Phytotaxa 334 (2): 183–194
http://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.334.2.7

Genetic diversity of the genus *Terfezia* (Pezizaceae, Pezizales): New species and new record from North Africa

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Abstract

Morphological and phylogenetic analyses of large ribosomal subunit (28S rDNA) and internal transcribed spacer (ITS rDNA) of *Terfezia* samples collected from several bioclimatic zones in Algeria and Spain revealed the presence of six distinct *Terfezia* species: *T. arenaria*, *T. boudieri, T. claveryi; T. eliocrocae* (reported here for the first time from North Africa), *T. olbiensis*, and a new species, *T. crassiverrucosa* sp. nov., proposed and described here, characterized by its phylogenetic position and unique combination of morphological characters. A discussion on the unresolved problems in the taxonomy of the spiny-spored *Terfezia* species is conducted after the present results.

Key words: desert truffles, *Pezizaceae,* phylogeny, taxonomy

Introduction

The genus *Terfezia* (Tul. & C.Tul.) Tul. & C. Tul. produce edible hypogeous ascomata growing mostly in arid and semi-arid ecosystems, although they can be found also in a wide range of habitats, such as temperate deciduous forests, conifer forests, prairies, or even heath lands (Moreno *et al.* 2014). The fructifications of these mushrooms are very appreciated in several Middle-Eastern, North-African and Mediterranean countries where they have a prominent place in the local diet and traditional medicine of native people and Bedouins (Alsheikh & Trappe 1983, Mandeel & Al-Laith 2007). Desert truffles establish mycorrhizal symbioses with specific host plants, with a remarkable preference for the *Cistaceae* family, and especially the genus *Helianthemum* spp. However, some species are often harvested under oaks and pine trees (Alsheikh 1994, Díez *et al.* 2002, Chevalier 2014). The species of *Terfezia* are exceptional in terms of mycorrhizae structural plasticity, as they are able to produce sheeting ectomycorrhizae, endomycorrhizae or even ectendomycorrhizae with *Cistaceae* hosts and Aleppo pine (Fortas & Chevalier 1992, Zitouni-Haouar *et al.* 2014).

The nomenclatural and taxonomic history of *Terfezia* is characterized by a great number of old species names lacking useful diagnostic features, most of them rarely cited after the first time (Tulasne and Tulasne 1851, Chatin 1891, 1892, 1893, 1895, 1896, 1897, Mattirolo 1887, 1900, 1906, 1922, Fischer 1897, 1938, Harkness 1899, Imai 1933, Gilkey 1947, Trappe 1971, 1979, Korf 1973, Marasas & Trappe 1973, Alsheikh 1994). The revisions made by molecular phylogeny highlighted several misidentification at the genus and species level and decreased considerably the *Terfezia* richness. Reexamination and molecular phylogenetic analyses of some herbarium specimens and personal collections from several countries around the world revealed their erroneous generic assignments and removed the current ambiguity around their taxonomic status. These molecular revisions increased also the geographic uniformity of the genus *Terfezia;* only species from the Mediterranean region and the Middle East being proven to belong to the *Terfezia* s.str. (Kovács *et al.* 2011b, Kovács & Trappe 2014). The phylogenetic analyses of Percudani *et al.* (1999) and Norman & Egger (1999) significantly supported the restoration of *Terfezia terfezioides* (Mattir.) Trappe as *Mattirolomyces terfezioides* (Mattir.). The South-African *Terfezia* species, *T. pfeilii* Henn. and *T. austroafricana* Marasas & Trappe were combined as *Kalaharituber pfeilii* (Henn.) Trappe & Kagan-Zur and *Mattirolomyces austroafricanus*

