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Two new *Terfezia* species from Southern Europe

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Abstract

Two new species of *Terfezia*, *Terfezia grisea* and *Terfezia cistophila*, are documented from Spain and Greece, based on morphology and ITS-rDNA sequence data. Macro and micro descriptions with illustrations and ITS phylogenetic data for the two species are provided, which are discussed in relation to similar species in this genus and their host plants.

Key words: desert truffle, hypogeous, mycorrhizal fungi, *Pezizaceae, Cistaceae*

Introduction

The genus *Terfezia* (Tul. & C.Tul.) Tul. & C.Tul. is included in the *Pezizaceae* (Laessøe & Hansen 2007) within the order *Pezizales*. The edible hypogeous ascomata of these fungi are known as "desert truffles" due to their habitat, which is typically arid and semi-arid ecosystems, mostly in the Mediterranean region (Morte *et al*. 2009, Zambonelli *et al*. 2014) and constitute an important economic resource for the local populations (Shavit 2014). Species of *Terfezia* have a long history of culinary and medical uses, because they are rich in nutrients and bioactive compounds (Shavit & Shavit 2014). While in some areas, desert truffles have been traditionally used as food, in most regions interest has only recently been increasing, and these fungi are now treasured for their economical and nutritional value and for research (Kagan-Zur *et al*. 2014).

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, Morte & Andrino 2014), and with trees from different phyla (Bordallo *et al*. 2013, Díez *et al*. 2002, Taylor *et al*. 1995). These plants and their associated fungi play a major role in the maintenance of Mediterranean shrub lands and xerophytic grasslands, and thus in preventing erosion and desertification (Honrubia *et al*. 2014). In fact, this mycorrhizal association is well adapted to semiarid climates through the physiological mechanism of drought avoidance (Morte *et al*. 2000, 2010, Turgeman *et al*. 2011). The soils of desert truffles show a remarkable variability that reflects the climatic conditions in which they form. *Terfezia* species (or their host) seem to be able to adapt to a wide range (high or relatively low) of soil pH, edaphic conditions and texture (Bonifacio & Morte 2014).

Some species have been successfully cultivated and new biotechnologies to increase their productive yield and to extend their cultivation areas have been developed (Morte *et al*. 2008, 2009, 2012, Slama *et al*. 2010, Honrubia *et al*. 2014).

Kirk *et al*. (2008) estimated that there are 12 species of *Terfezia* worldwide, while Index Fungorum (2015) lists 48 species. The application of novel molecular methods to hypogeous fungal group, on which desert truffles can be found, allows the discovery of new species. The finding of undiscovered species within the genera of desert truffles will rise throughout the coming years (Bordallo & Rodríguez 2014), similar to those carried out on genera like *Tuber* (Bonito *et al*. 2010).Recently, five species of *Terfezia* have been reported from the Iberian Peninsula (Bordallo *et al*. 2013) and one from the Canary Islands (Bordallo *et al*. 2012). The difficulty of sampling desert truffles implies their discovery only in specific locations. This allows the hypothesis that a thorough study of the same and other collection zones and during different seasons of the year would favour the discovery of new species (Claridge *et al*. 2000a, b, Henkel *et al*. 2012).

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

Material and Methods

Collecting sites and collections

Ascomata of *Terfezia* spp. were collected in different years and from different locations in Spain and Greece. Specimens used in this study are listed in Table 1. In the collection seasons of the year (from February to June), fresh specimens were photographed in the field, including the plants in the vicinity of where they were found, and brought to the laboratory for macro-morphological study. Collections were frozen at –20 ºC for DNA analysis and dried at 40 °C and stored in sealed plastic bags, labeled with collection details. The samples are deposited in the Herbarium of the University of Murcia (MUB), Spain.

TABLE 1. List of *Terfezia* collections used in this study.

