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Five new *Terfezia* species from the Iberian Peninsula

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Abstract — A phylogenetic analysis of Iberian *Terfezia* collections indicates eight clades, of which three correspond with previously described species (*T. alsheikhii*, *T. fanfani*, *T. olbiensis*) and five are described as new species — *T. albida*, *T. eliocrocae*, *T. extremadurensis*, *T. pini*, and *T. pseudoleptoderma*. These results are supported by the morphology of the examined *Terfezia* ascomata that were identified by their ecological patterns and chorology, as well as by phylogenetic analyses. Morphological features and ITS-rDNA sequence analyses from identified *Terfezia* specimens are described. A key to these *Terfezia* species is also provided.

Key words — desert truffle, hypogeous, mycorrhizal fungi, *Pezizaceae*

Introduction

The genus *Terfezia* (Tul. & C.Tul.) Tul. & C.Tul. includes twelve species (Kirk et al. 2008) of hypogeous ascomycetes formerly placed in the family *Terfeziaceae* within the order *Pezizales* (Trappe 1979). However, based on molecular and morphological characters, *Terfezia* is now accepted in the *Pezizaceae* (Norman & Egger 1999; Percudani et al. 1999; Laessøe & Hansen 2007). These hypogeous fungi are edible and are known as "desert truffles" due to their habitat in typically arid and semi-arid ecosystems, mostly in the Mediterranean region (Morte et al. 2009).

Most *Terfezia* species establish mycorrhizal symbiosis with plants from family *Cistaceae*, mainly with perennial and annual *Helianthemum* species (Dexheimer et al. 1985; Fortas & Chevalier 1992; Gutiérrez et al. 2003) or with trees from different phyla (Díez et al. 2002; Taylor et al. 1995). These plants and their associated fungi play a major role in maintaining Mediterranean shrub lands and xerophytic grasslands and thus in preventing erosion and

desertification (Honrubia et al. 1992). This mycorrhizal association is well adapted to semiarid climates through the physiological mechanism of drought avoidance (Morte et al. 2000, 2010).

Six *Terfezia* species have been reported so far from the Iberian Peninsula: *T. claveryi* Chatin, *T. boudieri* Chatin, *T. arenaria* (Moris) Trappe, *T. leptoderma* Tul. & C. Tul., *T. olbiensis,* and *T. alsheikhii*. *Terfezia claveryi*, *T. boudieri,* and *T. olbiensis* have been recorded from basic soils and *T. arenaria* from acid soils, but all four in mycorrhizal association with different *Helianthemum* species. However, *T. leptoderma* has been found in association with *Tuberaria guttata* L. Fourr. in acid soils, with *Cistus* in slate-derived soils, and with *Quercus ilex* L. and *Pinus halepensis* Mill. in basic soils (Díez et al. 2002). Isolations from *Cistus*, *Pinus*, and *Quercus* could belong to distinct species with different host or/and edaphic specialization. These authors emphasized that the sampling in these ecosystems is scanty and further study is necessary. Moreover, the small spores of *T. leptoderma* specimens collected under pine and evergreen holm oak fit those of *T. olbiensis,* which was described as morphologically similar to *T. leptoderma*, except for slightly smaller spores and shorter spines (Díez et al. 2002). There is a certain consensus that *T. olbiensis* is an immature form and a synonym of *T. leptoderma* (Díez et al. 2002; Moreno et al. 1986).

The main objective of this study was to describe five new *Terfezia* species. For this purpose, we conducted classical morphological studies complemented by phylogenetic analyses based on ITS-rDNA sequences from *Terfezia* specimens collected throughout the Iberian Peninsula.

Materials & methods

Fungal specimens

Ascomata of *Terfezia* spp. were collected in different years and from different locations in the Iberian Peninsula (Spain and Portugal). The ascomata were collected from cracks in the soil close to host plants. Characterized and sequenced specimens are listed in Table 1. Generally, three specimens per collection were used when the material was fresh. Furthermore, conserved dried material was processed. Soil characters were obtained from the Spanish Geological Map, according to FAO-CEE, 1998 from the "Atlas Nacional de España" (IGN). The different host plants were identified using Flora Ibérica keys (Muñoz-Garmendia & Navarro 1993).

External ascocarp characteristics (shape, colour, appearance) were recorded in detail. Ascomata were then cut and the morphology of the peridium and gleba was described. Asci and ascospores were examined using an Olympus BX51 microscope equipped with a digital camera (Canon PSpro1). A minimum of 100 asci, ascospores, and peridial warts was measured per species. The ascospores of *Terfezia eliocrocae* and *T. pseudoleptoderma* were stained with acid fuchsine solution (0.01% acid fuchsine in acetic acid, ethylene glycol, and lactic acid, 1:1:1, v/v/v) to improve visualization.

Samples of the collected specimens are stored in the Mycological Herbarium of the Botany Area of the University of Murcia (MUB).