Accepted by Wen-Ying Zhuang: 8 Jan. 2018; published: 16 Jan. 2018

(Marasas & Trappe) Kovács, Trappe & Claridge (Ferdman *et al.* 2005, Trappe *et al.* 2010a, 2010b). Genus *Terfezia* was shown to be absent from the American mycoflora after the four known American species *T. longii* Gilkey, *T. spinosa* Harkn., *"T. mexicana"* and *T. gigantea* Imai were checked by molecular phylogenetic analysis. These taxa were shown to deserve a different genus and combined or redescribed as *Stouffera longii* (Gilkey) Kovács & Trappe, *Mattirolomyces spinosus* (Harkn.) Kovács, Trappe & Alsheikh, *M. mexicanus* Kovács, Trappe & Alsheikh and *Imaia gigantea* (S. Imai) Trappe & Kovács, respectively (Kovács *et al.* 2008, Kovács *et al.* 2011a). Phylogenetic analysis of Spanish *Terfezia* collections have allowed the discovery and description of eight new *Terfezia* species from the Iberian Peninsula: *T. alsheikhii* Kovács, M.P. Martín & Calonge (Kovács *et al.* 2011b), *T. albida* Ant. Rodr., Mohedano & Bordallo, *T. eliocrocae* Bordallo, A. Morte & Honrubia, *T. extremadurensis* Mohedano, Ant. Rodr. & Bordallo, *T. pini* Bordallo, Ant. Rodr. & Mohedano, *T. pseudoleptoderma* Bordallo, Ant. Rodr. & Mohedano (Bordallo *et al.* 2013), *T. grisea* Bordallo, Kaounas & Ant. Rodr., *T. cistophila* Ant. Rodr., Bordallo, Kaounas & A. Morte (Bordallo *et al.* 2015), and one from the Canary Islands, *T. canariensis* Bordallo & Ant. Rodr. (Bordallo *et al.* 2012). Among the five most common *Terfezia* species in the Mediterranean region and the Middle East (Alsheikh 1994), *T. arenaria* (Moris) Trappe has the lowest intraspecific variability (Kovács *et al.* 2011b). In contrast, considerable genetic variation was reported in *T. boudieri* Chatin (Aviram *et al.* 2004, Ferdman *et al.* 2009, Sbissi *et al.* 2011), and *T. claveryi* Chatin (Sbissi *et al.* 2011). A high intraspecific and intrasporocarpic ITS variability was detected also among Spanish collections of *T. leptoderma* Tul. and *T. olbiensis* Tul. & C.Tul. (Kovács *et al.* 2011b). Thus, species boundaries in these groups are not fully clear yet (Alsheikh 1994, Díez *et al.* 2002, Kovács *et al.* 2011b).

The main purpose of the present study was to characterize the diversity of *Terfezia* in North Africa. Several collections of *Terfezia* ascomata harvested throughout Algeria, and two newly collected samples from Spain were analyzed. Macro- and micromorphological studies, as well as a phylogenetic analysis using ITS and 28S rDNA sequences were conducted.

Material and methods

Fungal samples

The specimens studied in this work were collected from a wide variety of habitats in Algeria and Spain. These regions belong to humid, semi-arid, arid and Saharan bioclimatic zones (Pouget 1980; Rivas Martínez *et al.* 2011), all of them characterized by xerophilous vegetation dominated by *Helianthemum* species. Sites of collection and ecological data of samples used in this study are listed in Table 1.

Morphological studies

Macromorphological characteristics of peridium and gleba were recorded from fresh specimens. Microscopic studies of peridium were conducted on rehydrated sections cut from dried specimens. Observations were performed in distilled water, 5% KOH, Melzer's reagent and cotton blue-lactophenol. Ascospore dimensions were measured in distilled water mounts on at least 50 randomly selected mature spores. Cell, asci and ascospores shape and sizes were determined using an Olympus CX22 microscope equipped with an ocular micrometer. Ascospore ornamentation was examined and photographed with a scanning electron microscopy (SEM) Zeiss DSM-950 instrument, at the University of Alcalá (Spain). Critical point drying technique was performed on the samples prior to mounting, following Moreno *et al.* (1995). Samples representing new species or new record of *Terfezia* were deposited in the Herbarium AH (University of Alcalá de Henares, Spain) under voucher specimens numbers indicated in the Table 1.

DNA extraction, PCR amplification and sequencing

DNA was extracted from approximately 25-40 mg of dried tissues. Ascomata were superficially disinfected and clean glebal tissues were removed from the inner part of ascocarps to be ground into a fine powder in liquid nitrogen. The total genomic DNA was extracted using GF-1 Plant DNA Extraction Kit (Vivantis Technologies, USA) following the manufacturer's instructions. Extracts were eluted in 50 µl of Elution Buffer supplied in the kit and stored at -20°C. DNA concentration was estimated using a Nano-Drop spectrophotometer (Thermo Scientific). The polymerase chain reaction (PCR) targeting ITS1-5.8S-ITS2 region of the rDNA was carried out using primers ITS1 and ITS4 (White *et al.* 1990). The large ribosomal subunit (28S rDNA) was amplified using the primers pair LR0R-LR5 (Vilgalys & Hester 1990, Cubeta *et al.* 1991). PCR amplifications were performed as described by Zitouni-Haouar *et al.* (2015). Amplification products were loaded in 1% agarose gels stained with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) and visualized with a UV transilluminator. PCR products were purified using the UltraClean PCR clean-up kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. Sequencing reactions were performed with one or more PCR primers using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the electrophoresis was carried out on ABI PRISM 3130XL Genetic Analyser (Applied Biosystems) at the Unit of Biochemistry and Molecular Biology of the University of Alcalá de Henares (Spain). Sequences generated in this study were deposited in GenBank under accession numbers reported in Table 1.

TABLE 1. Collection information of *Terfezia* specimen and sequences generated in this study.