Specimen	Genbank nº	Origin	Year	Collector
j327	KP189328	Burgos, Spain	2013	F. Sainz
j386	KP189329	Schinias Attica, Greece	2009	V. Kaounas
j388	KP189330	Schinias Attica, Greece	2011	V. Kaounas
j389	KP189331	Schinias Attica, Greece	2013	V. Kaounas
j476	KP189332	Schinias Attica, Greece	2014	V. Kaounas
j485	KP189333	Burgos, Spain	2013	F. Sainz
j34	KP728821	Badajoz, Spain	2010	A. García
j35	-	Badajoz, Spain	2010	A. García
j36	-	Badajoz, Spain	2010	A. García
j37	KP728822	Badajoz, Spain	2010	F. Camello
j38	-	Caceres, Spain	2010	F. Camello
j113	KP728824	Badajoz, Spain	2010	A. Rodríguez
j377	KP728825	Artemida Attica, Greece	2013	V. Kaounas
j384	KP728826	Rafina Attica, Greece	2013	V. Kaounas
j392	KP728827	Zagora Magnesia, Greece	2008	V. Kaounas
j473	-	Nea Makri Attica, Greece	2014	V. Kaounas
j474	-	Nea Makri Attica, Greece	2014	V. Kaounas
j475	-	Nea Makri Attica, Greece	2014	V. Kaounas
j477	KP728828	Zagora Magnesia, Greece	2014	V. Kaounas
j479	KP728829	Zagora Magnesia, Greece	2014	V. Kaounas

Morphological study

Morphological characters were described from fresh specimens. External ascocarp characteristics (shape, colour, appearance) were recorded in detail. Ascomata were then cut and the morphology of the peridium and gleba was described.

Microscopic study was performed in distilled water, KOH 5% and Melzer's reagent. Spores dimensions are based on at least 80 randomly selected spores outside asci in distilled water mount. Asci and ascospores were examined using an Olympus BX51 microscope equipped with a digital camera (Canon PSpro1).

For identification, ascomata were compared with descriptions from Bordallo *et al.* (2013). The descriptions of *Terfezia leptoderma* Tul., *T. fanfani* Mattir., *T. cadevalli* Font Quer, *T. hafizi* Chatin, *T. berberiodora* Lesp. ex Tul. & C.Tul., and *T. goffartii* Chatin were also checked.

Molecular study

DNA extraction. Genomic DNA was isolated from 150–200 mg of the outer 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.

PCR amplification and sequencing. The Internal Transcribed Spacer (ITS) region of the rDNA, including the 5.8S ribosomal gene, was amplified using the universal ITS1F and ITS4 primers (White *et al.* 1990, Gardes & Bruns 1993). All PCR amplifications were carried out in a final volume of 25 µL containing 0.2 mM of each dNTP, 0.4 µM of each primer, 5.2 mM $MgCl_2$, 0.625X PCR buffer and 1.25 U of Taq DNA polymerase (Bioline UK). 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, 45 cycles consisting of 30 s at 94 °C, 1 min at 60 °C, 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).

Sequence alignment and phylogenetic analysis. The ITS sequences of the fourteen samples of the *Terfezia* species (Table 1), and the closely related sequences from GenBank, were assembled with Clustal X (Thompson *et al.* 1997) followed by manual adjustment to improve the alignments. The phylogenetic analysis was carried out using MEGA4 (Tamura *et al.* 2007). The evolutionary history was inferred using the Neighbor-Joining method (NJ; Saitou & Nei 1987) and Maximum Parsimony method (MP; Eck & Dayhoff 1966), using a total of 41 taxa. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The NJ tree is 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 in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 446 positions in the final dataset.

The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 446 positions in the final dataset, out of which 84 were parsimony informative.

The sequences from *Tirmania nivea* (Desf.) Trappe, *Tirmania pinoyi* (Maire) Malençon, *Peziza depressa* Pers. and *Peziza ellipsospora* (Gilkey) Trappe were chosen as outgroup.

Results

Phylogenetic analysis

Sequence analyses of the ITS-rDNA from the examined samples produced two phylogenetic trees based on the Neighbor-Joining (NJ) and the Maximal Parsimony (MP) methods, both with a virtual sampling or bootstrap of 500 replicas (Fig. 1). The six sequences of the new *Terfezia grisea* clustered together forming a homogenous monophyletic clade, independently of their origin from Spain or Greece (Fig. 1). Similarly, the sequences of *T. cistophila* are distinguished by 90% (NJ) bootstrap supports with the closely related *T. fanfani* (Fig. 1).