Table 1. *Terfezia* collections analyzed

Clade	Collection* #	Origin ^ & Host	Year, Collector	Genbank nº
1	tO9*	Belvis (Cc). *Tuberaria guttata*	2009. J. Mohedano	HM056199
1	tO10*	Belvis (Cc). *T. guttata*	2009. J. Mohedano	HM056200
1	tO11*	Belvis (Cc). *T. guttata*	2009. J. Mohedano	HM056201
1	tO17*	Valdecañas (Cc). *T. guttata*	2009. J. Mohedano	HM056202
1	tO18	Valdecañas (Cc). *T. guttata*	2009. J. Mohedano	
1	tO19	Valdecañas (Cc). *T. guttata*	2009. J. Mohedano	
1	tO20*	Castuera (Ba). *T. guttata*	2009. M. Romero	HM056203
1	tO21	Castuera (Ba). *T. guttata*	2009. M. Romero	
1	tL9	Valdecañas (Cc). *T. guttata*	2007. J. Mohedano	
1	tL10*	Valdecañas (Cc). *T. guttata*	2007. J. Mohedano	HM056204
1	tL11	Valdecañas (Cc). *T. guttata*	2007. J. Mohedano	
1	tL12	Valdecañas (Cc). *T. guttata*	2007. J. Mohedano	
1	#*	C. Arañuelo (Cc). *T. guttata*	2002. J. Díez	AF276678
2	tO4*	Lorca (Mu). *Helianthemum*	2001. A. Morte	HM056205
2	tO7*	Lorca (Mu). *Helianthemum*	2007. A. Morte	HM056206
3	tTP1	Montesihno. Portugal. *Cistaceae*	2006. A. Rodríguez	
3	tTP2*	Montesihno. Portugal. *Cistaceae*	2006. A. Rodríguez	HM056207
3	tL13	Quintana (Ba). *Cistaceae*	2008. M. Romero	HM056208
3	#*		2011. G. Kovacs	HQ698098
3	#		2011. G. Kovacs	HQ698100
4	tO31	Sieteiglesias (Va). *Quercus*	2003. A. García	
4	tO32*	Ronquines (Va). *Pinus*	1998. A. García	HM056209
4	tO33	Ronquines (Va). *Pinus*	1998. A. García	
4	tO35	Cornudilla (Bu). *Pinus*	2008. F. Sainz	
4	tO36	Cornudilla (Bu). *Pinus*	2008. F. Sainz	
4	tO37	Cornudilla (Bu). *Pinus*	2008. F. Sainz	
4	tO40*	Cornudilla (Bu). *Pinus*	2008. F. Sainz	HM056210
4	#*	Madrid. *Pinus*	2008. Barriuso et al.	DQ386140
5	tL16*	Villafranca (Bu). *Cistaceae*	2008. F. Sainz	HM056211
5	tL17*	Villafranca (Bu). *Cistaceae*	2008. F. Sainz	HM056212
5	tL18	Villafranca (Bu). *Cistaceae*	2008. F. Sainz	
5	tO42*	Valdecañas (Cc). *Pinus*	2010. J. Mohedano	HM056213
5	#*	Madrid	2008. A. Rincón	FJ013064
6	tL1*	Belvis (Cc). *T. guttata*	2009. J. Mohedano	HM056214
6	tL2	Belvis (Cc). *T. guttata*	2009. J. Mohedano	
6	tL3	Belvis (Cc). *T. guttata*	2009. J. Mohedano	
—	tO22*	Castuera (Ba). *Cistaceae*	2009. M. Romero	HM056215
6	tO23*	Garrovilla (Cc). *T. guttata*	2004. A. Mateos	HM056216
6	tO24	Garrovilla (Cc). *T. guttata*	2004. A. Mateos	
6	tO30	Garrovilla (Cc). *T. guttata*	2004. A. Mateos	
6	tL6	Valdecañas (Cc). *T. guttata*	2001. J. Mohedano	
6	tL7*	Valdecañas (Cc). *T. guttata*	2001. J. Mohedano	HM056217
6	tL8*	Cortas Blas (Va). *Cistaceae*	1998. A. García	HM056218
6	tL14*	Quintana (Ba). *T. guttata*	2009. M. Romero	HM056219
6	tL15	Quintana (Ba). *T. guttata*	2009. M. Romero	
6	#*	Cañaveral (Hu). *Cistaceae*	2002. J. Díez	AF396862
6	#*	—	2002. J. Díez	AF276676

Table 1, concluded

Clade	Collection* #	Origin ^ & Host	Year, Collector	Genbank nº
6	#*	Madrid.	2008. A. Rincón	FJ013087
—	#*	Valencia. *Pinus*	2002. J. Díez	AF396864
7	tO15*	Ballestero (Ab). *Helianthemum*	2009. A. Rodríguez	HM056220
7	tO16*	Albacete (Ab). *Helianthemum*	2009. A. Rodríguez	HM056221
7	#*	Murcia. *Helianthemum*	2001. A. Gutiérrez	AF387655
8	tO12*	Masegoso (Ab). *Quercus*	2009. A. Rodríguez	HM056222
8	tO13	Masegoso (Ab). *Quercus*	2009. A. Rodríguez	
8	tO14*	Masegoso (Ab). *Quercus*	2009. A. Rodríguez	HM056223
8	tO34*	Santa Espina (Va). *Pinus*	1998. A. García	HM056224
8	tO39*	Onteniente (V). *Pinus*	2009. F. García	HM056225
8	#*	Murcia. *Pinus*	2001. Gutiérrez et al	AF387656
8	#*	—	2002. J. Díez	AF276677
8	#*	France. *Quercus*	2002. J. Díez	AF396863
—	#*	Jarada. Morocco	1994. M. Achouri	AF301421
—	#*	C. Arañuelo (Cc).	2002. J. Díez	AF276674
—	#*	Zeelim. Israel	1996. Bedouins	AF092098

* = included in the cladistic analysis; # = external sequence.

^ Spanish provinces: Ab: Albacete; Ba: Badajoz; Bu: Burgos; Cc: Cáceres; Hu: Huelva; Mu: Murcia; V: Valencia; Va: Valladolid.

Macroscopic and microscopic characterization

For identification, ascomata were compared with descriptions from Mattirolo (1900, 1906, 1907, 1922), Lázaro Ibiza (1908), Malençon (1938, 1973), Ceruti (1960), Cerutti et al. (2003) and keyed according to Montecchi & Lazzari (1993) and Montecchi & Sarasini (2000). Trappe (1971, 1979), Calonge et al. (1977) and Honrubia et al. (1992) were also taken into account. The descriptions of *Tuber lutescens* Lázaro Ibiza (≡ *Terfezia lutescens* (Lázaro Ibiza) Malençon) and *T. pallidum* Lázaro Ibiza (≡ *T. pallida* (Lázaro Ibiza) Malençon) were also consulted (Lázaro Ibiza 1908, Malençon 1938). In addition, descriptions of *T. leptoderma*, *T. fanfani*, *T. cadevalli* Font Quer, *T. hafizi* Chatin, *T. berberiodora* Lesp. ex Tul. & C.Tul., and *T. goffartii* Chatin were also checked.

DNA analyses

Genomic DNA was isolated from 150–200 mg of the inner gleba of the ascocarps using the E.Z.N.A. Fungal DNA kit (Omega Bio-Tek, Doraville, GA, USA) and following the manufacturer's instructions. The Internal Transcribed Spacer (ITS) region of the rDNA, including the 5.8S ribosomal gene, was amplified using the universal ITS5 and ITS4 primers (White et al. 1990). All PCR amplifications were carried out in a final volume of 25 µL containing 0.2 mM of each dNTP, 0.6 µM of each primer, 3.75 mM $MgCl_2$, 1X PCR buffer and 1.25 U of Taq DNA polymerase (Invitrogen, California, USA).

PCR reactions were performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: an initial denaturalization step for 2 min at 94°C, 30 cycles consisting of 30 s at 94°C, 1 min at 65°C, and 1 min at 72°C, and a final extension at 72°C for 4 min.

PCR products were purified using the E.Z.N.A. Cycle-Pure kit (Omega Bio-Tek) following the manufacturer's instructions. Clean PCR products were sequenced in both directions at the Molecular Biology Service (University of Murcia). The BioEdit (Hall

1999) program was used to obtain the consensus sequence of each sample. Sequences were deposited on GenBank (NCBI) under accession numbers indicated in Table 1.