Taxon	**Coll. N°**	**Origin, collection date**	**Host plant**	**Bioclimatic zone**	**GenBank accession numbers**	
					ITS	**LSU**
T. arenaria	LBMB 18	Mridima, El-Taref, Algeria, March 2013	*Helianthemum guttatum*	Humid	MF940175	MF940205
T. arenaria	LBMB 20	Hammamet, Tebessa, Algeria, April 2011	*H. guttatum*	Superior arid	MF940176	MF940206
T. arenaria	LBMB 22	Hammamet, Tebessa, Algeria, April 2011	*H. guttatum*	Superior arid	MF940177	MF940207
T. boudieri	LBMB 19	Oued Daoura, Bechar, Algeria, April 2012	*H. lippii*	Saharan	MF940178	-
T. boudieri	LBMB 25	Oued Daoura, Bechar, Algeria, April 2012	*H. lippii*	Saharan	MF940179	MF940208
T. boudieri	LBMB 32	Ain Sefra, Naama, Algeria, April 2012	*Helianthemum* sp.	Inferior arid	-	MF940209
T. boudieri	LBMB 36	Bou Saada, Msila, Algeria, May 2012	*Helianthemum* sp.	Medium arid	MF940180	MF940210
T. boudieri	LBMB 38	Bou Saada, Msila, Algeria, May 2012	*Helianthemum* sp.	Medium arid	MF940181	-
T. boudieri	LBMB 41	Moudjbara, Djelfa, Algeria, June 2011	*Helianthemum* sp.	Medium arid	-	MF940211
T. claveryi	LBMB1	Aïn Sekhouna, Saida, Algeria, March 2013	*H. hirtum*	Semi-arid	MF940182	MF940212
T. claveryi	LBMB 3	Bouchouat, Tiaret, Algeria, March 2013	*H. hirtum, H. salicifolium*	Medium arid	MF940183	MF940213
T. claveryi	LBMB 4	Mesbah, El-Bayadh, Algeria, April 2015	*Helianthemum* sp.	Superior arid	MF940184	MF940214
T. claveryi	LBMB 5	Bouchouat, Tiaret, Algeria, March 2013	*H. hirtum, H. salicifolium*	Medium arid	MF940185	MF940215
T. claveryi	LBMB 6	Mesbah, El-Bayadh, Algeria, April 2015	*Helianthemum* sp.	Superior arid	MF940186	MF940216
T. claveryi	LBMB 8	Aïn Sekhouna, Saida, Algeria, March 2013	*H. hirtum*	Semi-arid	MF940187	MF940217
T. claveryi	AH46139	Villacañas, Toledo, Spain, March 2017	*Helianthemum* sp.	Low dry	MF940188	MF940218
T. claveryi	LBMB 10	Bouchouat, Tiaret, Algeria, March 2013	*H. hirtum, H. salicifolium*	Medium arid	MF940189	MF940219
T. claveryi	LBMB 12	Mesbah, El-Bayadh, Algeria, April 2015	*Helianthemum* sp.	Superior arid	MF940190	MF940220
T. claveryi	LBMB 13	Kef El Ahmar, El-Bayadh, Algeria, April 2012	*H. hirtum*	Superior arid	MF940191	MF940221
T. claveryi	LBMB 15	Parc National d'El Kala, El-Tarf, Algeria, April 2011	*H. guttatum*	Humid	MF940192	-
T. claveryi	LBMB 17	Ain Sefra, Naama, Algeria, April 2012	*Helianthemum* sp.	Inferior arid	MF940193	MF940222
T. claveryi	LBMB 27	Mecheria, Naama, Algeria, March 2013	*Helianthemum* sp.	Medium arid	MF940194	MF940223
T. claveryi	LBMB 30	Kef El Ahmar, El-Bayadh, Algeria, April 2012	*H. hirtum*	Superior arid	MF940195	MF940224
T. claveryi	LBMB 31	Bouchouat, Tiaret, Algeria, March 2013	*H. hirtum, H. salicifolium*	Medium arid	MF940196	MF940225
T. claveryi	LBMB 34	Mecheria, Naama, Algeria, March 2013	*Helianthemum* sp.	Medium arid	MF940197	MF940226
T. claveryi	LBMB 39	Ksar Chellala, Tiaret, Algeria, March 2009	*H. hirtum*	Medium arid	MF940198	MF940227
T. claveryi	LBMB 42	Ksar Chellala, Tiaret, Algeria, April 2012	*H. hirtum*	Medium arid	MF940199	MF940228
T. claveryi	LBMB 43	Bou Saada, Msila, Algeria, May 2012	*Helianthemum* sp.	Medium arid	-	MF940229
T. eliocrocae	LBMB 33	Beni Abbes, Bechar, Algeria, April 2012	*H. lippii*	Saharan	MF940200	MF940230
T. eliocrocae	AH 46384	Bouchouat, Tiaret, Algeria, March 2013	*H. hirtum, H. salicifolium*	Medium arid	MF940201	MF940231
T. crassiverrucosa	LBMB 14	Chrea, Tebessa, Algeria, April 2011	*Helianthemum* sp.	Superior arid	MF940202	MF940232
T. crassiverrucosa	AH 46383	Chrea, Tebessa, Algeria, April 2011	*Helianthemum* sp.	Superior arid	MF940203	MF940233
T. olbiensis	AH46143	Tocón de Quéntar, Granada, Spain, March 2017	*Helianthemum* sp.	Low dry	MF940204	MF940234

