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RESEARCH ARTICLE

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Phylogeny of chrysosporia infecting reptiles: proposal of the new family *Nannizziopsiaceae* and five new species

A.M. Stchigel[1], D.A. Sutton[2], J.F. Cano-Lira[1], F.J. Cabañes[3], L. Abarca[3], K. Tintelnot[4], B.L. Wickes[5], D. García[1], J. Guarro[1]

Key words

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Abstract We have performed a phenotypic and phylogenetic study of a set of fungi, mostly of veterinary origin, morphologically similar to the *Chrysosporium* asexual morph of *Nannizziopsis vriesii* (*Onygenales*, *Eurotiomycetidae*, *Eurotiomycetes*, *Ascomycota*). The analysis of sequences of the D1-D2 domains of the 28S rDNA, including representatives of the different families of the *Onygenales*, revealed that *N. vriesii* and relatives form a distinct lineage within that order, which is proposed as the new family *Nannizziopsiaceae*. The members of this family show the particular characteristic of causing skin infections in reptiles and producing hyaline, thin- and smooth-walled, small, mostly sessile 1-celled conidia and colonies with a pungent skunk-like odour. The phenotypic and multigene study results, based on ribosomal ITS region, actin and β-tubulin sequences, demonstrated that some of the fungi included in this study were different from the known species of *Nannizziopsis* and *Chrysosporium* and are described here as new. They are *N. chlamydospora*, *N. draconii*, *N. arthrosporioides*, *N. pluriseptata* and *Chrysosporium longisporum*. *Nannizziopsis chlamydospora* is distinguished by producing chlamydospores and by its ability to grow at 5 °C. *Nannizziopsis draconii* is able to grow on bromocresol purple-milk solids-glucose (BCP-MS-G) agar alkalinizing the medium, is resistant to 0.2 % cycloheximide but does not grow on Sabouraud dextrose agar (SDA) with 3 % NaCl. *Nannizziopsis arthrosporioides* is characterised by the production of very long arthroconidia. *Nannizziopsis pluriseptata* produces 1- to 5-celled sessile conidia, alkalinizes the BCP-MS-G agar and grows on SDA supplemented with 5 % NaCl. *Chrysosporium longisporum* shows long sessile conidia (up to 13 μm) and does not produce lipase.

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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 rhexolytically (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 *Geomyces*, *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 *Gymnoascaceae* (*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 teleomorphs belonging to very diverse genera in the families *Arthrodermataceae* (Currah 1985), *Ascosphaeriaceae* (van Oorschot 1980), *Chaetomiaceae* (Vidal et al. 2000), *Gymnoascaceae* (van Oorschot 1980, Currah 1985), *Lasiosphaeriaceae* (Mouchacca & Gams 1993, Ueda 1994), *Onygenaceae* (van Oorschot 1980, Currah 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, Roilides 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, Hellebuyck 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

[1] Mycology Unit, University Rovira i Virgili and IISPV, C/ Sant Llorenç 21, 43201 Reus, Spain; corresponding author e-mail: jose.cano@urv.cat.
[2] University of Texas Health Science Center, Department of Pathology, San Antonio, USA.
[3] Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.
[4] Robert Koch Institute, FG16 Mycology, Berlin, Germany.
[5] Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas, USA.

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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.

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.

Table 1 Fungi included in this study.

Species[1]		Origin	GenBank accession no.			
			D1-D2	ACT	ITS	TUB
Nannizziopsis vriesii	RKI 04-0104	Human, brain abscess, Nigerian man, Germany	HF547853	HF547877	HF547869	HF547878
Nannizziopsis chlamydospora (*Chrysosporium* sp. 1)	UTHSC 04-2056	*Pogona vitticeps*, USA	HF547854	HF547879	HF547870	HF547880
Nannizziopsis chlamydospora (*Chrysosporium* sp. 1)	UTHSC 06-1419	*Pogona vitticeps*, USA	HF547855	HF547881	HF547871	HF547882
Nannizziopsis draconii (*Chrysosporium* sp. 2)	CCFVB CH12	*Pogona vitticeps*, Spain	HF547856	HF547883	EU883993*	HF547884
Nannizziopsis arthrosporioides (*Chrysosporium* sp. 3)	UTHSC R-4263	*Physignathus* sp. (water dragon), USA	HF547857	HF547885	HF547872	HF547886
Chrysosporium longisporum (*Chrysosporium* sp. 4)	UTHSC R-4380	Snake, multifocal dermatitis, USA	HF547858	HF547887	HF547873	HF547888
Nannizziopsis pluriseptata (*Chrysosporium* sp. 5)	UTHSC 10-1045	Skink lizard, USA	HF547859	HF547889	HF547874	HF547890
Chrysosporium ophiodiicola	CBS 122913[T]	Snake, subcutaneous granuloma, USA	EU15820*	HF547891	EU15819*	HF547892
Nannizziopsis vriesii	IMI 149994[T]	*Ameiva* sp., skin and lungs, USA	AY176715*	HF547893	AJ131687*	HF547894
Nannizziopsis guarroi	CBS 124553[T]	*Iguana iguana*, Spain	FJ839684*	HF547895	EU018451*	HF547896
	CCFVB CH11	*Iguana iguana*, Spain	HF547860			
	CCFVB CH14	*Iguana iguana*, Spain	HF547861			
	CCFVB CH15	*Iguana iguana*, Spain	HF547862			
	CCFVB CH16	*Iguana iguana*, Spain	HF547863			
	UTHSC R-4309	Snake, USA	HF547864			
	UTHSC 05-1370	*Pogona vitticeps* , USA	HF547865			
	UTHSC 06-3993	*Agama agama* , USA	HF547866	HF547897	HF547875	HF547898
	UTHSC 07-3227	*Pogona vitticeps*, USA	HF547867			
	UTHSC R-4317	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	MUCL 10068[T]	Woolen overcoat, Solomon Islands	AY176731*			
Aphanoascus mephitalis	ATCC 22144[T]	Wolf dung, Canada	AY176725*			
Chrysosporium keratinophilum	CBS 392.67	Soil, New Zealand	AY176730*			
Arthroderma cajetani	OMH H1-10	Human, Canada	AY176736*			
Arthroderma otae	UAMH 2338	Human, skin scrapings and hair, Canada	AY176735*			
Arthroderma ciferrii	ATCC 24447[T]	Soil, USA	EF413625*			
Ctenomyces serratus	CBS 187.61[T]	Soil, Australia	AY176733*			
Chrysosporium vallenarense	UAMH 6914	Dung of *Alopex lagopus*, Chile	AY176732*			
Gymnoascus littoralis	CBS 454.73	Conch shell, Canada	FJ358272*			
Gymnascus aurantiacus	ATCC 22394[T]	Soil, Russia	AY176747*			
Gymnascus ruber	CBS 352.90	Soil, England	AY176746*			
Ascosphaera subglobosa	Voucher A.A. Wynns 5004(C)	*Megachile rotundata*, USA	HQ540517*			
Ascosphaera apis	CBS 252.32	*Apis mellifica*, Denmark	AY004344*			
Paracoccidioides brasiliensis	IMTSP 556	Human, Brazil	U81263*			
Ajellomyces dermatitidis	ATCC 18187[T]	Human, USA	AY176704*			
Ajellomyces capsulatus	UAMH 7141	Soil, USA	AF038353*			
Byssochlamys nivea	CBS 100.11[T]	*Geastrum coronatum*, Sweden	AY176750*			
Eurotium herbariorum	ATCC 16469	Unpainted board, USA	AY176751*			
Petromyces alliaceus	ATCC 16891	Soil, Australia	AY176752*			
Arachnomyces nodosetosus	CCF 3957	Human, nail infection, Czech Republic	HM205103*			
Arachnomyces glareosus	CBS 116129	Human, thumb nail, Canada	FJ358273*			
Arachnomyces 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*			
Bettsia alvei	KVL 10-08	Pollen, Denmark	HQ540516*			