Phylogenetic analyses

Nucleotide Basic Local Alignment Search Tool (BLAST) searches (megablast) were used to compare the sequences obtained in this study with other DNA sequences in GenBank (NCBI, Altschul et al. 1997) to provisionally identify the specimens prior to phylogenetic analyses. The sequence alignment was carried out with ClustalW (MEGA software, version 4; Tamura et al. 2007), following the default options. A minimal manual adjustment was made with the MEGA v.4 editor. Alignments are available in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S10906?x-access-code=eadd311aaa59cd96424d699af26cb699&format=html)

Evolutionary history was inferred based on the Maximum Parsimony (MP; Eck & Dayhoff 1966) and the Minimum Evolution (ME; Rzhetsky & Nei 1992) methods using MEGA v.4. The closest species not belonging to the sister group were selected as outgroups. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. The branches corresponding to the partitions reproduced in less than 50% bootstrap replicates were excluded. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985).

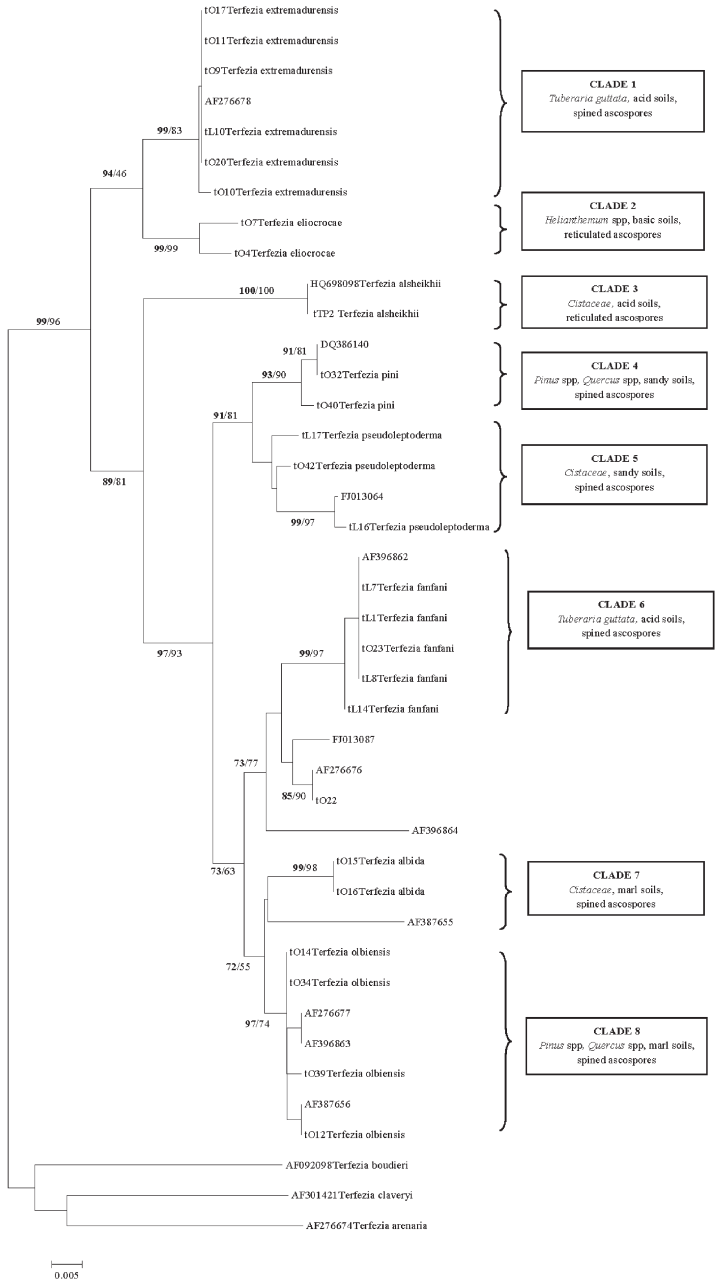
The MP tree (Plate 1) was obtained using the Close-Neighbour-Interchange algorithm at search level 3 with which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale, and branch lengths were calculated using the average pathway method (Nei & Kumar 2000). The number of changes over the whole sequence is presented in units. All positions with gaps and missing data were eliminated from the dataset (the Complete Deletion option). The final dataset contained a total of 429 positions, of which 69 were parsimony informative.

The ME tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are presented in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbour-Interchange (CNI) algorithm (Nei & Kumar 2000) at search level 1. The Neighbour-joining algorithm (Saitou & Nei 1987) was used to generate the initial tree. All the positions with gaps and missing data were eliminated from the dataset (the Complete Deletion option). The final dataset contained a total of 429 positions.

The sequences from *T. claveryi, T. boudieri* and *T. arenaria* were chosen as outgroup.

Phylogenetic results

Sequence analyses of the ITS-rDNA from the examined samples produced two trees based on the Maximal Parsimony (MP) and the Minimum Evolution (ME) methods, both with a virtual sampling or bootstrap of 1000 replicas. In the ME phylogeny, the 38 sequences are clearly distributed in eight well-separated lines (Plate 1) that match the eight phenetically described species.


tO17Terfezia extremadurensis
tO11Terfezia extremadurensis
tO9Terfezia extremadurensis
AF276678
tL10Terfezia extremadurensis
tO20Terfezia extremadurensis
tO10Terfezia extremadurensis
tO7Terfezia eliocrocae
tO4Terfezia eliocrocae
HQ698098Terfezia alsheikhii
tTP2 Terfezia alsheikhii
DQ386140
tO32Terfezia pini
tO40Terfezia pini
tL17Terfezia pseudoleptoderma
tO42Terfezia pseudoleptoderma
FJ013064
tL16Terfezia pseudoleptoderma
AF396862
tL7Terfezia fanfani
tL1Terfezia fanfani
tO23Terfezia fanfani
tL8Terfezia fanfani
tL14Terfezia fanfani
FJ013087
AF276676
tO22
AF396864
tO15Terfezia albida
tO16Terfezia albida
AF387655
tO14Terfezia olbiensis
tO34Terfezia olbiensis
AF276677
AF396863
tO39Terfezia olbiensis
AF387656
tO12Terfezia olbiensis
AF092098Terfezia boudieri
AF301421Terfezia claveryi
AF276674Terfezia arenaria
99/83
94/46
99/99
100/100
99/96
91/81
93/90
91/81
89/81
99/97
97/93
99/97
73/77
85/90
73/63
99/98
72/55
97/74
0.005
CLADE 1
Tuberaria guttata, acid soils,
spined ascospores
CLADE 2
Helianthemum spp, basic soils,
reticulated ascospores
CLADE 3
Cistaceae, acid soils,
reticulated ascospores
CLADE 4
Pinus spp, Quercus spp, sandy soils,
spined ascospores
CLADE 5
Cistaceae, sandy soils,
spined ascospores
CLADE 6
Tuberaria guttata, acid soils,
spined ascospores
CLADE 7
Cistaceae, marl soils,
spined ascospores
CLADE 8
Pinus spp, Quercus spp, marl soils,
spined ascospores

The MP phylogenetic topology is consistent with that of the ME analysis in which the same nodes and clusters are reproduced (Plate 1). The cladogram distinguishes eight clades (Table 2).

