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The diversity of *Terfezia* desert truffles: new species and a highly variable species complex with intrasporocarpic nrDNA ITS heterogeneity

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Abstract: Desert truffles belonging to *Terfezia* are well known mycorrhizal members of the mycota of the Mediterranean region and the Middle East. We aimed to test (i) whether the morphological criteria of *Terfezia* species regularly collected in Spain enable their separation and (ii) whether the previously hypothesized edaphic/biotic specificity of one group could be confirmed by study of a larger number of specimens. The species *T. arenaria* and *T. claveryi* can be identified unambiguously by morphological characters. We consider *T. leptoderma* as a distinct species while several lineages of similar spiny spored *Terfezia* truffles with cellular peridium were detected that have no obvious anatomical differences. Several species treated generally as synonyms of *T. olbiensis* have been described in this group, and because they cannot be unambiguously assigned to separate lineages we propose to consider the group as the *T. olbiensis* species complex. A high level of intrasporocarpic variation of the nrDNA ITS was detected in the *T. olbiensis* species complex, especially in one of its lineages. We detected no exclusive specificity to either plant associates or soil, except in *T. leptoderma*, which was associated with *Quercus* spp. and cistaceous plants on acidic soils. Nevertheless the clades showed a tendency either to associate with *Quercus/Helianthemum/Cistus* or *Pinus* hosts. Specimens having distinct anatomical features, reticulate spores and cellular peridium formed a separate group in the molecular phylogenetic analyses of nrDNA ITS and LSU regions; for these specimens we propose a new species, *Terfezia alsheikhii* sp. nov.

Key words: ascomycota, ascospores, concerted evolution, host specificity, ITS variability, Pezizaceae

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INTRODUCTION

Desert truffles are hypogeous ascomycetes living on several continents (Díez et al. 2002, Ferdman et al. 2005, Trappe et al. 2010a) where they play important roles as mycorrhizal partners of plants and their fruit bodies are a potentially important food source (Trappe et al. 2008a, b). A study revealed that fungi adapted to deserts evolved in several lineages of the Pezizaceae (Trappe et al. 2010a). Among the genera in these lineages *Terfezia* represents the best known and probably most frequently collected desert truffles (Díez et al. 2002, Læssøe and Hansen 2007). Although several members of the genus were described from different continents, recent molecular-taxonomic studies revealed that probably only the species from the Mediterranean region and the Middle East belong in *Terfezia* s. str. The genera *Imaia* (Kovács et al. 2008) and *Kalaharituber* (Ferdman et al. 2005) were introduced to accommodate respectively *Terfezia gigantea* Imai from North America and Japan and *Terfezia pfeilii* Henn. from South Africa. The American *Terfezia longii* Gilkey and *T. spinosa* Harkn. also belong in different genera (Kovács et al. 2011), while *Terfezia austroafricana* Marasas & Trappe from South Africa belongs in *Mattirolomyces* (Trappe et al. 2010a, b).

Five *Terfezia* species have been reported regularly from the Mediterranean region and the Middle East; they are *T. arenaria* (Moris) Trappe, *T. boudieri* Chatin, *T. claveryi* Chatin, *T. leptoderma* Tul. and *T. olbiensis* Tul. & C. Tul. (Montecchi and Sarasini 2000). Additional *Terfezia* species have been described from the region but are treated mostly as synonyms of these five species. Four of the five are common in Spain; the fifth, *T. boudieri*, commonly collected in North Africa, Middle East and southwestern Asia, also has been reported from the Iberian Peninsula (Moreno et al. 2002). The true *Terfezia* species are mycorrhizal and have been used for mycorrhizal experiments with plants in the Cistaceae, mainly *Helianthemum* spp. (see references in Kovács et al. 2003). Plantations of inoculated hosts have been established for cultivation of *Terfezia* species with economic importance (Morte et al. 2008). *Terfezia claveryi* Chatin and *T. boudieri* have been used as model organisms for molecular biological studies (Morte et al. 2008, Navarro-Ródenas et al. 2009, Zaretsky et al. 2006).

Terfezia arenaria, *T. claveryi* and *T. boudieri* can be easily separated by morphological characters, whereas

the separation of *T. olbiensis* and *T. leptoderma* are less clear, judging from keys to species of the genus (Montecchi and Sarasini 2000, Alsheikh 1994). *Terfezia olbiensis* and *T. leptoderma* and the other spiny spored *Terfezia* species have been considered as different developmental stages and treated as synonyms by several authors (see DISCUSSION).

Díez et al. (2002) studied Mediterranean *Terfezia* species by both morphological and molecular phylogenetic methods. The ITS regions of 11 specimens of four species were sequenced and analyzed (Díez et al. 2002). They found considerable variation of the ITS sequences of those *T. leptoderma* specimens and hypothesized this variation might relate to edaphic and/or biotic factors such as soil types and/or host plants. Nevertheless because of the small number of specimens studied the question was left unresolved. Intrahyphal, heterokaryotic variation of the nrDNA was found in *T. boudieri* (Aviram et al. 2004) and intraspecific genetic variability was interpreted as a cryptic speciation in the taxon (Ferdman et al. 2009).

We studied *Terfezia* specimens deposited in the Mycological Collection of the Herbarium of the Real Jardín Botánico, Madrid, (MA-Fungi) to test whether (i) the morphological criteria of species regularly collected in Spain enable their unambiguous separation and (ii) the previously hypothesized background of the intraspecific variation of the ITS region of nrDNA could be confirmed by study of a larger number of specimens. In addition, some specimens with distinctive anatomical features found during this research formed a distinct group in the molecular phylogenetic analyses; for these we propose a new *Terfezia* species.