[T] Ex-type strain.
* sequences retrieved from the GenBank database.
[1] ATCC: American Type Culture Collection, USA; CBS: 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, Bellaterra, 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; MUCL: 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.

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 *N. vriesii* and *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 in Table 1. Phylogenetic analysis of the D1-D2 encompassed representatives of the clinical isolates and reference strains of the families within the order *Onygenales* (*Ajellomycetaceae*, *Arachnomycetaceae*, *Arthrodermataceae*, *Ascosphaeraceae*, *Gymnoascaceae* and *Onygenaceae*) as well as some representatives of *Eurotiales*. *Eremascus fertilis* (HQ540515) and *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 *C. guarroi*, *C. ophiodiicola* and *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 Kornerup & 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 Slifkin (2000), incubating the Petri dishes at 30 °C for 14 d. Growth on dermatophyte test medium (DTM) and colour 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-MS-G) 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 *Chrysosporium* represented in GenBank, with the exception of eight reptilian isolates that showed 100 % identity with the type strain of *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 *Eurotiales* (97 % bs/1 pp), *Arachnomycetales* (97 % bs/1 pp) and *Onygenales* (72 % bs/1 pp), respectively. The *Onygenales* encompassed five well-supported clades, corresponding to the families *Ascosphaeraceae* (99/1), *Gymnoascaceae* (99/1), *Arthrodermataceae* (96/1) and *Onygenaceae* (90/1), and the fifth one (99/1) that embraced the type strains of *C. guarroi* and *N. vriesii*, and most of our clinical isolates, and a poorly-supported group (66/-) that included some members of the family *Ajellomycetacaeae*. This analysis did not resolve the taxonomic position of the type strain of *C. ophiodiicola* and of the clinical isolate UTHSC R-4380 (provisionally named *Chrysosporium* sp. 4), which consisted of two different branches between the *Onygenaceae* and the *Nannizziopsis* group.

In the combined ITS-ACT-TUB ML tree (Fig. 2) several terminal well-supported branches representing undescribed species were shown. These were *Chrysosporium* sp. 1, that included the isolates UTHSC 04-2056 and UTHSC 06-1419, *Chrysosporium* sp. 2 (isolate CCFVB CH12), *Chrysosporium* sp. 3 (isolate UTHSC R-4263) and *Chrysosporium* sp. 5 (isolate UTHSC 10-1045). Additionally, three strains of *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 *N. vriesii*, and a human clinical strain, morphologically identified as *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 *C. ophiodiicola* (CBS 122913) and the isolate UTHSC R-4380 (*Chrysosporium* sp. 4) were placed away from the others and in fact acted as outgroups.

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 chlamydospores was *Chrysosporium* sp. 1 (Fig. 3f, g). Single intercalary conidia were formed by all the isolates, with those