Table 2. Diagnostic characters of clades and *Terfezia* spp.

Clade	Species	Bootstrap %	Soil	Host
1	*T. extremadurensis*	94	Acid	*Tuberaria guttata*
2	*T. eliocrocae*	94	Alkaline	*Helianthemum* spp.
3	*T. alsheikhii*	89	Acid	*Cistaceae*
4	*T. pini*	91	Acid	*Pinus* spp., *Quercus* spp.
5	*T. pseudoleptoderma*	91	Acid	*Cistaceae*
6	*T. fanfani*	70	Acid	*Tuberaria guttata*
7	*T. albida*	70	Alkaline	*Helianthemum* spp.
8	*T. olbiensis*	70	Alkaline	*Pinus* spp., *Quercus* spp.

Taxonomy

The morphology and distributions of the examined specimens support eight phenetic species, described below (presented in clade order; Plate 1).

Terfezia extremadurensis Muñoz-Mohedano, Ant. Rodr. & Bordallo, **sp. nov.**

MycoBank MB561591 Plate 2a,b

Differs from all other spiny-spored *Terfezia* spp. by its larger spores with wider spines and its *Tuber*-like glebal morphology.

Type: Spain, Extremadura, Cáceres, Belvis de Monroy, 17 March 2009, leg Muñoz-Mohedano (**Holotype**, MUB Fung-0026).

Etymology: referring to Extremadura, the western Spanish region where the species was first identified

Ascomata hypogeous to partially emergent at maturity, 2–5 cm, subglobose, sometimes furrowed and nodulose, often cracked, often with a small basal depression, rarely with pseudostipe, cream colour at first, becoming brown, black spots on the sun-exposed parts or where handled, smooth. Peridium 300–600 μm thick, well-defined, concolorous with surface in cross section, pseudoparenchymatous, composed of subglobose cells, hyalines and thin-walled in the innermost layers, yellowish and with thicker walls in the outermost layers. Gleba solid, fleshy, succulent, whitish at first, soon becoming salmon pink, darkening with age, greenish grey at maturity, marbled with thin, white,

Plate 1. Minimum Evolution (ME) and Maximum Parsimony (MP) consensus tree showing evolutionary relationships of 38 sequences derived from the ITS-rDNA region. On each branch, % of 1000 bootstrapping replicates supported by ME is shown first (in bold) and by MP second (in plain font). Host plant, soil, and ascospore information is presented for each clade. Non-significant bootstrap values under 70 are not included.

meandering veins, sometimes arising from the base and inconspicuous in very mature specimens; frequently with small holes indicating mycophagous activity. Odour faint, not distinctive. Asci inamyloid, subglobose to ovate, sessile or short-stipitate, 60–80 × 50–65 µm, walls 1–2 µm thick, with 6–8 irregularly disposed spores, randomly arranged in the gleba. Ascospores globose, (21–) 22–26(–27) µm diam (median = 24 µm) including ornament [(16–)17–19(–20) µm (median = 18 µm) without ornament], hyaline, smooth and uniguttulate at first, by maturity yellow and ornamented with conical, blunt, thick spines, sometimes truncated, sometimes finger-like, often joined at the base, 3–4(–5) µm long, 1–3 µm wide at the base.

Ecology & distribution — Widely distributed in the western half of the Iberian Peninsula, common in grassland of Extremadura in sandy, acid soils, associated exclusively with *Tuberaria guttata*, from late winter to early spring. This is the earliest *Terfezia* species to appear (in March) associated with *T. guttata*.

Additional collections examined: **SPAIN**: **Extremadura**, Cáceres: Valdecañas, 2007, Muñoz-Mohedano (MUB Fung-0012); 2009, Muñoz-Mohedano (MUB Fung-0035); Badajoz: Castuera, 2009, M. Romero (MUB Fung-0036).

Comments — *Terfezia extremadurensis* differs from other spiny-spored *Terfezia* species in its *Tuber*-like gleba, with meandering veins of the gleba not completely surrounding the fertile tissue and not forming pockets as is typical for all other *Terfezia* species. *Terfezia fanfani*, described below, shares the same habitat but has reddish tones, a different gleba, and smaller spores (19–22 µm) with thin spines 1 µm wide at the base.

Clade 1 comprises *T. extremadurensis* and clearly differs from the other *Terfezia* species in the shape of the gleba (reminiscent of *Tuber*) and the ascospores with truncated crested spines. As far as we are aware, these two characteristics have not been described to date for *Terfezia*. The phylogenetic analyses clearly support with a high bootstrap value this taxon in an independent clade (Plate 1). Despite the similarity of *T. lutescens* (Malençon 1938) to *T. extremadurensis*, the absence of any description of spines with crossed crests (an undoubtedly visible character) by Lázaro Ibiza (1908) leads us to think that it is actually a different species. However, we were unable to find the original samples of Lázaro Ibiza to compare their ITS-rDNA sequences.

Terfezia eliocrocae Bordallo, Morte & Honrubia, **sp. nov.** Plate 2c,d

MycoBank MB561592

Differs from other reticulated spored *Terfezia* spp by its friable and crumbly gleba that remains milky white when exposed.

Type: Spain, Murcia, Lorca, Zarzadilla de Totana, 21 April 2007, leg A. Morte (**Holotype**, MUB Fung-0015).

Etymology: referring to Eliocroca, old Roman name for the city of Lorca (Murcia, Spain), and its surrounding area, where the type specimen was collected.