Taxonomy

Terfezia grisea Bordallo, V. Kaounas & Ant. Rodr., sp. nov. Fig. 2
MycoBank 810936

Type:—GREECE, Attica, Schinias, 12 April 2011, leg V. Kaounas (**Holotype**, MUB Fung-j388).

Ascomata hypogeous to partially emergent at maturity, solitary, 1–2.5 cm in size, tuberiform, subglobose, often conical sterile base, initially pale rusty, in places spotted whitish, later brown, rusty or ochraceous brownish, in places blackish brown to almost black, smooth (fig. 2 A–D). *Peridium* not separable from gleba, 200–400 µm thick, poorly delimited, 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 with greyish pockets at first (Fig. 2 A&B), maturing to blackish gray pockets of fertile tissue separated by whitish, sterile veins (Fig. 2 C&D). Faint odour, no distinctive. Mild taste.

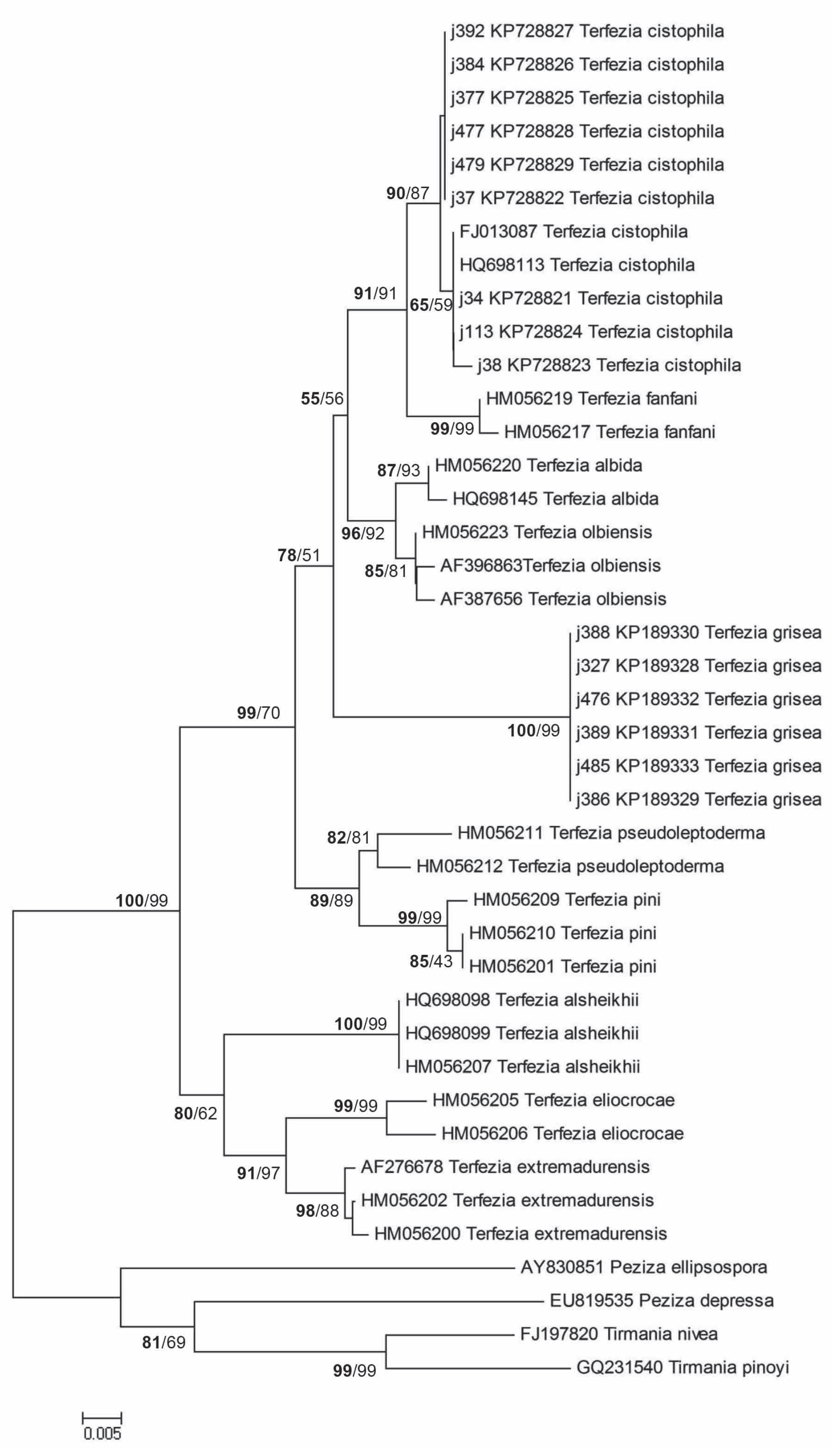

FIGURE 1. Neighbor-Joining (NJ) consensus phylogenetic tree based on ITS sequences. Bold numbers are percentages of 1000 bootstrapping replicates supporting the NJ tree presented here. Non-bold numbers are percentages of 500 bootstrapping replicates supporting the same node by the MP method.