Phylogenetic analysis

ITS and 28S rDNA sequences were first compared with sequences deposited in public databases using the BLAST algorithm (Altschul *et al.* 1997) to determine the closest relatives, and then aligned with them. A single combined ITS-28S rDNA alignment was built using the MUSCLE algorithm in MEGA 7.0. software (Kumar *et al.* 2016). Each DNA sequence was assembled and edited manually if needed. Reference sequences were selected to represent most of the currently accepted *Terfezia* species from Díez *et al.* (2002), Ferdman *et al.* (2005), Hansen *et al.* (2005), Kovács *et al.* (2011b), Bordallo *et al.* (2012, 2013, 2015). *Peziza depressa* Pers. and *Tirmania pinoyi* (Maire) Malençon sequences were chosen as outgroup. Two phylogenetic analysis were performed on the final alignment employing Maximum likelihood and Bayesian methods. For the best-scoring maximum likelihood tree, analyses were performed in RAxML-HPC2 on XSEDE (Miller *et al.* 2010) using 1.000 bootstrap replications and GTR+GAMMA model. The best models (HKY+G for ITS and HKY+I for LSU) were determined with jmodeltest (www.jmodeltest.org) and implemented in MrBayes 3.2.6 (Ronquist *et al.* 2012), where a Bayesian analysis was performed (four chains, 10.000.000 generations, first 25% sampled trees discarded, sampling every 1000th generation). Only high branch support is shown at the nodes, maximum likelihood bootstrap support (MLB) ≥ 70% and Bayesian posterior probability (PP) ≥ 0.95.

Results

Phylogenetic analysis

The total lengths of ITS and 28S rDNA sequences of *Terfezia* species in the final alignment were 612 and 847 bp, with 201 and 51 variable positions, respectively. The phylogenetic inference of the 33 specimens of *Terfezia* studied suggested the existence of 14 major clades in *Terfezia* (I–XIV on Fig. 1). The most basal branches of the genus were represented by *T. alsheikhii* species which formed a significantly supported group distinctly separated from the other *Terfezia* species. The new *T. claveryi* sequences generated during this study formed a homogeneous highly supported clade. Very strong support (98% MLB and 1.0 PP) was achieved for the clade II comprising two sequences deposited initially as *T. claveryi* in GenBank (derived from Spanish specimens) and two new sequences obtained from the Algerian samples studied in the present work, showing a specific combination of anatomical features not encountered neither in *T. claveryi* species nor in other *Terfezia* taxa. The phylogenetic analyses of both the ITS and LSU sequences and the morphological characteristics provided strong support to recognize these samples as type collections for a novel *Terfezia* species. Accordingly, we propose *T. crassiverrucosa* sp. nov. as new species to accommodate the *Terfezia* taxa of the clade II. The *T. boudieri* clade was robustly supported and bifurcated into two lineages. The sequences of *T. boudieri* from the present study clustered together in a single lineage while some other *T. boudieri* GenBank sequences formed a distinct lineage in this group. The *T. arenaria* sequences obtained in this study and the reference sequences formed a highly supported group with insignificant intraspecific variability. Two samples collected from Algeria (LBMB 33 and AH 46384) were significantly related (100% MLB and 1.0 PP) with *T. eliocrocae*, representing the first record of this species from North-Africa, although sample LBMB 33 was significantly different from all others. The Spanish specimen AH46143 nested in the *T. olbiensis* clade with a significant support (86% MLB and 1.0 PP). The remaining seven major clusters were formed by species of: *T. canariensis, T. albida, T. grisea, T. pseudoleptoderma* and a mixture of different *Terfezia* taxa including *T. extremadurensis–T. olbiensis* group 4 (TLO-4), *T. olbiensis* group 1 (TLO-1)–*T. cistophila–T. fanfani–T. leptoderma* (TLO-1a) and *Terfezia pini–T. olbiensis* group 3 (TLO-3) (Fig. 1).

FIGURE 1. Maximum likelihood (ML) tree inferred from the combined ITS and 28S rDNA sequences of *Terfezia* species with *Peziza depressa* and *Tirmania pinoyi* as outgroups. The sequences obtained in the present study are highlighted in bold. The first value on the branches represent the ML bootstrap proportions (≥ 70%) and the value after the slash shows the posterior probability calculated by Bayesian analysis (≥ 0.95). Bar = 1 changes /100 characters.

Taxonomy

Terfezia crassiverrucosa Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla, sp. nov. Fig. 2

MycoBank MB 822869

Type:—Algeria. Tebessa: Chrea, 1 April 2011, Z. Fortas (**Holotype**, AH 46383).