Ascomata more or less crushed shapes, roughened, embossed, >5 cm. Cream colour, blackish aspect when ripe with a rough, cracked texture. Sticky mycelial remains and pseudo-stipe. Peridium thin, well delimited, inseparable from the gleba, cream to blackish-brown colour, partially rough when ripe; pseudoparenchymatous, ≤200 µm thick, cells round, 30 × 15 µm; external layer with hyphal extensions. Gleba intensely white, remaining white and not darkening when mature or in contact with air; both fertile and sterile tissues milky white with small islets of fertile tissue surrounded by very thin ramified sterile veins. Asci 8-spored, subglobose, ovoid or pyriform elongated. Ascospores spherical with a well-developed reticulum, regular, 17–19 µm diam. including ornamentation; at first hyaline and smooth, in age becoming yellow with a very marked (≤1 µm tall) reticulum.

Habitat — Growing in grasslands on calcareous soils with *Helianthemum almeriense* and *H. violaceum* (*Cistaceae*).

Additional collections examined: **SPAIN**: **Murcia**: Totana, 2001, A. Morte (MUB Fung-0005); 2007, A. Morte (MUB Fung-0016).

Comments — *Terfezia eliocrocae* (Clade 2), distinguished by inner milky white colour that persists in contact with air, differs from *T. berberiodora* (characterized by hazelnut-shaped ascocarps, an atypical gleba in islets, and a characteristic flavour of *Berberis vulgaris*) in its smaller spores and larger ascocarps (Castro & Freire 1982). *Terfezia hafizi* also produces slightly reticulate but larger (18–20 µm) ascospores (Chatin 1892). The markedly reticulate ascospores distinguish *T. eliocrocae* from *T. hafizi* and *T. alsheikhii* (see below).

Terfezia alsheikhii Kovács, M.P. Martín & Calonge, Mycologia 103: 848. 2011.

Plate 2e,f

Ascomata regular, globose, 0.5–1.5 cm, smooth, firm; no strong odour detected. Peridium smooth, thin, inseparable from the gleba; brown-ochre; pseudoparenchymatous throughout, with thick-walled cells ≤50 µm in diameter. Gleba formed by big pinkish islets of fertile tissue, varying in size and surrounded by a scarce sterile white tissue, stained by brown-reddish zones when in contact with air. Asci primarily 8-spored, subglobose, ovoid, pyriform, 60–100 × 40–70 µm, sessile or with a short thick peduncle. Ascospores spherical, reticulate, 15–18 µm diam. (including ornamentation); at first hyaline, yellow when mature; decorated by an irregular, well-developed reticulum with thickish (nearly 1 µm) net; very mature spores with roundish to flat warts ≤2 µm × 2 µm.

Habitat — *Terfezia alsheikhii* grows in sandy acidic soils associated with cistaceous plants.

Collections examined: **SPAIN**: **Extremadura**, Badajoz: Castuera, 2009, M. Romero (MUB Fung-0034). **PORTUGAL**: Braganza: Portelo, Montesinho Natural Park, 2006, A. Rodríguez (MUB Fung-0009).

Comments — Gregarious specimens have been found by raking under *Cistus monspeliensis* L. on the border of a forest track.

Terfezia pini Bordallo, Ant. Rodr. & Muñoz-Mohedano, **sp. nov.** Plate 2G,H

MycoBank MB561594

Differs from *T. fanfani* and *T. pseudoleptoderma* by its spores with long spines joined at their bases to form a pseudo-reticulum.

Type: Spain, Burgos, Cornudilla, 8 March 2008, leg F. Sáinz (**Holotype**, MUB Fung-0027).

Etymology: referring to pinewoods, its preferred habitat.

Ascomata globose, round, regular, <2 cm diam., smooth, base often with residual mycelium. Peridium smooth, slightly tomentose, thin, not clearly delimited and difficult to separate from the gleba, 200–400 µm thick; at first cream colour, becoming ochre, grey in cross-section grey, partially covered by a whitish film; pseudoparenchymatous, cells thick-walled, ≤40 µm diam., pigmented in the outer part. Gleba initially whitish; when mature, the fertile tissue forming round islets of various sizes, initially pale pinkish becoming greenish brown and greyish when very mature and always surrounded by a sterile tissue of white veins. Asci 6–8-spored, ovoid, ellipsoid, subglobose, sessile, 60–90 × 45–60 µm, walls 1µm thick. Ascospores spherical with spines, 20–23(–25) µm (including ornamentation); initially hyaline and smooth with a big central drop, when mature, yellowish-ochre and decorated by cylindrical spines (3–4(–5) × 1 µm) with a round tip, joined basally to form crests, often in a pseudo-reticulum.

Ecology — The species grows mainly in sandy pine forests from November to May. It is rarely observed on the surface and is often found at a depth of 3–5 cm, usually under mosses.

Additional collections examined: **SPAIN**: **Castile & León**, Valladolid: Sieteiglesias, 2003, Aurelio García (MUB Fung-0007); Ronquines, 1998, Aurelio García (MUB Fung-0010).

Comments — *Terfezia pini* (represented by Clade 4) is diagnosed by its occurrence on burnt areas under pine and oak and lack of association with cistaceous plants and its spore ornamentation comprising long spines joined at the bases to form a pseudo-reticulum. A high bootstrap value in the phylogenetic analyses strongly supports its separation from other species (Plate 1).

The spine crests of *T. pini* resemble the lamelliform folds of the non-reticulate episporium of *T. cadevalli* (Solá 1925), which is distinguished in that it forms neither a reticulum nor clearly identifiable spines). The larger ascospores of