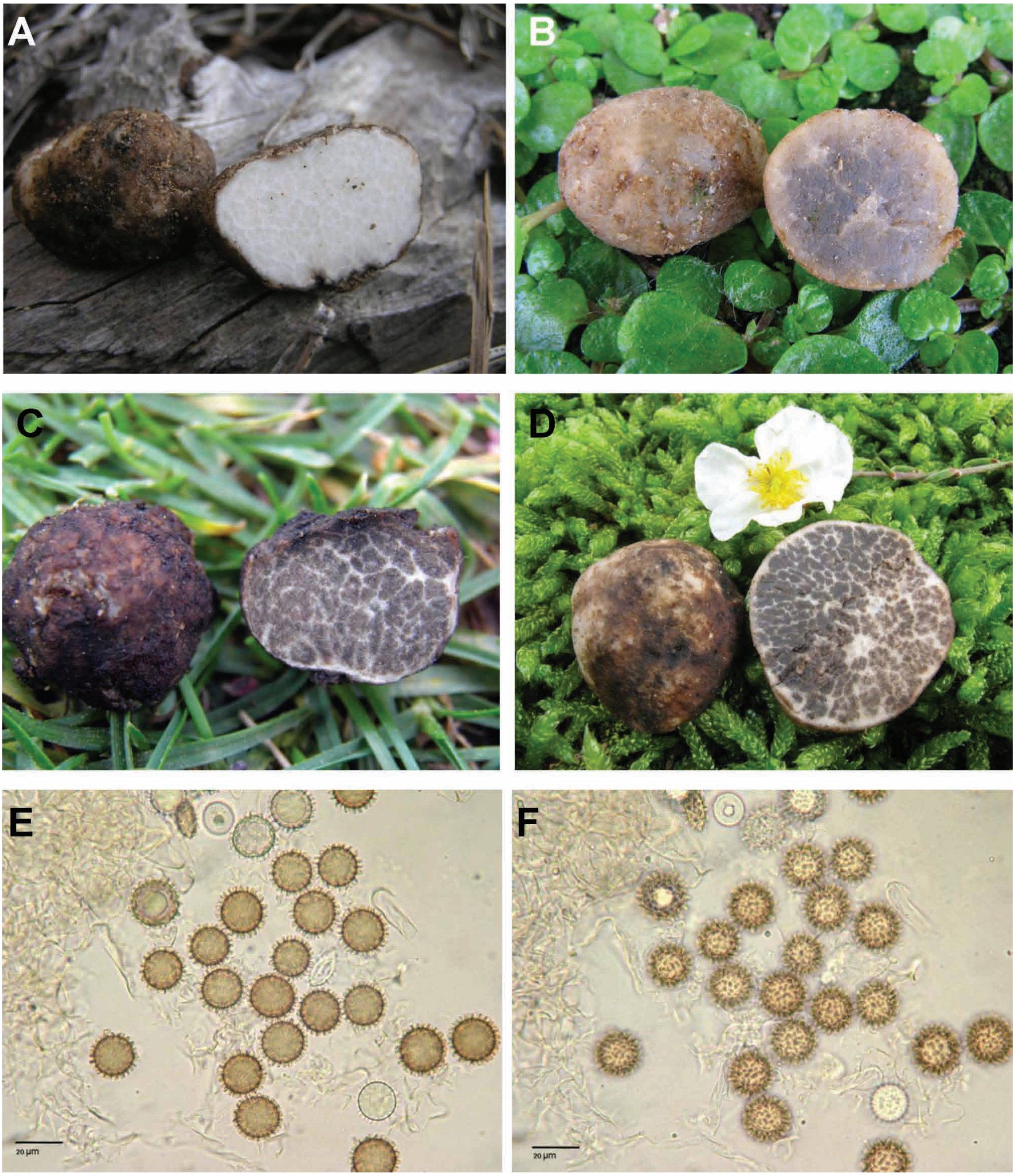

FIGURE 2. *Terfezia grisea*: (A) inmature ascocarp, (B) semi-mature ascocarp, (C, D) mature ascocarps, (E, F) ascospores. Bars: 20 µm.

Asci inamyloid, subglobose to ovate, sessile or short-stipitate, 60–80 x 40–60 µm, walls 1 µm thick, with 6–8 irregularly disposed spores, randomly arranged in the gleba. *Ascospores* globose, (18–)19–21(–22) µm diam (mean= 21 µm) including ornament, (15–)16–17(–18) µm (mean= 16 µm) without ornament, hyaline, smooth and uniguttulate at first, by maturity yellow ochre and ornamented with conical, sometimes truncated, separate, blunt spines, 2–3 µm long, 1–2 µm wide at the base (Fig. 2 E&F).

Ecology and Distribution:—alkaline, sandy soils, in a coastal pine forest in Greece, in grassland areas without trees in Spain, associated with *Helianthemum* spp., from March to June in Spain, March to April in Greece.

Etymology:—referring to its grey appearance gleba.

TABLE 2. Synopsis of the main taxonomic characteristics of spiny-spored *Terfezia* species, according to their original descriptions.

Species	*T. grisea*	*T. cistophila*	*T. extremadurensis*	*T. pini*	*T. pseudoleptoderma*	*T. fanfani*	*T. albida*	*T. olbiensis*	*T. leptoderma*
Ascomata size (cm across)	1–2.5	0.5–2	2–5	<2	≤2	2–5	2–4	2–5	<2
Peridium (µm)	200–400, poorly delimited, whitish at first, becoming blackish brown	150–400, poorly delimited, whitish at first, becoming intense black	300–600, well-defined, cream colour at first, becoming brown	200–400, smooth, slightly tomentose, not clearly delimited, cream colour, becoming ochre and grey	200–400, smooth or slightly rough, cream colour, darkening where exposed to air	200–700, initially white, soon becoming reddish brown, darkening in maturity with black maculae	200–500, poorly delimited, white at first, becoming light cream, greenish with age on injured areas	300–500, not clearly delimited; initially cream, becoming brown, frequently with black maculae	Slightly tomentose, not clearly delimited. Greyish