MATERIALS AND METHODS

Microscopic study.—Examination of dried specimens by light microscopy was conducted with rehydrated freehand sections cut from ascomata. Cell, spore and ascus sizes were measured with the aid of an ocular micrometer. Micrographs were obtained with Nikon microscopes equipped with digital cameras. Ascospores were studied by scanning electron microscopy (Hitachi S-3000N SEM) after gold coating.

DNA analyses.—A small piece (~ 15 mg) taken from one place of the dry ascoma was used to extract total genomic DNA with an E.Z.N.A. Fungal DNA Extraction Kit (Omega) following the manufacturer's instructions with small modifications. The ITS region of the nrDNA was amplified and sequenced as described by Martín and Winka (2000). The LR0R-LR5 region was amplified and sequenced as described by Kovács et al. (2008). The electropherograms were checked and assembled with the Staden program package (Staden et al. 2000).

When intrasporocarpic ITS variability was detected during the verification of electropherograms after direct sequencing (see below) the ITS region was amplified again, but with a high fidelity *Pfu* DNA polymerase (MBI Fermentas, Vilnius, Lithuania). Before cloning into a pGEMT Easy Vector system (Promega, Madison, Wisconsin) the amplicons purified after gel electrophoresis (Gel-M Kit, Viogene, Hong-Kong) were A-tailed with a normal *Taq* polymerase and dATP (MBI Fermentas, Vilnius, Lithuania) and purified again. The plasmids were transformed into chemically competent JM109 *E. coli* cells (Promega, Madison, Wisconsin) following manufacturer's instructions. Positive clones were selected and purified for sequencing as described by Kovács et al. (2007). Twelve positive clones of each amplicon were sequenced by LGC Genomics (Berlin, Germany) with universal primers. We deposited the LSU and ITS sequences obtained either from the clones or the directly sequenced amplicons in GenBank (HQ698054–HQ698149, SUPPLEMENTARY TABLE I).

Phylogenetic analyses.—Sequences were compared with sequences deposited in public databases by BLASTn (Altshul et al. 1990; http://www.ncbi.nih.gov/blast/). LSU sequences were included in a Pezizaceae dataset (Læssøe and Hansen 2007, Trappe et al. 2010a) to analyze the position of the new species. Several datasets of ITS sequences were analyzed. Here we present the results of two of them. Group 1 contains all ITS sequences except the cloned sequences of ITS types from one ascoma, plus ITS sequences of *Terfezia* species deposited in GenBank, and *Peziza depressa* as outgroup. Group 2 contains all ITS sequences and ITS sequence types of the *T. leptoderma*-*T. olbiensis* group with *T. arenaria* and *T. claveryi* ITS sequences as outgroup. In this dataset we used only the sequences obtained in the present study.

Sequences were aligned with Clustal X (Thompson et al. 1997) and MAFFT (Katoh et al. 2002, Katoh and Toh 2008). Alignments were checked and adjusted manually with ProSeq 2.9 (Filatov 2002) and deposited in TreeBASE (S11098). The best-fit nucleotide substitution model was selected with the program jModelTest (Posada 2008) applying the Akaike information criterion (AIC). This model was used to calculate distances for a neighbor joining (NJ) analyses with the PAUP* 4.0b10 software package (Swofford 2003). We verified branches with a NJ bootstrap (NJB) analysis with 1000 replicates. Phylogenies also were inferred by parsimony analyses with PAUP heuristic search for the most parsimonious (MP) trees. Gaps were handled either as missing characters or as fifth characters with MULTREES in effect, and TBR was used as branch-swapping algorithm. We tested branch supports by parsimony bootstrap (PB) with a fast heuristic search with 1000 replicates. Maximum likelihood (ML) phylogenetic analyses were carried out with the online version of PHYML 3.0 (Guindon and Gascuel 2003). We used the GTR nucleotide substitution model with ML estimation of base frequencies. The proportion of invariable sites was estimated and optimized. Six substitution-rate categories were set, and the gamma distribution parameter was estimated and optimized. We used bootstrap analysis with 1000 replicates

(MLB) to verify branches. The same substitution model was used in Bayesian analyses performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) with the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/index.aspx). Four Markov chains were run 5 000 000 generations, sampling every 100 steps, with a burn-in at 7500 sampled trees. Phylogenetic trees were viewed and edited by Tree Explorer, part of the MEGA 4.0 program (Kumar et al. 2004) and a text editor.

RESULTS

Four species (*T. arenaria*, *T. claveryi*, *T. leptoderma* and *T. olbiensis*) were represented in the herbarium of the Madrid Royal Botanical Garden. We studied the anatomical characters of 110 specimens. Some specimens were found to be misidentified (SUPPLEMENTARY TABLE I), but most of these were immature or in bad condition. Some specimens deposited as *T. claveryi* showed distinctive anatomical characteristics. Although their ascospores had an incomplete reticulum resembling *T. claveryi*, their peridium was clearly cellular (pseudoparenchymatic). This character combination, different from any described *Terfezia* species, suggested those collections represented a new *Terfezia* species.

After anatomical study, 71 specimens representing the four known and the potential new species were chosen for subsequent molecular phylogenetic analyses. The ITS region of 67 samples were amplified and 62 were successfully sequenced (SUPPLEMENTARY TABLE I).

LSU sequences of representatives of groups formed in the ITS analyses were analyzed together with a subset of family Pezizaceae. *Terfezia* spp. formed a monophyletic group with moderate support (FIG. 1) within the *Peziza depressa-Ruhlandiella* lineage of the family (FIG. 1) similar to phylogenetic analyses of Læssøe and Hansen (2007) and Trappe et al. (2010a). The LSU sequences of specimens with the distinctive combination of pseudoparenchymatic peridium and spores with incomplete reticulum grouped into genus *Terfezia* but separated from all other *Terfezia* species (FIG. 1) into a well supported distinct group.