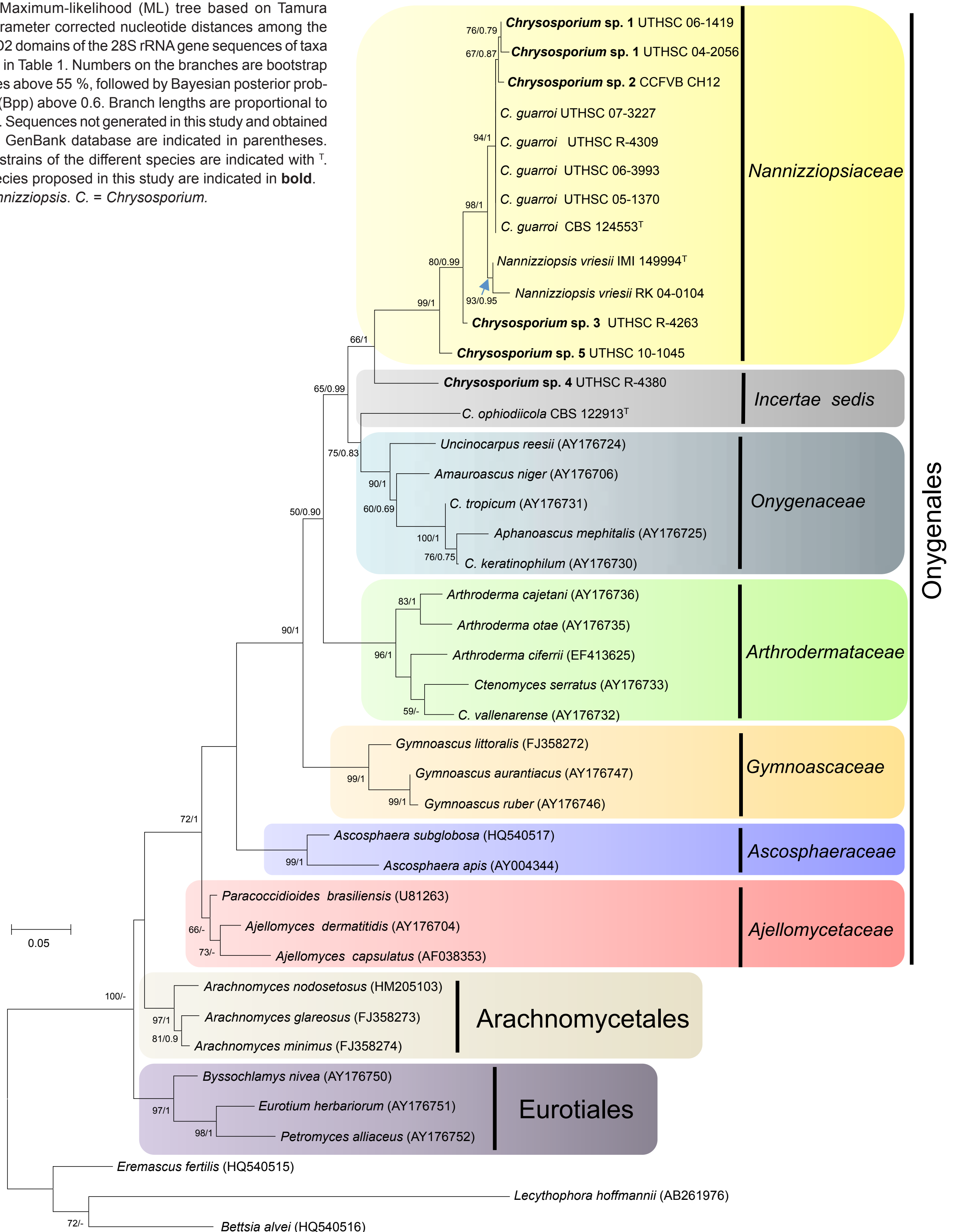

Fig. 1 Maximum-likelihood (ML) tree based on Tamura three-parameter corrected nucleotide distances among the D1 and D2 domains of the 28S rRNA gene sequences of taxa included in Table 1. Numbers on the branches are bootstrap ML values above 55 %, followed by Bayesian posterior probabilities (Bpp) above 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. ophiodiicola*, all

Table 2 Key physiological features of fungi included in this study.

Fungi	BCP-MS-G agar			Hemolysis (on blood agar)	Lipase	SDA plus 3 % NaCl	SDA plus 5 % NaCl	Cycloheximide tolerance (SDA plus 0.2 %)	Growth on PYE at 15 °C	Growth on PYE at 40 °C
	Milk solids hydrolysis	pH reaction								
		Acidification	Alkalisation							
Nannizziopsis vriesii IMI 149994[T]	–	+	–	+	+	+	(+)	+	–	–
Nannizziopsis vriesii RKI 04-0104	(+)	+	–	+	+	+	(+)	–	+	+
Nannizziopsis guarroi CBS 124553[T]	–	–	+	+	+	–	–	+	+	–
Nannizziopsis guarroi UTHSC R-4309	–	–	+	+	+	–	–	+	+	–
Nannizziopsis guarroi UTHSC 05-1370	–	–	+	+	+	–	–	+	+	–
Nannizziopsis guarroi UTHSC R-4262	–	–	+	+	+	–	–	+	+	–
Nannizziopsis guarroi UTHSC R-4317	(+)	–	+	+	+	+	–	+	+	+
Chrysosporium ophiodiicola CBS 122913[T]	(+)	+	–	–	+	+	(+)	+	+	–
Nannizziopsis chlamydospora UTHSC 04-2056 (*Chrysosporium* sp. 1)	(+)	–	+	+	+	+	–	+	+	+
Nannizziopsis chlamydospora UTHSC 06-1419 (*Chrysosporium* sp. 1)	(+)	–	+	+	+	+	–	+	+	+
Nannizziopsis draconii CCFVB CH12 (*Chrysosporium* sp. 2)	+	–	+	+	+	–	–	+	+	–
Nannizziopsis arthrosporioides UTHSC R-4263 (*Chrysosporium* sp. 3)	(+)	+	–	+	+	+	(+)	+	+	+
Chrysosporium longisporum UTHSC R-4380 (*Chrysosporium* sp. 4)	–	+	–	+	–	+	(+)	+	+	–
Nannizziopsis pluriseptata UTHSC 10-1045 (*Chrysosporium* sp. 5)	+	–	+	+	+	+	+	+	+	+

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

the strains included in the study produced hemolysis on blood agar (Fig. 3a–9a). All of them, with the exception of *Chrysosporium* sp. 4, showed lipolytic activity (Fig. 3c–6c, 8c, 9c). The isolates were grown on BCP-MS-G agar to test the acidification or alkalisation of this medium, and the milk solids hydrolysis. *Nannizziopsis vriesii*, *C. ophiodiicola*, *Chrysosporium* sp. 3 and *Chrysosporium* sp. 4 acidified the medium, whereas *C. guarroi*, *Chrysosporium* sp. 1, *Chrysosporium* sp. 2 and *Chrysosporium* sp. 5 produced alkalisation, and only *Chrysosporium* sp. 2 and *Chrysosporium* sp. 5 showed a strong hydrolysis of milk solids. On SDA medium with 3 % NaCl, *Chrysosporium* sp. 2 did not grow and nor did four isolates of *C. guarroi*. On the same medium with 5 % NaCl, growth was scarce for all these isolates, except for *Chrysosporium* sp. 5 that showed good growth.