Gleba color	whitish with greyish pockets at first, maturing to blackish gray pockets	whitish with greyish pockets at first, maturing to light ochre, darkening to pale brown at maturity	whitish at first, soon becoming salmon pink, darkening with age, greenish grey at maturity	initially whitish; round islets initially pale pinkish becoming greenish brown and greyish at maturity	initially whitish, with the fertile tissue forming translucent greyish-blue islets surrounded by white veins	initially white, then fertile tissue in islets becoming pale pink, then olive green, finally blackish grey at maturity	white at first, maturing to greyish green pockets	initially white, then fertile tissue forming small grey - greenish grey islets	Whitish even at maturity
Odour	no distinctive	spermatic	no distinctive	no distinctive	no distinctive	no distinctive	spermatic	no distinctive	no distinctive
Asci size (µm)	60–80 x 40–60	55–65 x 45–50	60–80 × 50–65	60–90 × 45–60	60–85 x 45–85	70–80 × 55–70	70–85 × 55–70	60–90 × 50–60	60–80 × 50–60
Spore size (µm) (including spines)	(18–)19–21(–22)	(16–)17–20(–21)	(21–)22–26(–27)	20–23(–25)	19–23	19–23(–25)	(18–)19–22(–23)	15–19	19–24
Spine size (µm) (long x base wide)	2 –3 x 1–2	1.5 –2.5 x 1	3–4(–5) x 1–3	3–4(–5) x 1	2–5 x ≤1	(2–)3–4(–5) x 1	2–3 x 1–2	1–2 (–2.5) x 1	-
Soil pH	alkaline	acid	acid	acid	acid	acid	alkaline	alkaline	acid
Host plant	*Helianthemum* spp	*Cistus* spp	*Tuberaria guttata*	*Pinus* spp, *Quercus* spp	*Cistaceae*	*Tuberaria guttata*	*Helianthemum* spp	*Pinus* spp, *Quercus* spp	*Pinus* spp