Diagnosis:—*Ascomata* hypogeous, subglobose, substipitate with short basal attachment, <8 cm broad (Fig. 2a). *Peridium* smooth, light to dark brownish, 100–200 µm thick with emergent hyphae 6–10 µm broad at septa, pseudoparenchymatous, composed of subglobose, polygonal to irregularly oblong cells (9–) 12.5–38 × 15–60(–62) µm, with cell walls 1–2 µm thick. *Gleba* solid, fleshy, light pink to pale yellow with subglobose to elongate light pink to pale gray pockets of fertile tissue, separated by light yellow to pale orangish yellow sterile veins (Fig. 2b). *Asci* (4–6)–8– spored, hyaline, often ellipsoid to ovoid or at times subglobose, 51–60 × 60–81 µm, non-stalked (Fig. 2 c & d). *Ascospores* globose, first hyaline, turning light yellowish to bright orange with age, measuring (16–) 17–21 (–24) µm in diameter including ornamentation, consisting in more or less broad flat truncate to round-tipped warts (0.5–) 1–1.5 × 2–4.5 (– 6) µm, and relatively elongated rounded elements measuring 1–1.5 × 1.5–3 µm. The prominent, crowded ornamentation hides a fine reticulum on the spore surface formed between the warts (Fig. 2 c–h).

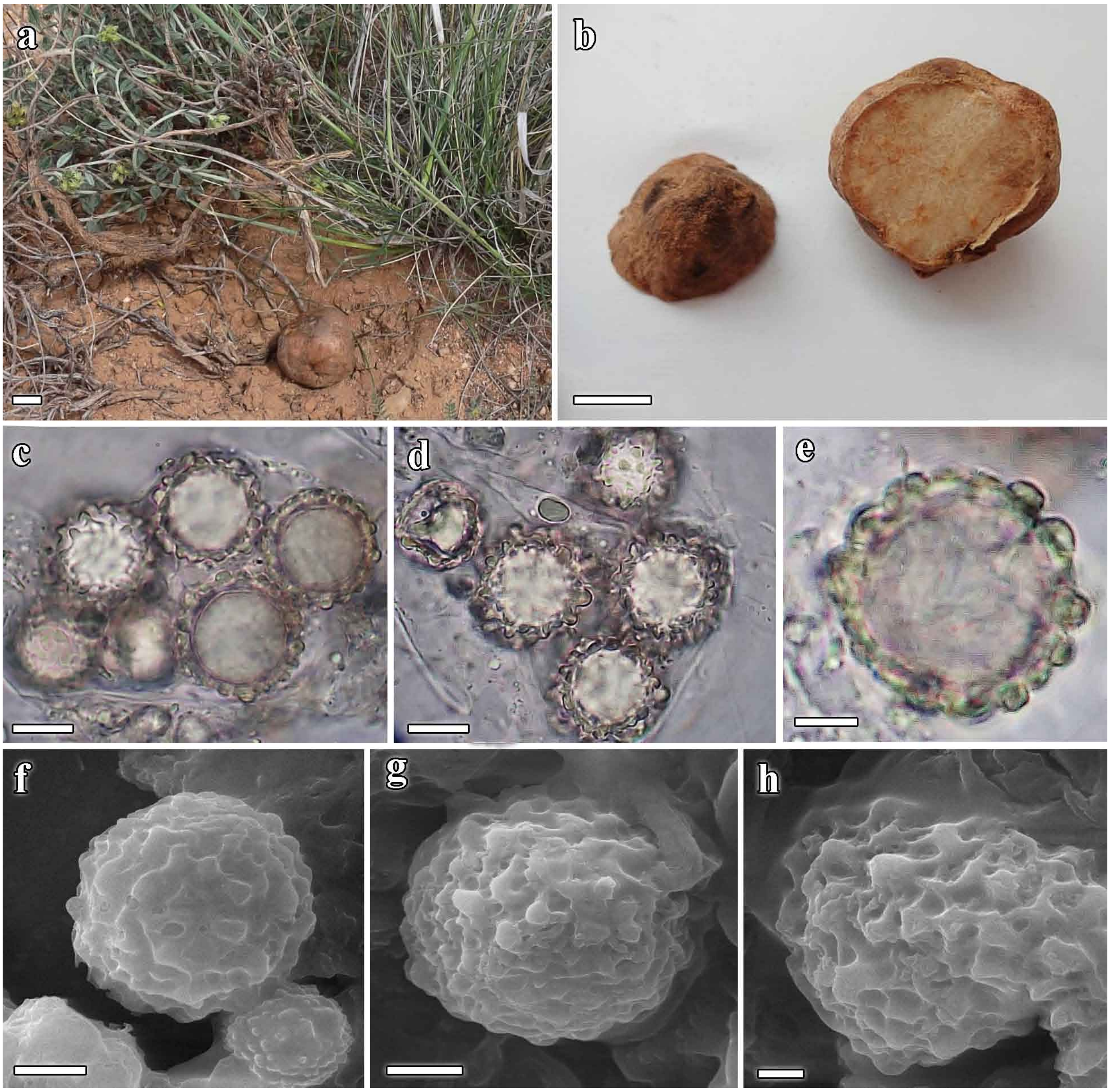

FIGURE 2. Macro- and microscopic characters of *Terfezia crassiverrucosa.* a. ascocarp collected under *Helianthemum hirtum.* b. gleba, cross section. c,d. asci with spores. e–h. ascospores (f–h. scanning electron micrograph). Bars: a–b = 1 cm, c–d = 10 µm, e = 5 µm, f–g = 5 µm, h = 2 µm.