The combination of both phenotypic and molecular results demonstrated that five *Chrysosporium* species (*Chrysosporium* sp. 1, *Chrysosporium* sp. 2, *Chrysosporium* sp. 3, *Chrysosporium* sp. 4 and *Chrysosporium* sp.5) represent new species. The type species of the genus *Chrysosporium*, *C. merdarium*, is phylogenetically unrelated to *N. vriesii* (Vidal et al. 2000), and phenotypically very different; it is not keratinolytic, produces very variable colonies (yellow, pink or green), and sparsely echinulate, subglobose conidia. Therefore, we propose to accomodate the species *Chrysosporium* sp. 1, *Chrysosporium* sp. 2, *Chrysosporium* sp. 3, and *Chrysosporium* sp. 5, which in the combined tree were placed in the *Nannizziopsis* clade, in the genus *Nannizziopsis*, while *Chrysosporium* sp. 4 is described here as a new species of *Chrysosporium.*

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

Etymology. From Greek *chlamydos*-, 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, straight or twisted, 1–3(–4) µm wide. *Conidia* unicellular, 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. Fetid (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* dermal lesion, holotype CBS H-21115, cultures ex-type CBS 133985, UTHSC 04-2056, FMR 10835; ex *Pogona vitticeps* dermal lesion, UTHSC 06-1419.

Nannizziopsis draconii J. Cabañes, 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. 1A2), felted, slightly elevated at centre, with regular margin; reverse yellowish white (M. 2A2) to pale yellow at centre (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,

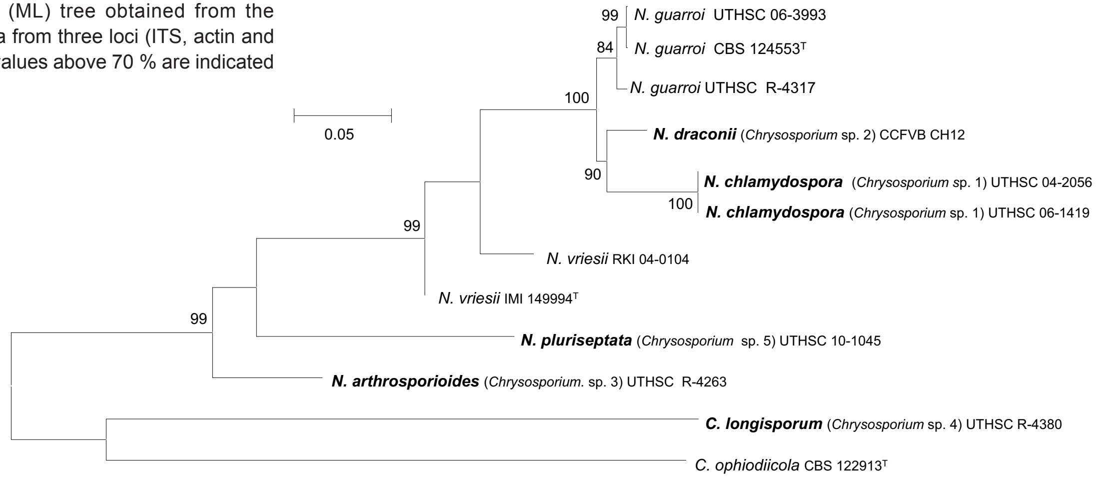

Fig. 2 Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, actin and β-tubulin). Bootstrap support values above 70 % are indicated at the nodes.

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).