Terfezia arenaria.—The ITS sequences of *T. arenaria* formed a well supported group with low intragroup variability (FIG. 2). The robustly warty spores of the species can be easily identified; small differences in wart shape and spore size of different specimens could simply represent variation of the character. Intraspecific ITS variation was detected in one specimen that contained a TT/TTT indel variant at the end of the ITS2 region.

Terfezia claveryi.—The clade of ITS sequences of *T. claveryi*, received 100% support (FIG. 2) and comprised two well supported subclades, TC-1 and TC-2, both of which contained sequences from the present study and GenBank (FIG. 2). No obvious anatomical difference between the two groups was detected, nor did they exhibit geographic/edaphic or biotic differences, although most herbarium entries had information only on the collection locality. No intrasporocarpic ITS variability was found in any *T. claveryi* specimens.

The Terfezia leptoderma/Terfezia olbiensis *group.*—The anatomical study of the specimens designated as either *T. leptoderma* or *T. olbiensis* led us to consider specimens with spiny spores plus a cellular (pseudoparenchymatic) peridium as belonging to the *T. leptoderma*/*T. olbiensis* group. The length, size and density of spines of the ascospores varied considerably, sometimes even within a sporocarp (FIG. 3).

Analyses of the ITS sequences revealed a surprisingly high diversity in this complex. Sequences from the present study together with ones deposited either as *T. leptoderma* or *T. olbiensis* in GenBank formed four main groups (FIG. 2). Groups TLO-1, TLO-2 and TLO-3 formed a strongly supported clade with a separate lineage with 100% support of an apparently undescribed *Terfezia* species placed between this group and TLO-4 (FIGS. 2, 4). The four TLO groups and the new species formed a well supported group in the phylogenetic analyses of the ITS sequences (FIG. 2). This branching of the groups was different from the topology obtained during analyses of the LSU sequences (FIG. 1).

TLO-1 contained 15 sequences from the present study. There was a highly supported subgroup, TLO-1a, with almost no intragroup variation (FIG. 2). Information about the habitats of 11 specimens of group TLO-1 was found and 10 were collected under *Quercus* spp. or *Cistus*/*Helianthemum* spp. on acidic soil, whereas one of the two GenBank entries forming the distinct long branch within the group was collected from alkaline soil. The specimens within the well supported group TLO-1a also had one common anatomical characteristic not detected in other specimens in the *T. leptoderma* / *olbiensis* group, inflated, globose elements in the pseudoparenchymatic peridium. The ornamentation of their mature ascospores was formed by broad-based, long (up to 4–5 μm) fine, tapering spines. Considerable variation of both the length and the density of these spines could be detected, even within one ascoma (FIG. 3). TLO-1 specimens not belonging to subgroup TLO-1a had no swollen elements in their pseudoparenchymatic peridium and the fine spines of the ascospores were generally shorter (1–3 μm) than those in TLO-1a