Additional collections examined:—GREECE: ATTICA, Schinias, 2009, V. Kaounas (MUB Fung-j386). Same locality, 2013, V. Kaounas (MUB Fung-j389); 2014, V. Kaounas (MUB Fung-j476). SPAIN: CASTILLA Y LEÓN, BURGOS: Llano de Bureba, 2013, F. Sáinz (MUB Fung-j327); Solduengo, 2013, F. Sáinz (MUB Fung-j485).

Notes:—*Terfezia grisea* is a spiny-spored *Terfezia* species characterized by its ochraceous brownish, almost black peridium, blackish gray gleba and growing in alkaline sandy soils associated with *Helianthemum spp*. *T. albida* and *T. olbiensis* also grow in alkaline but clayey soils. Moreover, *T. albida*, although associated with *Helianthemum spp*., has larger ascomata, white peridium, grayish green gleba and spermatic odour. And for its part, *T. olbiensis* has larger ascomata and smaller spores than *T. grisea* (Table 2). In addition, the phylogenetic analysis distinguished the new taxon from the other species (Fig. 1).

Terfezia cistophila Ant. Rodr., Bordallo, V. Kaounas, & Morte, sp. nov. Fig. 3
MycoBank 811777

Type:—GREECE, Magnesia, Zagora, 25 April 2014, leg V. Kaounas (**Holotype**, MUB Fung-j477).

Ascomata hypogeous to partially emergent at maturity, solitary or gregarious, 0.5–2 cm in size, subglobose, often basal depression with a mycelial tuft, sometimes rounded sterile base, light beige at first, becoming dark reddish brown, with black spots, with some pitting at maturity, smooth (Fig. 3 A&B). *Peridium* poorly delimited, 150–400 µm thick, pseudoparenchymatous, composed of subglobose cells, 10–60 µm diam, hyalines and thin-walled in the innermost layers, yellowish and with thicker walls, up to 2,5 µm thick, in the outermost layers. *Gleba* solid, fleshy, succulent, whitish with greyish pockets at first, maturing to light ochre, darkening to pale brown at maturity, pockets of fertile tissue separated by whitish, sterile veins, sometimes with pink salmon spots (Fig. A–B). Faint odour, spermatic, more remarkable in young specimens. Mild taste.

Asci nonamyloid, subglobose to ovate, sessile or short-stipitate, 55–65 x 45–50 µm, walls 1 µm thick, with 6–8 irregularly disposed spores (Fig. 3 E), randomly arranged in the gleba. *Ascospores* globose, (16–)17–20(–21) µm diam (mean = 18.5 µm) including ornament, 13–16 µm (mean= 14.5 µm) without ornament, hyaline, smooth and uniguttulate at first, by maturity yellow ochre and ornamented with conical, separate, pointed, sometimes truncated spines, 1.5 –2.5 µm long, 1 µm wide at the base (Fig. 3 E&F).

Ecology and Distribution:—*Terfezia cistophila* grows in acid soils, associated with *Cistus monspeliensis* L. and *Cistus creticus* L., from February to April, in Greece and associated with *Cistus ladanifer* L., from April to May in Spain, Extremadura.

Etymology:—referring to its host plants affinity, which are mainly *Cistus* species.