Etymology:—The epithet (Latin *crassiverrucosa*) refers to the large warts covering the spore surface of this species.

Habitat:—Algerian arid area. In high steppe plains south of Constantine (Chrea, Tebessa), associated with *Helianthemum* sp. In high steppe plains south of Algiers (Bouchouat, Tiaret), found under *Helianthemum hirtum* and *H. salicifolium* associated mostly with *Stipa parviflora* on sandy loam alkaline soil, in sites also producing *T. claveryi* and *T. eliocrocae*. March, April.

Additional specimen examined:—ALGERIA. Tiaret: Bouchouat, March 2013, F.E.-H. Zitouni-Haouar.

Notes:—*T. crassiverrucosa* clearly differs from its closest relatives *Terfezia* species with warty-reticulate spores, *T. boudieri var. arabica* Chatin and *T. mellerionis* Chatin, by the shape of its asci (often ellipsoid to ovoid), the number of ascospores per asci (mostly 4–6, very rarely 8 spored) and the size of its spores which is considerably smaller (17–21 µm) (Chatin 1893, 1896). *T. metaxasi* Chatin produce ascomata with darker peridium (very dark brown to near-black) and much larger spores (30-32 µm) with darker color (dark brown) and more elongated papillae (up to 5µm tall) than *T. crassiverrucosa* (Chatin 1892, Alsheikh 1994). *T. aphroditis* Chatin differs from *T. crassiverrucosa* in having darker peridium (brownish black) and gleba (sooty-black irregularly marbled) and much bigger (28-32 µm) and darker (brown-sooty black) ascospores (Chatin 1897). The reddish brown peridium and the exclusively short, round-tipped papillae on the spore surface of *T. hispanica* Lazaro Ibiza differentiate that species from *T. crassiverrucosa* (Lazaro é Ibiza 1908). *T. hafizi* Chatin is also clearly distinguished from *T. crassiverrucosa* by its white gleba, globose stalked asci and purely reticulate ascospores (Chatin 1892).

Terfezia eliocrocae: Bordallo, Morte & Honrubia, Mycotaxon 124:196–197 (2013) Fig. 3

Ascomata hypogeous, 1–6 cm below soil surface, globose to subglobose, substipitate with short basal attachment, 15–50 × 20–55 mm, (6–) 9–57 g fresh weight (Fig. 3b-c). *Peridium* thin (0.1 mm thick), smooth, light brownish yellow to brown mottled with black patches. *Gleba* soft and friable, light yellowish white with creamy gray subglobose to elongated pockets of fertile tissue surrounded by pale yellow sterile veins. *Asci* eight-spored, hyaline, often ovoid to clavate or subglobose, (37–) 44–61 × 54–78 (–85) µm, astipitate or occasionally substipitate (Fig. 3d). *Ascospores* globose, (15–) 16–19 (–20) µm broad including ornamentation with prominent irregular reticulum. The young spores are hyaline and smooth, becoming at maturity pale yellow to light orange and ornamented with a well-developed reticulum. The reticular walls are 0.5–1 µm thick (Fig. 3 d–g).

Habitat:—In Algerian steppic plains (Bouchouat, Tiaret) (Fig. 3a) collected under *Helianthemum hirtum* and *H. salicifolium* in association with several neighboring plants: *Stipa parviflora, Astragalus mareoticus*, *Noaea mucronata, Atractylis serratuloides, Artemisia herba-alba, Thymelaea hirsutae* and *Schismus barbatus* on alkaline calcareous sandy loam soil*;* March*.* In desertic areas (Northwestern Algerian Sahara, Bechar) growing on calcareous alkaline sandy soil under *H. lippii;* April.

Specimens examined:—ALGERIA: Tiaret: Bouchouat, March 2013, F.E.-H. Zitouni-Haouar (AH 46384); Bechar: Beni Abbes, April 2012, F.E.-H. Zitouni-Haouar.

Discussion

The results of the phylogenetic analyses based on ITS and 28S rDNA sequence data and the morphological analyses revealed that the specimens investigated group in six distinct lineages. Three of them represent the most frequently recorded *Terfezia* species from the Mediterranean region, North Africa and Middle East: *T. claveryi, T. boudieri* and *T. arenaria*. These species are clearly discriminated from each other by their typical ascospore ornamentation, warty in *T. arenaria,* reticulate in *T. claveryi*, and warty-reticulate in *T. boudieri*. In fact, spore characters have proved to be the most reliable micromorphological features to delimit *Terfezia* species. However, some morphological similarities recorded in the same global type of the ascospore ornamentation complicate the identification process and lead to serious misidentifications. The reticulate spores of *T. eliocrocae*, a *Terfezia* species newly described from the Iberian Peninsula by Bordallo *et al.* (2013) and reported for the first time from North-Africa in this study, can be misidentified as *T. claveryi*, but the large epispore reticulum and the pseudoparenchymatic peridium of *T. eliocrocae* separates this species from the *T. claveryi* group. Kovács *et al.* (2009, 2011b) highlighted also that samples of *Mattirolomyces terfezioides* and *Terfezia alsheikhii* were originally deposited as *T. claveryi* in the herbarium of the Royal Botanical Garden (Madrid), and *T. canariensis* has been also mistaken with *T. claveryi* in the past (Bordallo & Rodríguez 2014). The morphological investigations performed on collections of the significantly independent lineage II from *T. claveryi*,