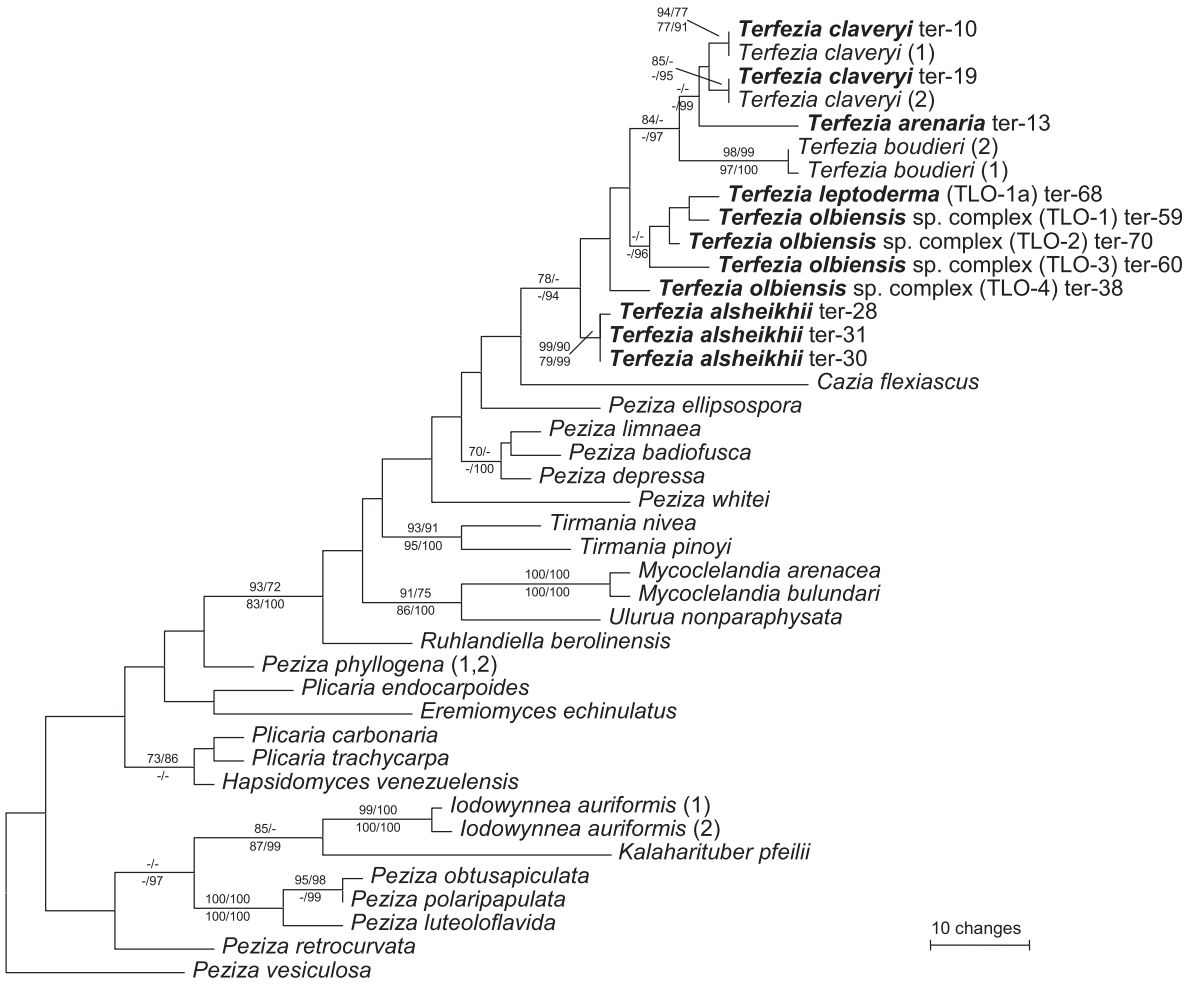

FIG. 1. One of three most parsimonious trees showing the position of *Terfezia* species within the *Peziza depressa-Ruhlandiella* lineage of the Pezizaceae. The tree was inferred from a 663 character alignment of partial nrDNA LSU sequences. *Peziza vesiculosa* was used as outgroup. Above the branches the first value shows the parsimony bootstrap and the value after the slash is the neighbor joining bootstrap. Below the branches the first value shows the maximum likelihood bootstrap, and the value after the slash is the posterior probability calculated by Bayesian analysis as percentage (not shown below 90%). The bootstrap values are shown as percentages, with values below 70% not shown. Bar = 10 changes.

(FIG. 3). No intrasporocarpic ITS variability was detected in the specimens of TLO-1.

TLO-2 contained four samples from the present study, one (*ter-69*) with intraspecific ITS variability (TABLE I, FIG. 4, not presented in FIG. 2). In this clade only this specimen had mature ascospores; the ornamentation was formed by short (2–3 μm), somewhat robust spines (FIG. 3).

ITS sequences of 11 specimens of the present study formed the well supported group TLO-3 (FIGS. 2, 4); nevertheless six of those specimens showed intrasporocarpic ITS variation (TABLE I, FIG. 4). The length and density of the spines of ascospores were variable within the group, sometimes within one ascocarp. Spores with long spines but also with shorter, less dense and more robust spines could be detected (FIG. 3). Information on the neighboring plants of nine specimens was recorded; six were collected under *Pinus*, two under *Quercus* and one under *Cistus* spp. (SUPPLEMENTARY TABLE I).

TLO-4 included five specimens, one with and four without intrasporocarpic ITS variability (FIGS. 2, 4). GenBank sequences grouping into this clade came from specimens collected in acidic soil. Only one specimen we studied had notes about the neighboring plants, which were *Cistus* species. Its ascospores had short (2–3 μm), somewhat robust, uncrowded cylindrical spikes (FIG. 3).

Intrasporocarpic ITS variability.—Different ITS types were detected in 10 specimens during verification of electropherograms produced in direct sequencing of ITS amplicons. Double peaks and shifts made the electropherograms unreadable upward from a certain