Additional collections examined:—GREECE: ATTICA, Artemida, 2013, V. Kaounas (MUB Fung-j377) ; Rafina, 2013, V. Kaounas (MUB Fung-j384); Nea Makri, 2014, V. Kaounas (MUB Fung-j474) ; MAGNESIA, Zagora, 2009, V. Kaounas (MUB Fung-j392). Same locality, 2014, V. Kaounas (MUB Fung-j479). SPAIN: EXTREMADURA, BADAJOZ: San Vicente de Alcántara, 2010, A. García (MUB Fung-j034). Same locality, 2010, F. Camello (MUB Fung-j037). CÁCERES: Cedillo, 2010, F. Camello (MUB Fung-j038); Aliseda, 2010, A. Rodríguez (MUB Fung-j113).

Notes:—*Terfezia cistophila* is a spiny-spored *Terfezia* species characterized by its intense blackening of peridium, light ochre gleba, spermatic odour and growing in acid soils associated with *Cistus* spp. It differs from *T. albida*, the other spiny-spored species with spermatic odour, in growing in alkaline clay soils, has larger ascomata, white peridium, grayish green gleba and larger spores. *T. fanfani*, *T. pseudoleptoderma*, *T. extremadurensis*, *T. pini* and *T. leptoderma*, the other spiny-spored species growing in acid soil, have larger spores with distinctly longer spines than *T. cistophila* and no distinctive odour (Table 2). Moreover the new taxon is distinguished from the other species based on ITS sequence identity (Fig. 1).

Discussion

According to the results of the phylogenetic tree, *T. grisea* have a 100% (NJ) bootstrap support and different to other spiny-spored *Terfezia* species previously described (Bordallo *et al.* 2013, Moreno *et al.* 1986, Tulasne & Tulasne 1851). Similarly, *T. cistophila* have a 90% (NJ) bootstrap support and with other two sequences, FJ013087, from uncultured Pezizae from *Pinus pinaster* root tips (Rincón & Pueyo 2010) and HQ698113, catalogued as *Terfezia* aff. *olbiensis* (Kovacs *et al.* 2011), both previously obtained from Spain. The morphological characters of *T. grisea* and *T. cistophyla* (Table 2), together with the molecular analysis data (Fig. 1) provide strong support to recognize as two distinct new species. We compared our new species with all morphologically and phylogenetically similar taxa (Table 2) and elucidated *T. grisea* and *T. cistophila* to be distinct species. Moreover, although other factors might also play a role, host specialization and edaphic tolerances (fungus and/ or host tolerances) might be the key in the species diversity of *Terfezia* genus (Díez *et al.* 2002). In this sense, *T. grisea* shares same characteristics of alkaline soils and *Helianthemum* species as host plants with *T. albida*, but the gleba of the latter is less greyish and it has a spermatic odour that is missing in *T. grisea*. In the case of *T. cistophila*, it shares soil characteristics and some host plant species with *T. pseudoleptoderma* but differs in most of the taxonomic characteristics (Table 2) and phylogenetic distance (Fig. 1).