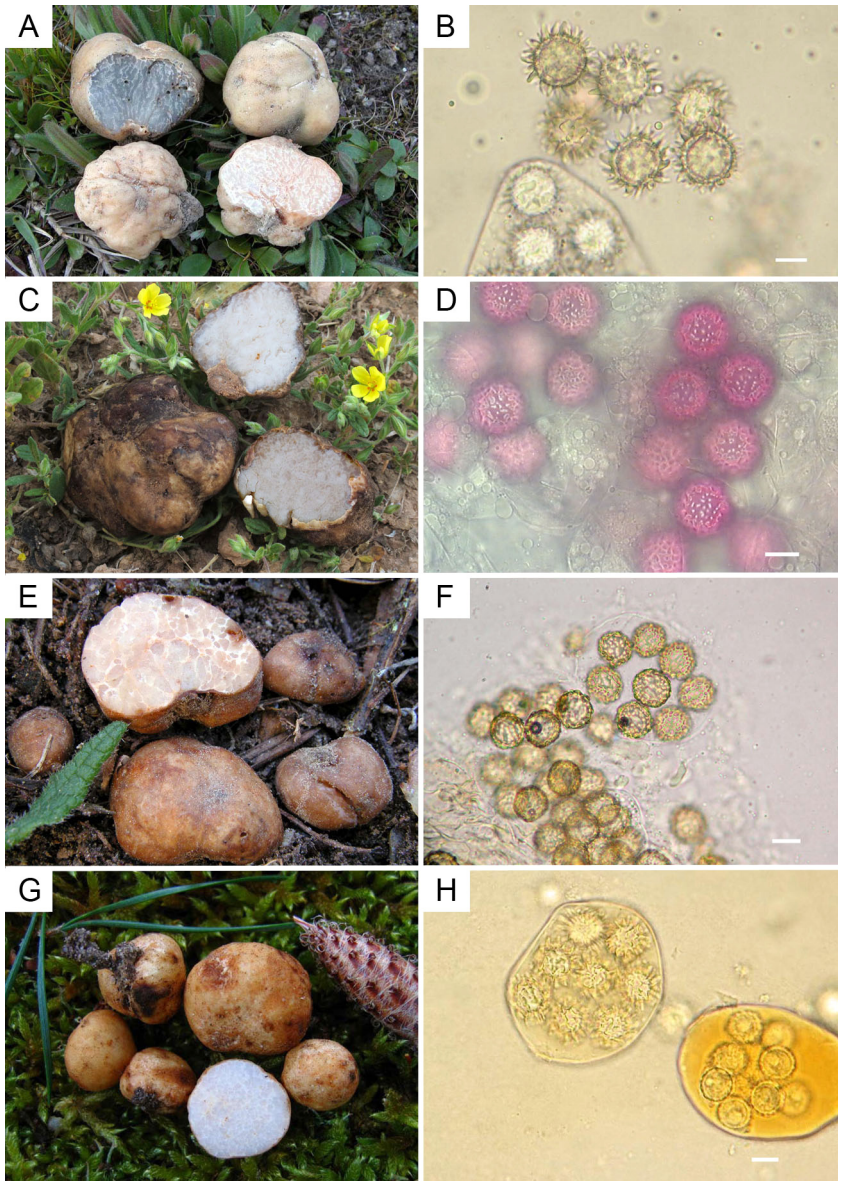


Plate 2. ***Terfezia extremadurensis*** (holotype, MUB Fung-0026): A, ascocarps; B, ascospores. ***T. eliocrocae*** (MUB Fung-0016): C, ascocarps; D, ascospores. ***T. alsheikhii*** (MUB Fung-0009): E, ascocarps; F, ascospores. ***T. pini*** (holotype, MUB Fung-0027): G, ascocarps; H, ascospores. Bars = 10 µm.

T. goffartii (25 µm without ornamentation) differentiates that species from *T. pini*. Despite the ascocarps of *T. pini* and *T. leptoderma* resemble each other, their spore ornamentations differ: the Tulasne brothers described the spicules of *T. leptoderma* as isolated (Tulasnes & Tulasne 1951), not crested nor forming a sub-reticulum on the spore surface. Moreover, the exclusive preference for burned oak or pine habitats has not been described for *T. leptoderma*.

Terfezia pseudoleptoderma Bordallo, Ant. Rodr. & Muñoz-Mohedano, **sp. nov.**
MycoBank MB561595 Plate 3a,b

Differs from *T. leptoderma* by its spore spicules with symmetric bases.

Type: Spain, Burgos, Villafranca Montes de Oca, Puerto de la Pedraja, 12 May 2008, leg F. Sáinz (**Holotype**, MUB Fung-0028).

Etymology: referring to the morphological similarity with *Terfezia leptoderma.*

Ascomata globose, round, quite regular, ≤2 cm diam., smooth when immature, partially rough when mature; initially cream colour, maturing to reddish-brown. Peridium smooth or slightly rough, inseparable from the gleba; cream colour, darkening where exposed to air. Gleba initially whitish, with the fertile tissue forming translucent greyish-blue islets surrounded by white veins or sterile tissue. Asci 8-spored; globose to ovoid. Ascospores spherical, 19–23 µm diam. (including ornamentation), spines separate, blunt, 2–5 µm long with bases asymmetric, ≤1 µm diam.; initially hyaline, yellowing in age.

Habitat — associated with cistaceous plants near pine and oak forests.

Additional collection examined: **SPAIN**: **Extremadura**, Cáceres: Valdecañas, 2010, Muñoz-Mohedano (MUB Fung-0039).

Comments — The ascocarps of *T. pseudoleptoderma* (Clade 5) are similar in size to *T. leptoderma,* which differs in the symmetric bases of the spore spicules.

In general, we noticed considerable confusion among authors who identify specimens as *T. leptoderma* that are much bigger than those described by Tulasne brothers.

Terfezia fanfani Mattir., Malpighia 14: 71. 1900. Plate 3c,d

Ascomata globose, round, regular, occasionally lobed, usually with mycelial remnants, 2–5 cm diam., smooth or slightly rough; firm; usually odourless. Peridium smooth, slightly rough, inseparable from the gleba, thin, 200–700 µm thick; initially white, soon becoming reddish brown, darkening in maturity with black maculae present in sun-exposed zones exposed; white in cross-section; pseudo-parenchymatous structure formed by rounded prismatic cells. Gleba initially white, then fertile tissue in islets becoming pale pink, then olive green, finally blackish grey when very mature. The gleba is always surrounded by white sterile tissue. Asci primarily 8-spored, sessile; subglobose, elongated

and ovoid, 70–80 × 55–70 µm diam. with 1 µm thick walls. Ascospores spherical with spines, 19–23(–25) µm diam. (including ornaments), initially hyaline, smooth, with a large central drop; in age ochre yellow and decorated with sharp thin elongated conic spines (2–)3–4(–5) µm long with 1 µm diam. bases (the spines not joined through the bases).

Habitat — acidic grasslands associated with *Tuberaria guttata* from the end of March to the end of April.

collections examined: **SPAIN**: **Extremadura**, Cáceres: Belvis, 2009, Muñoz-Mohedano (MUB Fung-0032); Garrovilla, 2004, A. Mateos (MUB Fung-0008); Valdecañas, 2001, Muñoz-Mohedano (MUB Fung-0004); Badajoz: Quintana de Serena, 2009, M. Romero (MUB Fung-0033); **Castile & León**, Valladolid: Cortas de Blas, 1998, Aurelio García (MUB Fung-0011).

Comments — *Terfezia fanfani* is recognized by its reddish colour, its growth with *Tuberaria guttata,* and its spores decorated with long and isolated spines. Initially hypogeous, it later it rises to the surface. Sharing both habitat and fruiting season with *T. arenaria*, *T. fanfani* fruits before *T. arenaria,* usually preferring saltier and less deep soils.