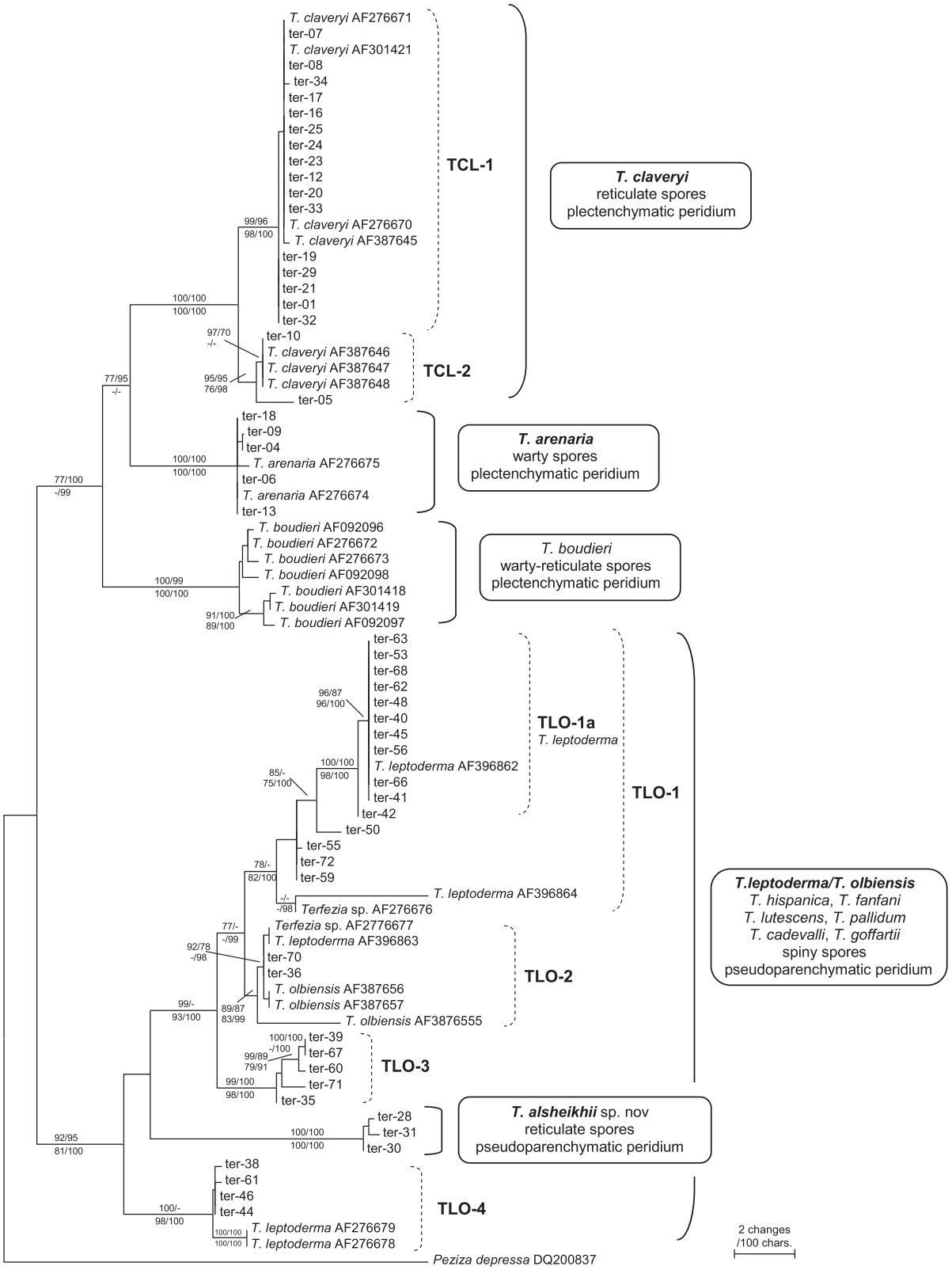

FIG. 2. The maximum likelihood (ML) tree of *Terfezia* species. The ML tree inferred from nrDNA ITS sequences with *Peziza depressa* as outgroup. We added GenBank accession numbers of data from earlier studies to sequences from public databases; sample names appear as given in GenBank. We based the analysis on an alignment of 637 characters. Above the branches the first value is the ML bootstrap and the value after the slash is the parsimony bootstrap. Below the branches the first value shows the neighbor joining bootstrap, whereas the value after the slash is the posterior probability calculated by Bayesian analysis as percentage (not shown below 90%). The bootstrap values are shown as percentages, with values below 70% not shown. Bar = 2 changes/100 characters.

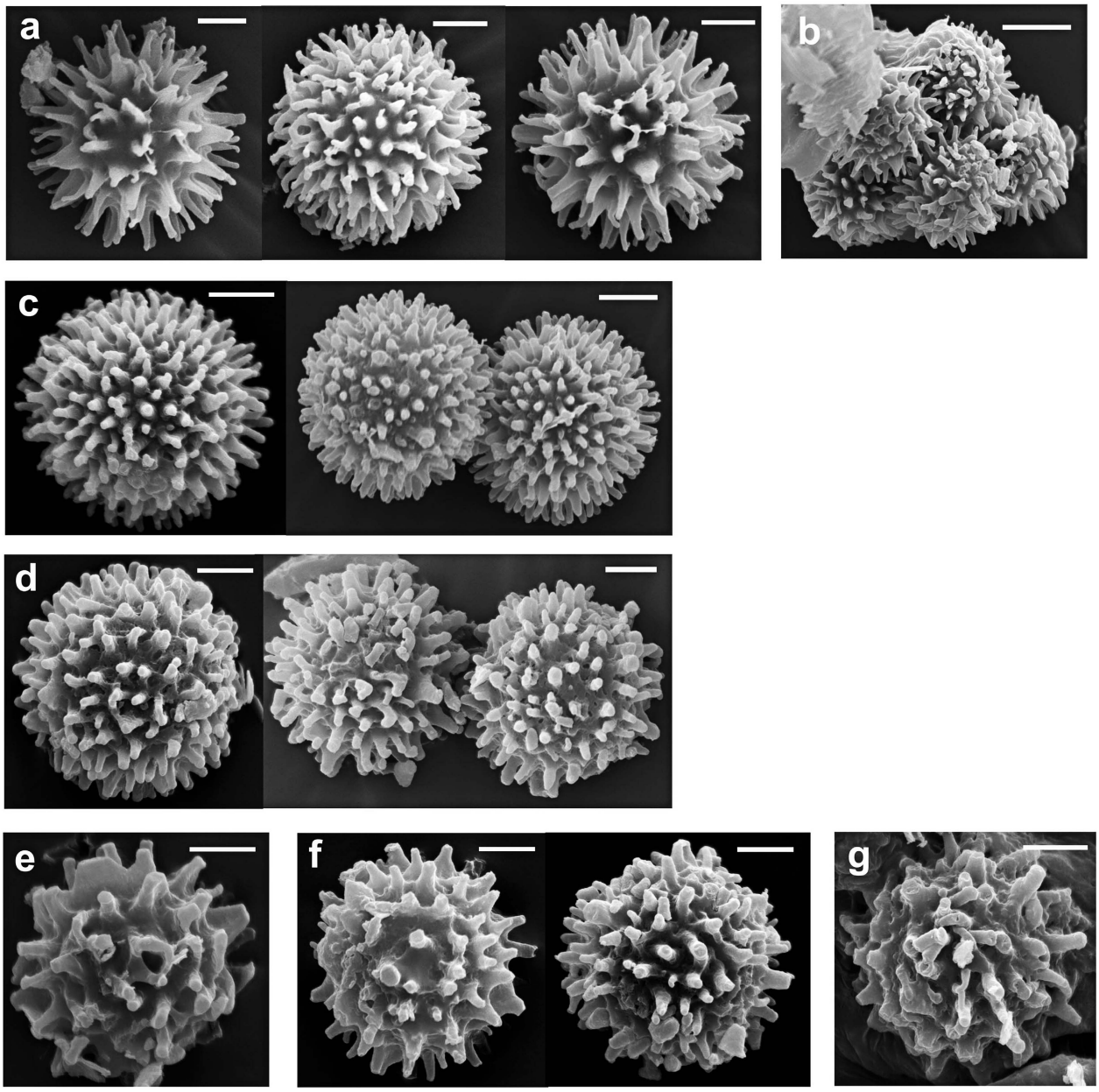

FIG. 3. Scanning electron micrographs of ascospores from the *Terfezia leptoderma/T. olbiensis* group. a. *T. leptoderma*, group TLO-1a, *ter-41* (MA-Fungi 28367). b. *T. leptoderma*, group TLO-1a, *ter-42* (MA-Fungi 24272). c. *T. olbiensis* species complex, group TLO-1, *ter-55* (MA-Fungi 47184-2). d. *T. olbiensis* species complex, group TLO-2, *ter-69* (MA-Fungi 54675). e. *T. olbiensis* species complex, group TLO-3, *ter-35* (MA-Fungi 5407). f. *T. olbiensis* species complex, group TLO-4, *ter-52* (MA-Fungi 41322). g. *T. olbiensis* species complex, group TLO-4, *ter-61* (MA-Fungi 47421). Spores in a, c, d, and f are from the same respective ascomata. Bars: a, c–g = 5 µm; b = 10 µm.