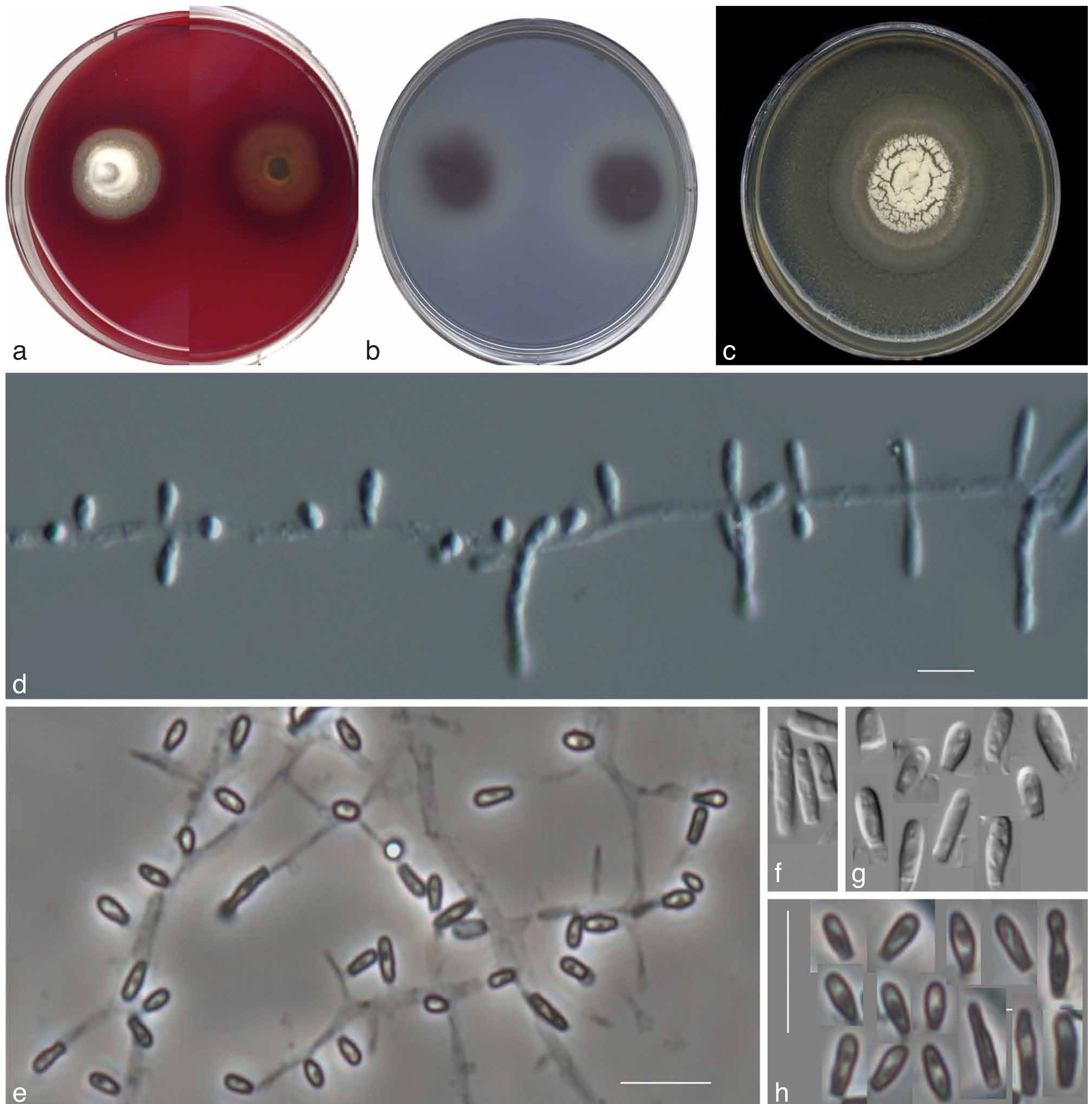

Fig. 4 *Nannizziopsis draconii* CCFVB CH12 (= *Chrysosporium* sp. 2). a. Colony on blood agar (surface and reverse); b. colonies on BCP-MS-G (reverse); c. colony on TOTM; d, e. conidiophores bearing sessile and terminal conidia; f. arthroconidia; g, h. sessile and terminal conidia. — Scale bars = 10 µm (d, f, g, differential interference contrast; e, h, phase contrast).

or terminal, hyaline, thin- and smooth-walled, claviform or cylindrical, 4–7 × 1.5–2(–2.5) µm; intercalary conidia scarce, cylindrical, 4–9 × 1.5–2 µm; arthroconidia catenate, mostly cylindrical 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. *Chlamydospores* 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.

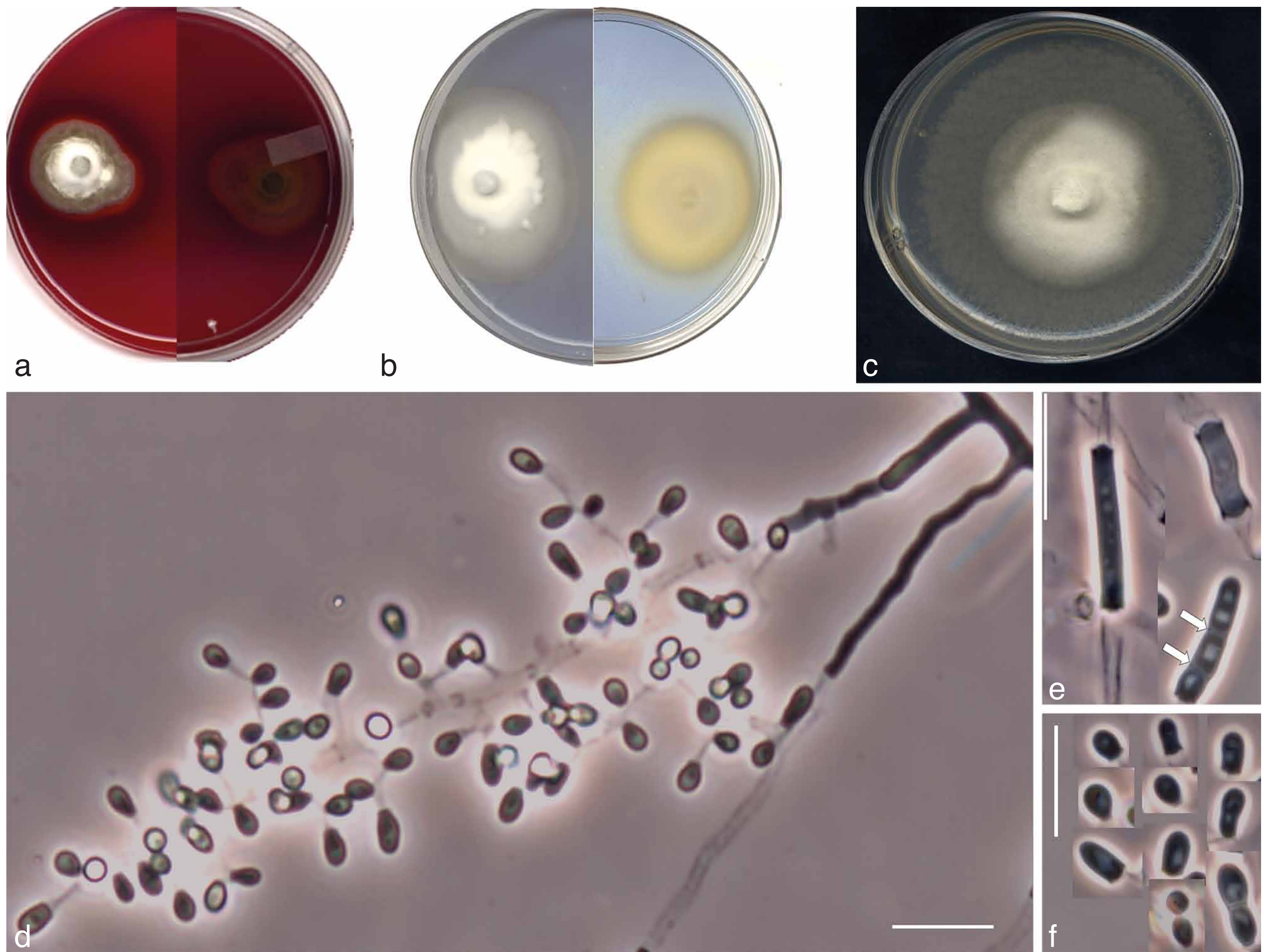

Fig. 5 *Nannizziopsis arthrosporioides* UTHSC R-4263 (= *Chrysosporium* sp. 3). a. Colony on blood agar (surface and reverse); b. colony on BCP-MS-G (surface and reverse); c. colony on TOTM; d. fertile hyphae bearing sessile conidia; e. two singly intercalary conidia and a terminal chain of arthroconidia (white arrows show the septa); f. sessile (some 2-celled) conidia. — Scale bars = 10 µm (d–f, differential interference contrast).

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 32–36 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 (*Erpeton tentaculatum* Lacépède), holotype CBS H-21139, cultures ex-type CBS 133990, UTHSC R-4380, FMR 10617.