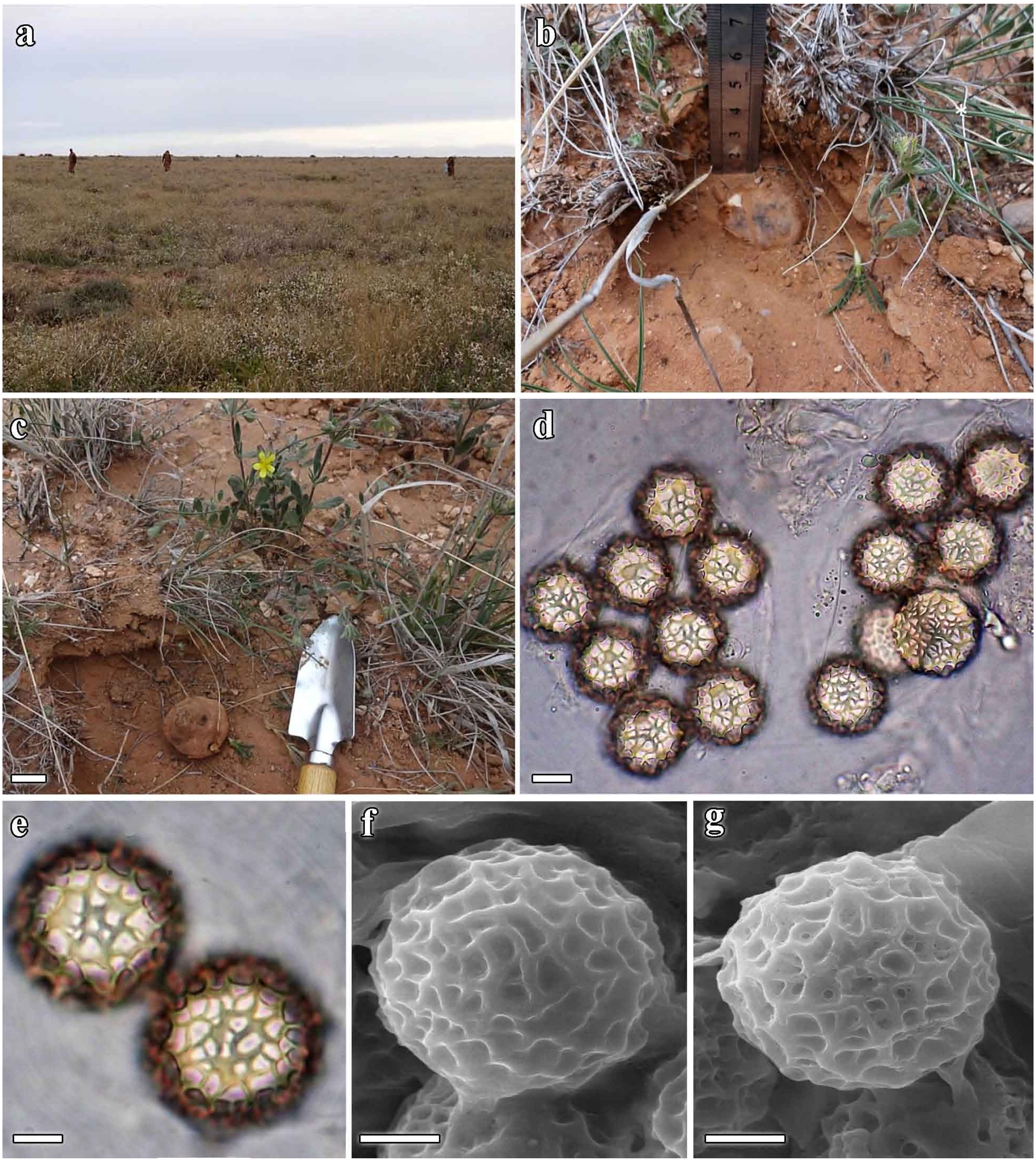

FIGURE 3. Macro- and micromorphological characteristics of *Terfezia eliocrocae*. a. the steppic habitat with Bedouins desert truffles harvesters. b,c. ascomata collected under *Helianthemum salicifolium*. d. asci and spores. e–g. ascospores (f,g. scanning electron micrograph). Bars: c = 2 cm, d = 10 µm; e–g = 5 µm.