position on both forward and reverse reads. This revealed respectively substitution and indel variation. We found these in one *T. arenaria* and one old specimen in poor condition deposited as *T. claveryi* from the Canary Islands. The intrasporocarpic ITS variation of these two specimens were not analyzed further.

Eight specimens with intrasporocarpic ITS variability belonged to the *T. leptoderma/olbiensis* group; one belonged to TLO-2, six to TLO-3 and one to TLO-4. The number of ITS types detected in single specimens were 2–7 and the maximum number of different characters between ITS types were 1–12 (TABLE I, FIG. 4). The ITS types originating from one fruit body formed either distinct (e.g. *ter-58* and *ter-64*) or mixed groups (e.g. *ter-57* and *ter-65*) (FIG. 4) when all ITS sequence types obtained from the *T.*

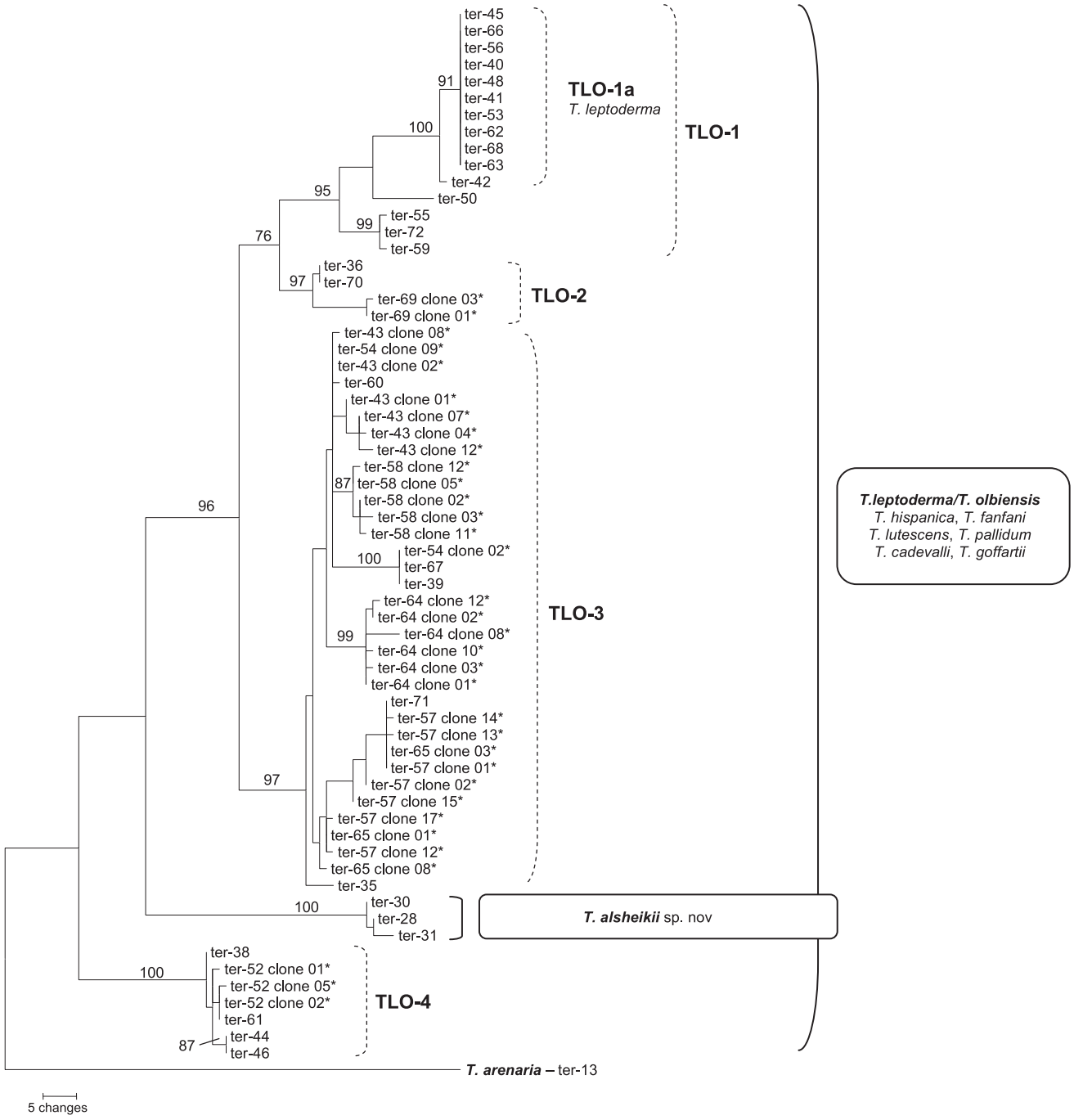

FIG. 4. One of the 899 most parsimonious phylogenetic trees inferred from a dataset of all nrDNA ITS sequence types of the *Terfezia leptoderma/T. olbiensis* group and *T. alsheikhii*. The analysis was based on an alignment 598 characters long. We included all variant intrasporocarpic ITS sequence types from each ascoma in the analyses. Where ITS polymorphism was detected, the sequences are referred to as ''clones'' and marked with asterisks. We obtained all sequences in the present study. *Terfezia arenaria* was used as outgroup. The parsimony bootstrap (PB) values are above the branches as percentages. Values below 75% not shown. Bar = 5 changes.