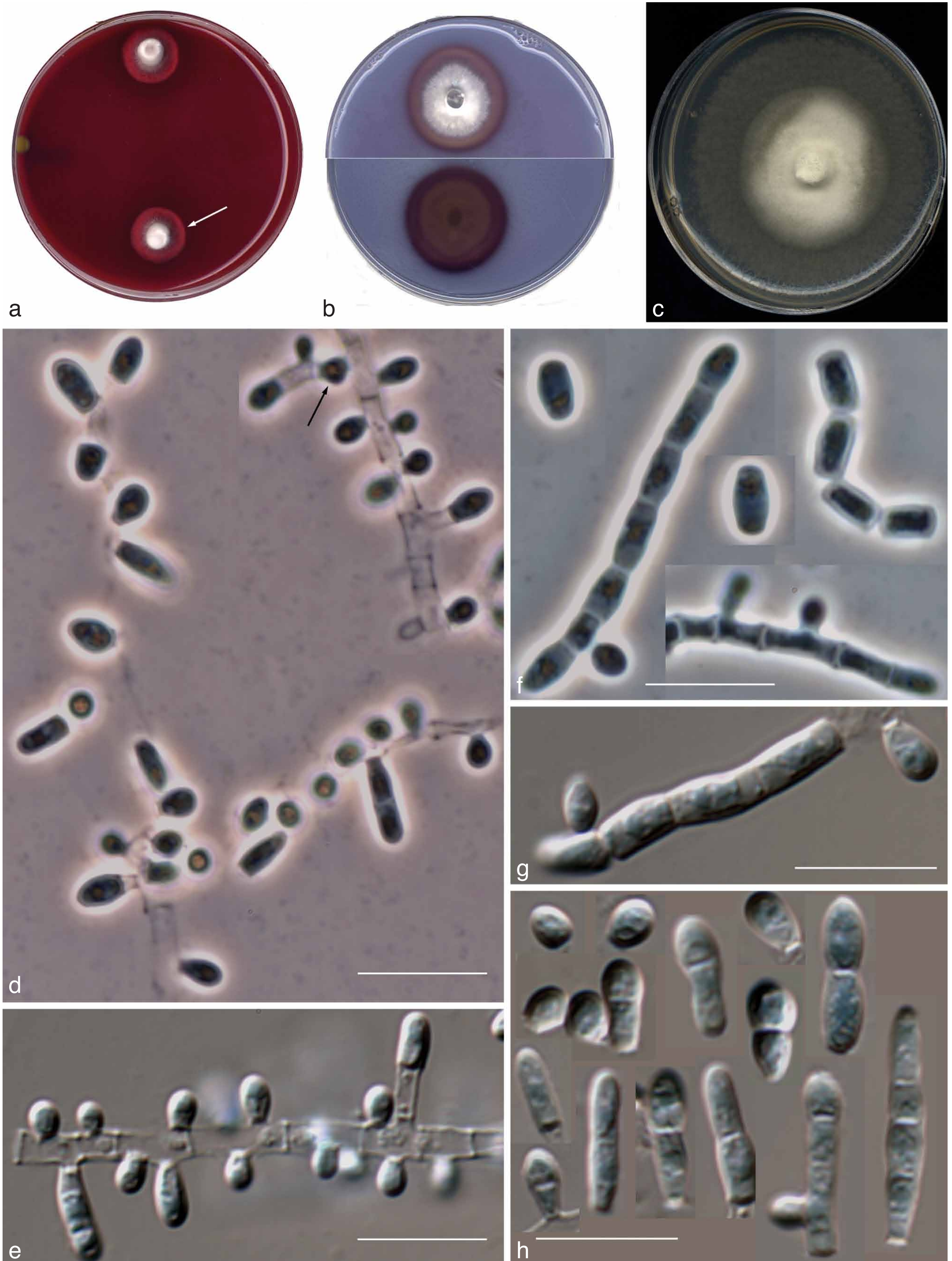

Fig. 6 *Nannizziopsis pluriseptata* UTHSC 10-1045 (= *Chrysosporium* sp. 5). a. Colonies on blood agar (the arrow shows the b-hemolysis halus); b. colony on BCP-MS-G (surface and reverse); c. colony on TOTM; d, e. fertile hyphae bearing mostly sessile conidia (arrow showing a intercalary conidium); f, 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).

Fig. 7 *Chrysosporium longisporum* UTHSC R-4380 (= *Chrysosporium* sp. 4). a. Colony on blood agar (surface and reverse); b. colony on BCP-MS-G (surface and reverse); c. colony on TOTM; d. fertile hyphae bearing mostly sessile conidia; e. sessile and intercalary (arrow) conidia. — Scale bars = 10 μm (d, e, phase contrast).

Nannizziopsis guarroi (J.Cabañes & Abarca) J.Cabañes, Abarca, Guarro, Stchigel & Cano, *comb. nov.* — MycoBank MB801991; Fig. 8a–g

Basionym. *Chrysosporium guarroi* J.Cabañes & 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, spherical, whitish, with a peridium composed of a network of loosely interwoven, verrucose, hyaline hyphae which are constricted at the septa. *Asci* 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,