shown that they represent a separate species, *T. crassiverrucosa*, characterized by a pseudoparenchymatic peridium and warty reticulate spores partially similar to those found in *T. boudieri* and *T. arenaria* at the same time. ITS sequences from these samples were significantly similar to GenBank accessions HQ698074 and AF387647 (Kovács *et al.* 2011b; Gutiérrez, unpublished data), both obtained from samples collected in Spain (Sierra Espuña and Zarzadilla de Totana, Murcia respectively) identified as *T. claveryi*. Kovács *et al.* (2011b) reported in their work that the specimen MA-FU 24303 (GenBank sequence HQ698074) was initially deposited in the Herbarium of the Real Jardín Botánico of Madrid (MA-Fungi), as *T. arenaria*. The fairly broad warts observed in the samples of *T. crassiverrucosa* studied in the present work and resembling in part those of *T. arenaria* could be the reason behind this previous misidentification. ITS and 28S rDNA sequences of *T. arenaria* generated in the present work suggested that this species is genetically conserved, in agreement with previous results (Kovács *et al.* 2011b). By contrast, Díez *et al.* (2002) detected a polymorphism in RFLP profiles of *T. arenaria*. Samples of *T. boudieri* studied in the present work showed no significant morphological differences. However, some GenBank ITS rDNA sequences (AF301418, AF301419, AF092097) deposited as *T. boudieri* (Aviram *et al.* 2004, Ferdman *et al.* 2005, 2009) formed a well-supported divergent lineage, suggesting that this lineage could represent a cryptic species within *T. boudieri* complex (Ferdman *et al.* 2009) or else match one of the several *Terfezia* species erected in the past and treated later as forms or developmental stages of *T. boudieri* (*T. boudieri var. arabica* Chatin, *T. boudieri var. pedunculata* Pat., *T. aphroditis* Chatin and *T. deflersii* Pat.) (Alsheikh, 1994), due to the lack of diagnostic morphological features allowing their separation. A similar situation affects *T. olbiensis*, which was considered previously by several authors as synonym or developmental stage (immature form) of *T. leptoderma* (Fogel 1980, Malençon 1984 *in* Moreno *et al.* 1986, Alvarez *et al.* 1993, Díez *et al.* 2002). However, combined molecular and morphological investigations suggested that *T. olbiensis* is a distinct valid species (Kovács *et al.* 2011b, Bordallo *et al.* 2013). The new DNA sequence produced in the present work coming from a spiny-spored *Terfezia* species originating from Spain clustered within a homogeneous lineage composed of exclusively *T. olbiensis* GenBank sequences derived from the works of Kovács *et al.* (2011b) and Bordallo *et al.* (2013). However, the clear distinction between *T. olbiensis, T. leptoderma* and other valid *Terfezia* species with spiny-spores is seemingly steel not reached. The sequences from *T. fanfani* and two new *Terfezia* species, *T. extremadurensis* and *T. pini,* reported recently from the Iberian Peninsula by Bordallo *et al.* (2013) nested in the clades of some lineages of the *T. olbiensis-T. leptoderma* species complex, TLO4, TLO1a and TLO3 respectively, proposed by Kovács *et al.* (2011b). Delimitation of species boundaries in this species complex is difficult due to the lack of clear distinctive morphological and anatomical features especially the spores characters with polymorphic spiny ornamentation even within the same specimen (Fogel 1980, Alsheikh 1994, Kovács *et al.* 2011b) which might be the principal reason behind misidentifications. Finally, collections of *T. claveryi*, *T. boudieri* and *T. arenaria* harvested from different bioclimatic zones were almost similar intraspecifically, suggesting that aridity has no influence on the phylogenetic structure of these taxa. The genetic pattern within the *Terfezia* species seems to correlate rather well with the phenotypic traits. The host specificity hypothesis of *Terfezia* species proposed previously by Díez *et al.* (2002) could not be confirmed with the present results as probably all specimens were found associated with exclusively *Helianthemum* spp.

The present research has highlighted the considerable progress which has been made in the taxonomy of the *Terfezia* species but raised at the same time many questions in need of thorough investigations. Further works with exhaustive sampling from new geographic areas as North Africa or Middle East are therefore required in order to construct a conventional reliable key for the delimitation of the spiny-spored *Terfezia* species. Finally, the strong mycorrhizal relationship linking the desert truffles to their putative host plant should not be overlooked as an important factor contributing substantially to the understanding of these fungi. The comparison of the phylogenies of *Helianthemum* and its *Terfezia* symbionts could help to elucidate the factors behind their speciation processes. Additionally, a combined simultaneous sequencing of the whole genome of some desert truffles species with their putative phytosymbionts could represent a valuable tool leading to the discovery of special genes which might guarantee more accurate species identification of these fascinating fungi.

Acknowledgments

We kindly thank A. Priego and J.A. Pérez from the University of Alcalá De Henares (Spain) for the valuable assistance in the SEM observations and Dr. J. Rejos, curator of the AH Herbarium (University of Alcalá). Dr. Shaun Pennycook (Landcare Research, New Zealand) is acknowledged for nomenclatural advice.

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