leptoderma/T. olbiensis group were analyzed together. The majority of ITS sequence types grouped into TLO-3 (FIG. 4). The same ITS type was found for example in *ter-57* (clone 01), *ter-65* (clone 03) and was identical to the ITS of *ter-71* (FIG. 4). These three specimens were collected in the same region but in different areas and from different plants (SUPPLEMENTARY TABLE I). Identical ITS types also were found in

TABLE I. Specimens with intrasporocarpic ITS heterogeneity in the *Terfezia olbiensis* species complex and the level of ITS variation

Specimens in *Terfezia olbiensis* complex	Group	Number of different ITS types	Maximum number of differing characters of two ITS types
ter-43	TLO-3	6	5
ter-52	TLO-4	3	2
ter-54	TLO-3	2	2
ter-57	TLO-3	7	12
ter-58	TLO-3	5	4
ter-64	TLO-3	6	7
ter-65	TLO-3	3	9
ter-69	TLO-2	2	1

specimens from distant regions, as in the case of *ter-54* (clone 02), *ter-39* and *ter-67* or *ter-54* (clone 09) and *ter-43* (clone 02) (FIG. 4, SUPPLEMENTARY TABLE I).

New species.—The phylogenetic analyses of both the ITS and LSU sequences separated the specimens with distinctive anatomical characteristics from the other *Terfezia* sequences (FIGS. 1, 2, 4), although the placement of the lineage with other Terfezia species differed depending on the analysis. The morphological characteristics and the molecular phylogenetic analyses provided strong evidence that these specimens represent a new *Terfezia* species.

TAXONOMY

Terfezia alsheikhii Kovács, M. P. Martín & Calonge sp. nov. FIG. 5

MycoBank MB519343

Ascomata hypogaea, 1–4 cm lata. Peridium 100–180 μm crassum, in ascomatibus immaturis stratis duobus: stratum exterius compactum, brunneolum per microscopium luce transmissa, manifeste pseudoparenchimatum; stratum duo partim cellulis oblongis, partim cellulis globosis; in ascomatibus maturis strato tertio, prosenchymato. Asci globosi vel subglobosi, 40–60 × 65–80 μm, plerumque 8-spori. Ascosporae globosae, (15–)16–18(–20) μm latae, irregulariter reticulatae, in sporis junioribus reticulum incompletum.

Ascomata hypogeous, 1–4 cm broad. Peridium 100–180 μm thick with two layers in immature ascomata: outer layer compact, brownish in light microscopy, clearly pseudoparenchymatic; layer 2 partly of angular oblong cells, partly of globose cells. The cells are 25–40(–50) × 20–30 μm. The cell walls are 1–2 μm thick. Mature ascomata with a third layer of interwoven hyphae 7–10 μm diam; this layer missing or very thin in younger ascomata. Asci globose to subglobose, 40–60 × 65–80 μm, generally eight-spored. Ascospores globose, (15–)16–18(–20) μm broad, irregularly reticulate, in younger spores the reticulum incomplete.

Etymology. Named after Dr Abdulmagid M. Alsheikh, whose well detailed, highly reliable monographic work on *Terfezia* sensu lato (Alsheikh 1994) is an essential starting point and continuous help in any work on these truffles.

Distribution, habitat and season. In Spain found under Cistaceae plants (*Tuberaria lignosa* and *Helianthemum salicifolium*) in Arroyomuerto (Salamanca) and Castrillino (León) and under *Pinus sylvestris* in Vinuesa (Soria) and Pena de Francia (Salamanca); March, July, September and December.

Collections examined. Type: SPAIN: Salamanca: Arroyomuerto, *T. Pérez Jarauta*, 5 Dec 1992 (HOLOTYPE MA-Fungi, MA-32251); other collections: SPAIN: Salamanca: Pena de Francia, *T. Pérez Jarauta* 5 Dec 1992 (MA-Fungi); León, Castrillino, *T. Pérez Jarauta*, 5 Mar 1992 and 2 Jul 1992 (MA-Fungi); Soria: Vinuesa, *J. C. Santos*, 15 Sep 1991 (MA-Fungi).

DISCUSSION

This study of *Terfezia* desert truffles collected on the Iberian Peninsula and Canary Islands revealed the high diversity of these fungi. This was especially prevalent within the *T. leptoderma*/*T. olbiensis* clade. The literature generally distinguishes five species in the Mediterranean region (Díez et al. 2002, Montecchi and Sarasini 2000). Comparisons of our data with the sequences available in public databases show that three *Terfezia* spp. can be reliably identified by morphological analyses. *Terfezia arenaria* with robust warty spores and prosenchymatous peridium can be identified easily, and its nrDNA ITS sequences showed low variation. Reticulate spores and prosenchymatous peridium are good characters to identify *T. claveryi* in combination; however identification on reticulate spores alone can be misleading. A collection of *Mattirolomyces terfezioides*, originally deposited as *T. claveryi* (in MA-Fungi Real Jardín Botánico) might have been misidentified due to the similarly reticulate spores of both species (Kovács et al. 2009). The newly described *Terfezia alsheikhii* also has reticulate spores, but its peridial structure is very different.