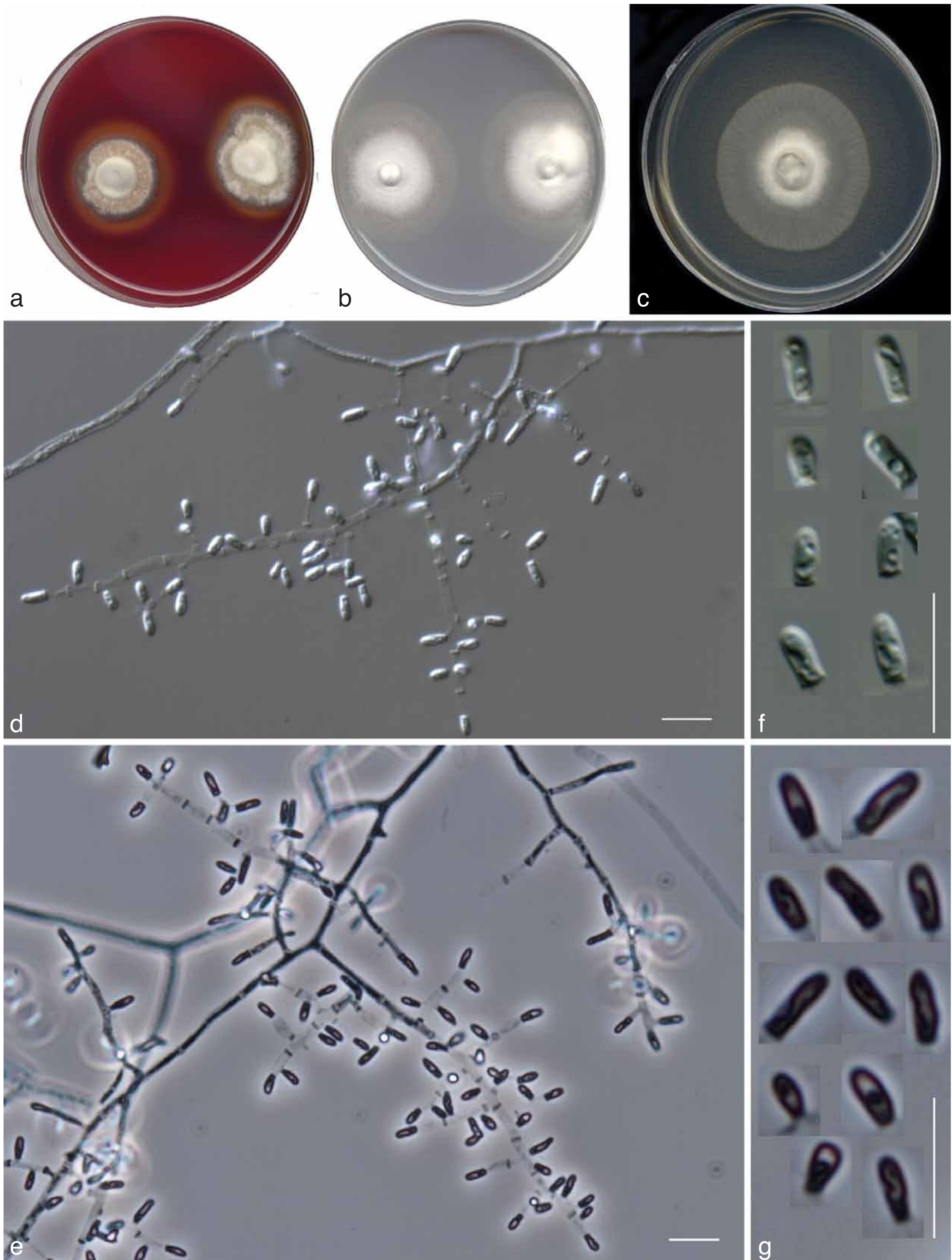

Fig. 8 *Nannizziopsis guarroi* CBS 124553. a. Colonies on blood agar; b. colonies on BCP-MS-G; c. colony on TOTM; d, e. fertile hyphae bearing sessile conidia; f, g. sessile conidia. — Scale bars = 10 µm (d, f, differential interference contrast; e, g, phase contrast).

