermatophytes Trichophyton erinacei, Trichophyton thuriniense and Trichophyton terrestre (family Arthrodertmataceae). However, the macroconidia of T. erinacei (20–50 × 5–7 μm), T. thuriniense (8–30 × 3–5 μm) and of T. terrestre (9–50 × 4–5 μm) are larger than those of N. pluriseptata (5–15 × 1.5–2.5 μm). Furthermore, N. pluriseptata presents terminal and lateral chains of arthroconidia, which are absent in Trichophyton spp. Chrysosporium longisporum is morphologically similar to the species of Nannizziopsis but it is characterized by producing long sessile conidia (up to 13 μm), and because is the only species unable to produce lipases.

The clinical isolate RKI 04-0104 and the type strain of N. vriesii only produced the Chrysosporium anamorph in culture, which is easily recognized by the production of very narrow sessile conidia (2–3 μm). Its teleomorph was only obtained in the original description of the species (Curreh 1985, Guarro et al. 1991). In our study, all the attempts to induce the formation of ascocoma on numerous media containing different sterile vegetable materials and horse hairs failed. The production of asperulate hyphae in culture, similar to those that constitute the ascomatal peridium, and considered typical of this species (Thomas et al. 2002), was also negative in our study. Although in the combined dataset tree, the clinical isolate of N. vriesii was separated from the type strain (IMI 149994) of this species, it was considered as belonging to that species. Both strains were morphologically very similar and the ACT and TUB sequences of the two strains were practically identical. Their separation in the phylogenetic tree was due to the presence of some differences (mainly insertions) in the ITS region.

Nannizziopsis is considered a primary pathogen causing dermal infections in different classes of reptiles, such as chameleons (Paré et al. 1997, 2006), crocodiles (Thomas et al. 2002), lizards (Martel et al. 2006, Mitchell et al. 2006, Bowman et al. 2007, Abarca et al. 2008, 2009, 2010, Han et al. 2010, Hedley et al. 2010, Hellebuyck et al. 2010, van Waeyenberghe et al. 2010, Johnson et al. 2011), and snakes (Nichols et al. 1999, Bertelsen et al. 2005, Rajeev et al. 2009, Eatwell 2010, Allender et al. 2011). The infections have consisted of single cases in pets or captive individuals but also in free-living animals, although different outbreaks in different species of reptiles have also been identified. The infection generally starts on the skin and progress rapidly involving subcutaneous soft tissues causing cutaneous ulcers and granulomas with infection of deeper tissues. Finally, the fungus can disseminate producing a fatal outcome. Cases involving these fungi have been reported in Australia, Belgium, Canada, Spain, UK and USA. Occasionally, Nannizziopsis spp. can infect humans causing severe lesions, as the case of lung infiltration and brain abscess described in a Nigerian man by the strain RKI 04-0104 included in this study (Steininger et al. 2005).

Various treatment options for these fungi include the use of itraconazole, ketoconazole or terbinafine combined with surgical debridement or amputation. The most promising treatment, however, appears to be voriconazole, which has demonstrated efficacy both in humans (Steininger et al. 2005) and in reptiles (Hellebuyck et al. 2010, van Waeyenberghe et al. 2010). Most of the infections caused by these fungi have been described in the last 10 yr, and is unclear if this could be attributed to recent climatic changes that could have affected the environment where these animals live or that previous infections had been overlooked or misidentified. It has been suggested that the different species of Nannizziopsis are associated with specific hosts (Bertelsen et al. 2005, Bowman et al. 2007). However, our study seems to not confirm this hypothesis, because, for example, N. guarroi infected lizards as well as snakes. Further studies utilizing more clinical isolates are required to more fully assess the host boundaries for these species.

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