In size, *T. fanfani* seems better to match the Clade 6 specimens that we examined as well as those cited by other authors (Calonge et al. 1977; Ceruti 1960; Janex-Favre et al. 1988; Moreno et al. 1986). Thus for both phylogenetic and morphological reasons, we believe this species is independent.

Terfezia albida Ant. Rodr., Muñoz-Mohedano & Bordallo, **sp. nov.** Plate 3e,f

MycoBank MB561596

Differs from all other spiny-spored *Terfezia* spp. in its larger ascomata, its white peridium, and its spermatic odour.

Type: Spain, Albacete, El Ballestero, 2 May 2009, leg A. Rodríguez (**Holotype**, MUB Fung-0029).

Etymology: referring to the external whitish colour.

Ascomata hypogeous to partially emergent at maturity, 2–4 cm across, 3–4 cm high, subglobose to turbinate, pulvinate, often with tapered, sterile base, white at first, becoming light cream, often black spots on the sun-exposed parts or where handled, greenish with age on injured areas, smooth. Peridium 200–500 µm thick, poorly delimited, white in cross section, pseudoparenchymatous, composed of subglobose cells of variable size, hyaline and thin-walled in the innermost layers, yellowish and with thicker walls in the outermost layers. Gleba solid, fleshy, succulent, white at first, maturing to grayish green pockets of fertile tissue separated by whitish, sometimes with pink spots, sterile veins. Spermatic odour, stronger in young specimens. Asci inamyloid, subglobose to ovate, sessile or short-stipitate, 70–85(–90) × 55–70 µm, walls 1 µm thick, with 6–8 irregularly disposed spores, randomly arranged in the gleba. Ascospores

globose, (18–)19–22(–23) µm diam (median = 20 µm) including ornament, 14–17(–18) µm (median = 16 µm) without ornament, hyaline, smooth and uniguttulate at first, by maturity yellow ochre and ornamented with conical, blunt, straight spines, sometimes cylindrical and curved, sometimes truncated, separate, 2–3 µm long, 1–2 µm wide at the base, sometimes connected to form a pseudo-reticulum.

Ecology & distribution — southeastern Iberian Peninsula, limited to arid and semiarid areas in calcareous alkaline soils, associated with *Helianthemum* spp., from late April to mid May.

Additional collections examined: **SPAIN**: **Castile–La Mancha**, Albacete: Albacete, 2009, A. Rodríguez (MUB Fung-0037).

Comments — *Terfezia albida* (Clade 7) differs from other spiny-spored *Terfezia* species in its larger average size, white peridial colour, and spermatic odour. It is the only spiny-spored *Terfezia* species associated with *Helianthemum* spp. in alkaline soils. *Terfezia eliocrocae* (described above) and *T. claveryi* share the same habitat but differ in their reticulate spores.

Clade 7 includes taxa with different morphologies, distributions, and host plants from its sister clade, Clade 8 (where *T. olbiensis* is found). The two clades are separated by a 72% bootstrap value (Plate 1). Only after wide sampling we were able to place *T. albida* in Clade 7 (associated with cistaceous plants) and *T. olbiensis* in Clade 8 (associated with pine and oak hosts).

Terfezia olbiensis Tul. & C. Tul., G. Bot. Ital.1(2(7–8)): 60. 1845. Plate 3g,h

Ascomata globose, round, regular, rarely presents pseudostipe, 2–5 cm across, smooth. Peridium smooth, inseparable from the gleba, thin, 300–500 µm thick, not clearly delimited; initially cream, becoming brown, frequently with black maculae where exposed to the sun or bruised, white in cross-section; pseudoparenchymatous structure formed by ± rounded thin-walled hyaline cells of different sizes that yellow and become prismatic towards the periphery. Gleba initially white, then fertile tissue forming small grey (later greenish grey) islets surrounded by salmon-tinged white sterile tissue. Asci sessile to occasional on a short thick peduncle, 8-spored, ellipsoidal to ovoid, citrus-shaped, 60–90 × 50–60 µm with 1–2 µm thick walls; dextrinoid when immature. Ascospores spherical and spiny, 15–19 µm (including ornament) diam., initially hyaline, smooth, and with a great central drop, when mature ochre yellow and covered by pointy thin conical 1–2 (–2.5) µm long (base = 1 µm) spines (some truncated) that are sparse and not joined through the base.

Habitat — *Terfezia olbiensis* grows in limestone and clayey pine and oak woodlands without *Helianthemum* spp. from mid-March to mid-April. It is the first *Terfezia* species to produce ascocarps in limestone soils.