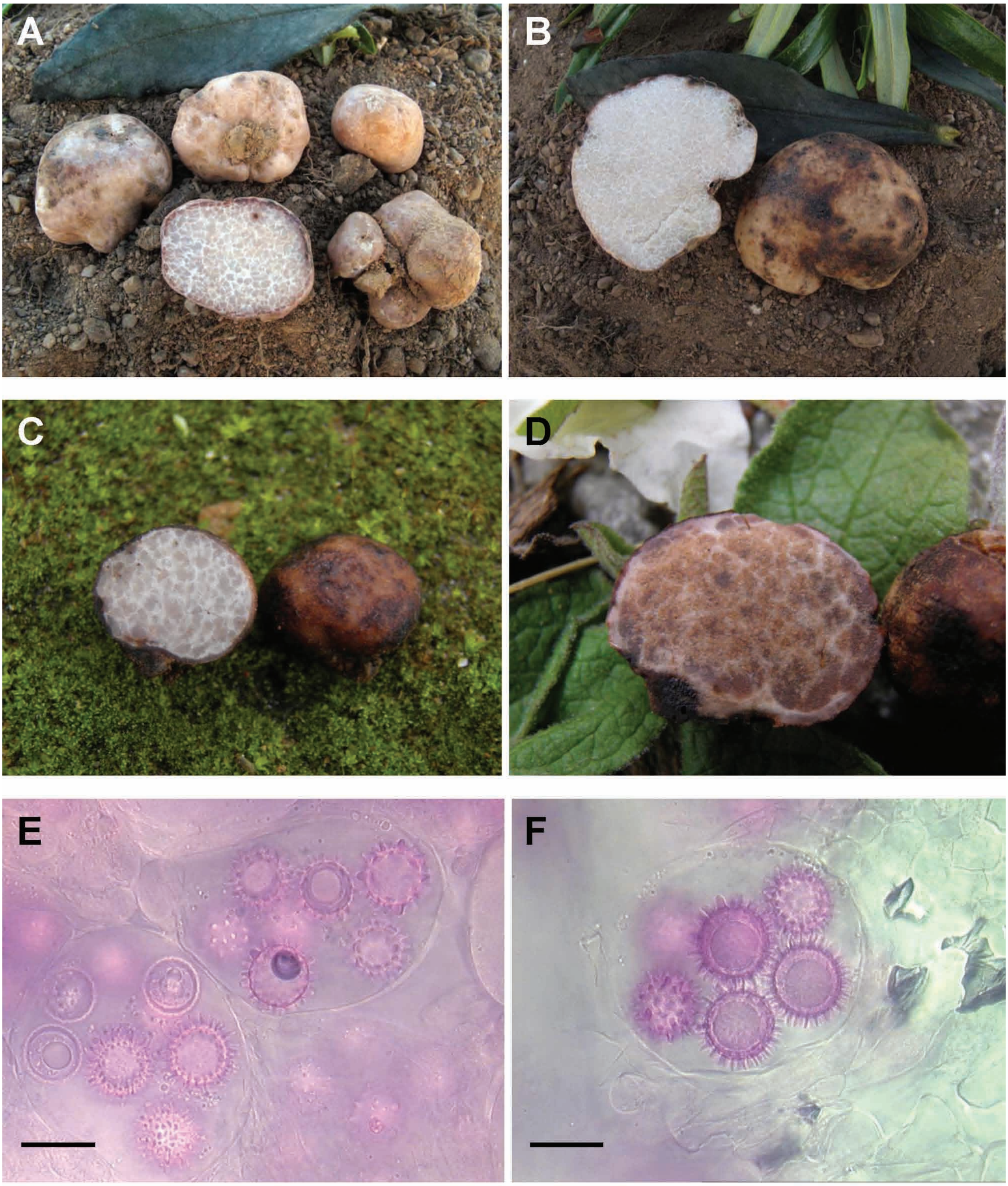

FIGURE 3. *Terfezia cistophila*: (A, B) inmature ascocarp, (C) semi-mature ascocarp, (D) mature ascocarps, (E, F) asci with ascospores. Bars: 20 µm.

The southern European and Mediterranean countries host a high diversity of *Cistaceae* species associated with a high number of mycorrhizal fungal species (Bordallo *et al*. 2013, Oria de Rueda *et al*. 2008, Comandini *et al*. 2006, Gutiérrez *et al*. 2003, Torres *et al.* 1995). However, most of the biogeographical and evolutionary studies that address *Cistaceae* colonization in the Mediterranean (Civeyrel *et al.* 2011, Falchi *et al.* 2009, Guzmán *et al*. 2009, Guzmán & Vargas 2009a, b) did not approach the possible interaction of the mycorrhizal character of these *Cistaceae* species with their distribution pattern. A high mycorrhizal dependence has been observed in some *Cistaceae* species (Morte *et al*. 2010, Honrubia *et al.* 2014), which depend on the presence of a fungal symbiont in their root for survival. Therefore, studies combining evolutionary studies on mycorrhizal fungi and their host plants are needed and they could help to explain the success of new species in different Mediterranean areas. In fact, Ascomycetes, particularly *Tuberaceae* and *Pezizales*, were significantly overrepresented on sampling in burned sites after fire in Mediterranean forest (Rincón *et al.* 2014), where *Pinaceae, Fagaceae* and *Cistaceae* species are the most abundant host plants.

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