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

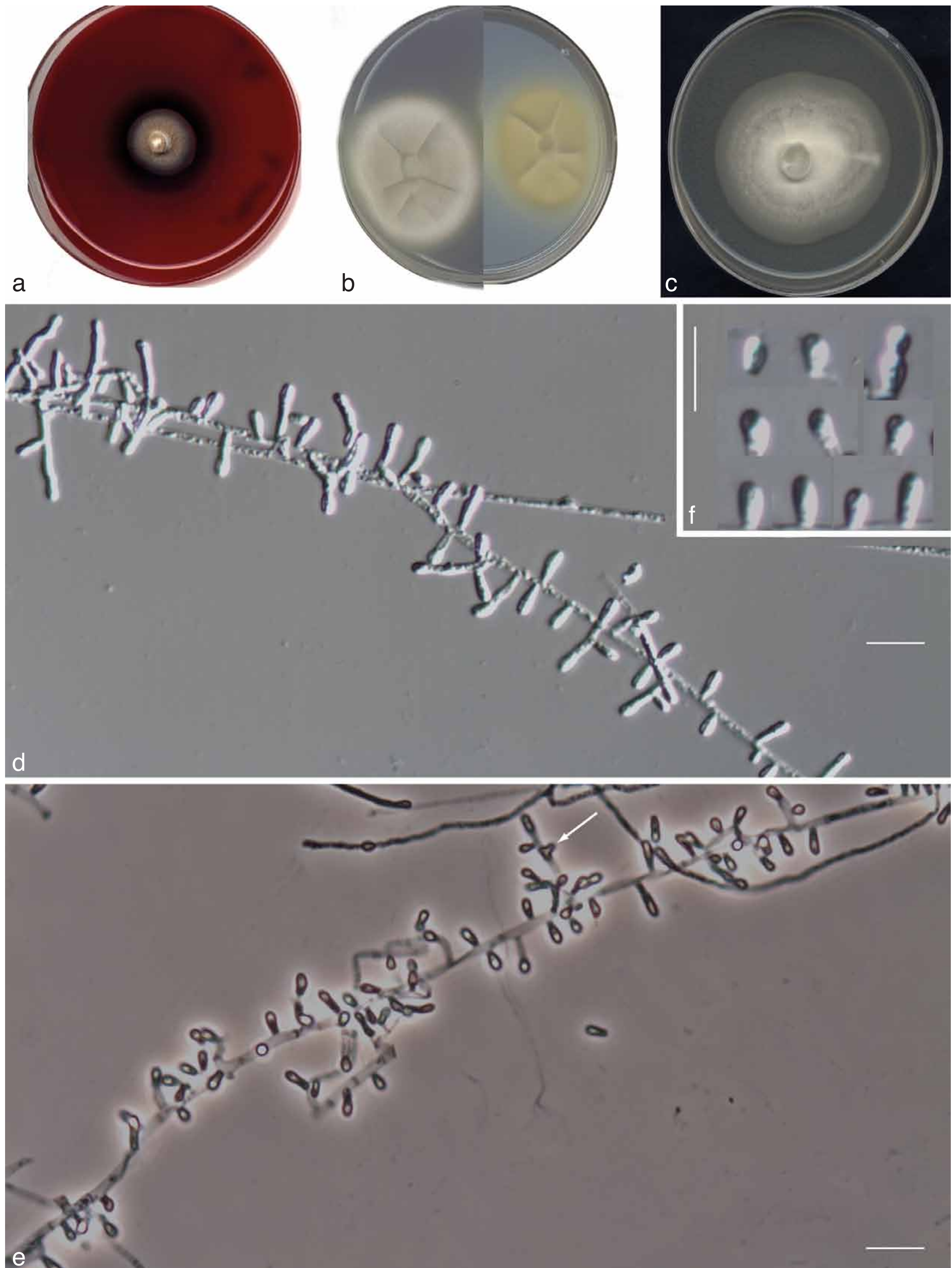

Fig. 9 *Chrysosporium* anamorph of *Nannizziopsis vriesii* IMI 149994. a. Colony on blood agar; b. colony on BCP-MS-G (surface and reverse); c. colony on TOTM; d, e. fertile hyphae bearing mostly sessile conidia (arrow shows an intercalary conidium); f. sessile conidia. — Scale bars = 10 µm (d, f, differential interference contrast; e, phase contrast).

of *Ascosphaera* (*Ascosphaerales*), i.e., *A. apis* and *A. subglobosa*, 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 *Ascosphaera*. 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 *Nannizziopsis*, with the type species *N. vriesii*, *C. guarroi*, which is here included in the

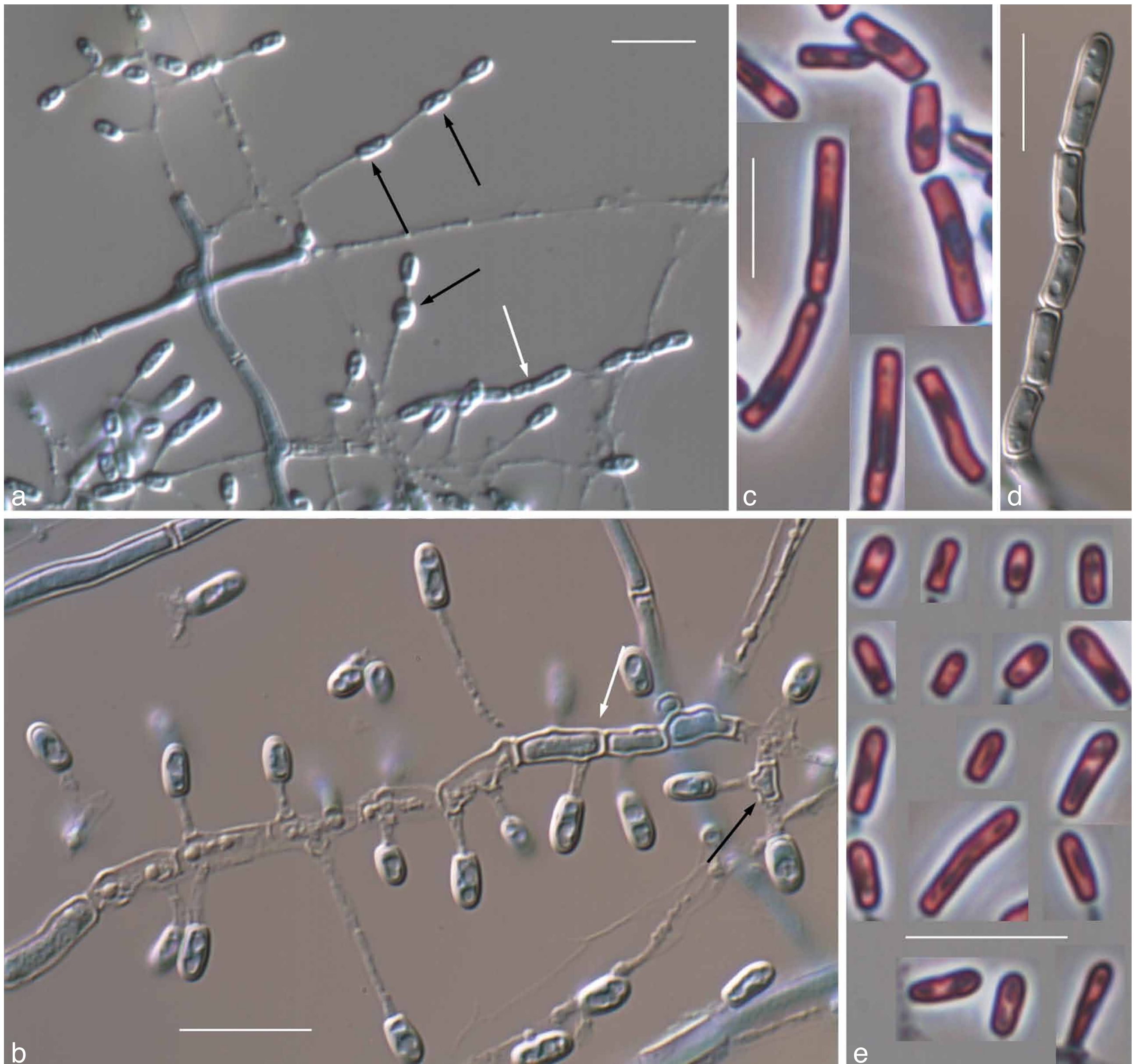

Fig. 10 *Chrysosporium ophiodiicola* CBS 122913. a, b. Fertile hyphae bearing sessile conidia, intercalary conidia (black arrows) and intercalary chains of arthroconidia (white arrows); c, d. arthroconidia; e. sessile conidia. — Scale bars = 10 µm (a, b, d, differential interference contrast; c, e, phase contrast).

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. ophiodiicola* (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 *Amauroascus*.

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 draconii* can be differentiated from the other species by the combination of several features, i.e. the ability to grow on BCP-MS-G agar alkalinizing 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, alkalinizes 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 thuringiense* and *Trichophyton terrestre* (family *Arthrodermataceae*). However, the macroconidia of *T. erinacei* (20–50 × 5–7 µm), *T. thuringiense* (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 (Currah 1985, Guarro et al. 1991). In our study, all the attempts to induce the formation of ascomata 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 utilising more clinical isolates are required to more fully assess the host boundaries for these species.

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