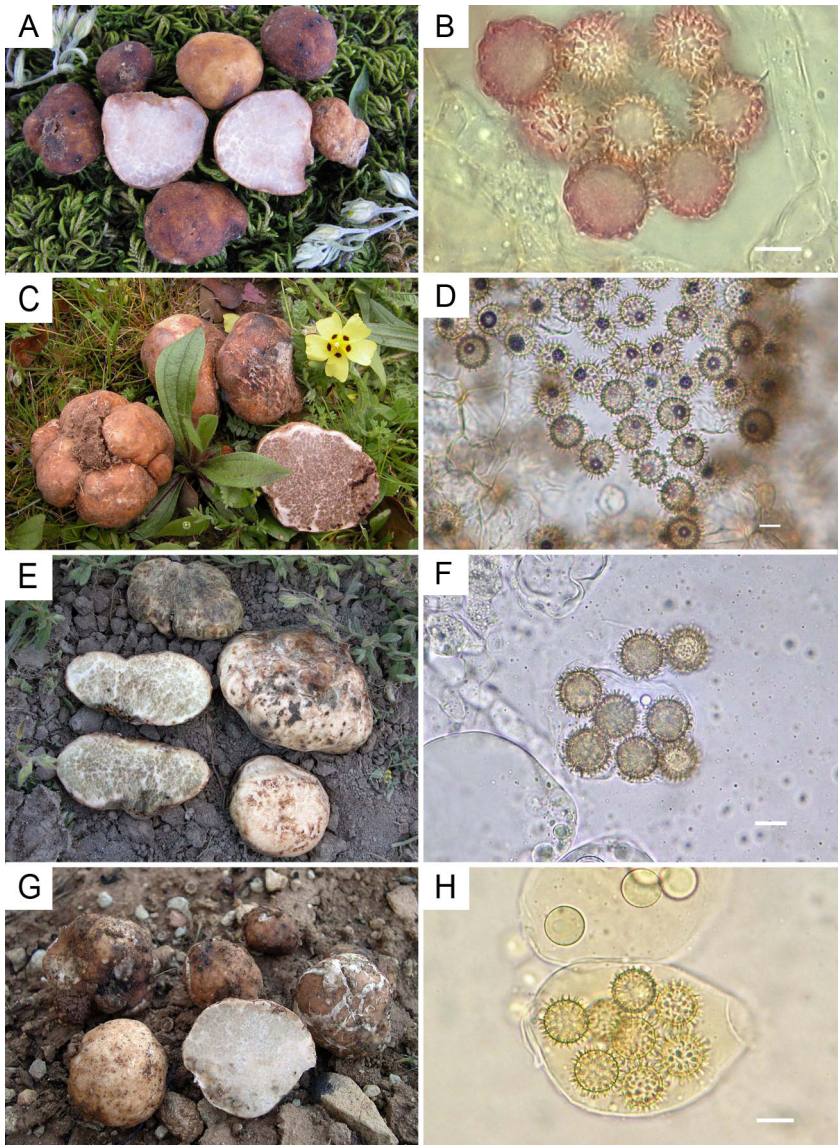


Plate 3. ***Terfezia pseudoleptoderma*** (holotype, MUB Fung-0039): A, ascocarps; B, ascospores. ***T. fanfani*** (MUB Fung-0032): C, ascocarps; D, ascospores. ***T. albida*** (holotype, MUB Fung-0029): E, ascocarps; F, ascospores. ***T. olbiensis*** (MUB Fung-0031): G, ascocarps H, ascospores. Bars = 10 μm.

COLLECTIONS EXAMINED: **SPAIN**: **CASTILE–LA MANCHA**, ALBACETE: Masegoso, 2009, A. Rodríguez (MUB Fung-0031); **CASTILE & LEÓN**, VALLADOLID: Santa Espina, 1998, Aurelio García (MUB Fung-0013); **VALENCIA**: Onteniente, 2009, F. García (MUB Fung-0030).

COMMENTS — Initially hypogeous, *T. olbiensis* later rises to the surface, where it remains firm. The fruitbodies usually are parasitized underground by larvae or eaten by rabbits, probably because they appear early in the year, when there is greater humidity and less sunlight. The odor is distinctive (somewhat rotten); the flavour is less than other edible *Terfezia* species. The most distinguished characteristic is the reduced length of its spines, always shorter than 2.5 µm.

Although similar to *T. leptoderma, T. olbiensis* has a bigger ascocarp (hazelnut- to walnut-sized) and smaller ascospores. Clade 8 taxa have different morphological and distribution characteristics from the other clades, which is also supported by the phylogenetic analyses with a 72% bootstrap value (PLATE 1). Therefore, we conclude that Clade 8 is formed by *T. olbiensis*.

Discussion

All the species described herein produce similar sized spiny to reticulate ascospores, which has led to the belief that they represent only one species. Our exhaustive comparison of the ascocarps and ascospores and comprehensive ITS-rDNA sequence analyses support recognition of several species.

Our results agree with those of Díez et al. (2002), who recognized three clades separated by a high statistical support (89%, 98%) based on morphological (gleba structure, size of ascospore spicules, ascospore size) and biological (acidic vs. alkaline soils, cistaceous vs. pine–oak hosts) characters. We propose Clade 4 for *Terfezia pini* based on data from Barriuso et al. (2008; see GenBank DQ386140). Moreover, a new sequence deposited by Rincón (unpublished data: GenBank FJ013064) belongs to Clade 5. In their later phylogenetic analyses of *Terfezia* spp., Ferdman et al. (2005) clearly separate DNA sequences AF276679, AF276678, AF387657, and AF387648 into two strongly supported different clades (100/99 bootstrap value) as *T. leptoderma* and *T. olbiensis* (Clade 8). This scenario is contrary to Malençon's opinion (see Díez et al. 2002, Moreno et al. 1986) that *T. olbiensis* represents an immature form of *T. leptoderma*.

The group covering species diagnosed by a spiny episporium (Clades 1, 4, 5, 6, 7 and 8) is paraphyletic, suggesting that episporium ornamentation is not a homologous character. Therefore, we do not consider episporium ornamentation a good taxonomic character, in contrast to other authors such as Mattirolo (1900).

Moreover, although AF396864 was classified as *T. leptoderma* (Díez et al. 2002), our analyses place it in a different clade by a relatively high bootstrap value (73/77). More sequences are needed to confirm its identity.

Our phylogenetic analyses corroborate the conclusion by Díez et al. (2002) that fungus-host specialization and soil tolerance are the keys that allow us to separate different species (clades). The monophyly of the proposed clades (species) (Plate 1) is congruent with the morphological (Plates 1 and 2) and biological (Table 1) data reported for the studied taxa.

Distributed throughout temperate and subtropical regions of the northern hemisphere, *Cistaceae* show the highest genus and species diversity in the Mediterranean floristic region (Guzmán & Vargas 2009). The Iberian Peninsula hosts a high diversity of *Cistaceae* and high number of mycorrhizal fungal species. Recent studies suggest that *Helianthemum almeriense* strongly depends on the presence of a fungal symbiont in its roots for survival (Morte et al. 2010). The Iberian Peninsula seems to be a desert truffle reservoir and further study and sampling are necessary in these ecosystems.

As recently highlighted by Bidartondo et al. (2008), we also emphasize the importance of an accurate identification of specimens whose DNA sequences have been subsequently registered in public databases, such as GenBank. Mistakes made in species identification can lead to erroneous and false conclusions. Checking the databases with contrasted data is necessary to prevent the perpetuation of errors when identifying new specimens.

Key to examined *Terfezia* species

Ascospore measurements include ornamentation.

1a. Fertile tissue does not create islets or reticulate ascospores 2
1b. Fertile tissue creates islets and spiny ascospores 3
2a. Ascospores with spines ..... ***T. extremadurensis*** (Clade 1)
2b. Ascospores reticulate 4
3a. Ascospores <20 µm and spines <2.5 µm ..... ***T. olbiensis*** (Clade 8)
3b. Ascospores ≥20 µm 5
4a. White gleba ..... ***T. eliocrocae*** (Clade 2)
4b. Pink gleba ..... ***T. alsheikhii*** (Clade 3)
5a. Spines <3 µm ..... ***T. albida*** (Clade 7)
5b. Spines >3 µm 6
6a. Crested spines ..... ***T. pini*** (Clade 4)
6b. Isolated spines 7
7a. Spines blunt with asymmetric base; ascomata <2 cm diam ..... ***T. pseudoleptoderma*** (Clade 5)
7b. Spines pointed with symmetric base; ascomata 2–5 cm diam .. ***T. fanfani*** (Clade 6)

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