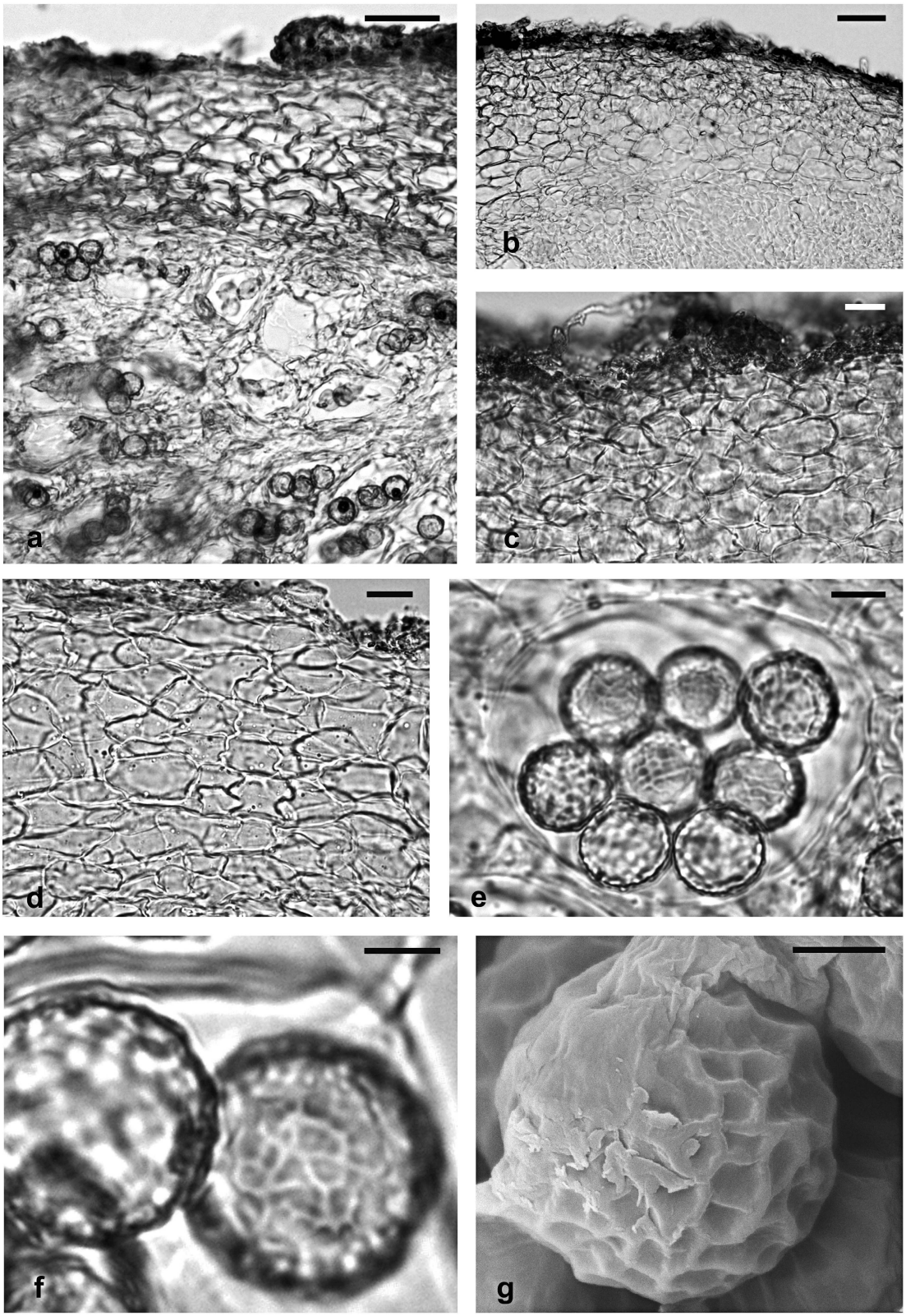

FIG. 5. Anatomical characteristics of *Terfezia alsheikhii*. a. Part of a cross section of an ascoma showing the peridium and the gleba with asci (MA-Fungi 32552). b, c. Cross sections of the peridium of a mature ascoma (MA-Fungi 29268). d. Cross section of the peridium. e–g. ascospores (g. scanning electron micrograph) of MA-Fungi 29680. Bars: a, b = 50 µm; c, d = 20 µm; e = 10 µm; f, g = 5 µm.

Terfezia leptoderma and *T. olbiensis* are the most widely used names for spiny spored *Terfezia* species, although several species have been described with this characteristic (see below). Some authors have treated these two species as synonyms after Fogel (1980) concluded that they represent different developmental stages of the same species. Alvarez et al. (1993) said, ''… we have found sporocarps intermediate in morphology between those reported for *T. olbiensis* and *T. leptoderma*… Most differences appear to be a matter of developmental stage…''. Díez et al. (2002) accepted both *T. olbiensis* and *T. leptoderma* but suggested they could represent the same species at different developmental stages. Janex-Favre et al. (1988), Alsheikh (1994) and Montecchi and Sarasini (2000) all considered these two as separated species.

Alsheikh (1994) noted ''inflated, spherical'' cells in the inner peridium of *T. leptoderma*. We detected such cells only in the well supported group TLO-1a and believe it represents *T. leptoderma*, which, according to available information, is associated with *Quercus*, *Helianthemum* and *Cistus* species on acidic soils.

Our analyses of the ITS sequences revealed at least four well supported lineages of the spiny spored *Terfezia* species in addition to *T. leptoderma* (TLO-1a). Although *T. olbiensis* is the most widely known species, in the literature six similar *Terfezia* species generally are considered taxonomic synonyms of *T. olbiensis*. Although Alvarez et al. (1993) for example regarded *T. hispanica* Lázaro Ibiza as a separate species, they noted it was ''…maybe an aberrant collection of *T. olbiensis*''. In addition to *T. hispanica* there are *T. fanfani* Mattir., *T. lutescens* (Lázaro Ibiza) Malençon, *T. pallida* (Lázaro Ibiza) Malençon, *T. cadevalli* Font Quer and *T. goffartii* Chatin. The types of *T. cadevalli*, *T. hispanica*, *T. lutescens* and *T. pallidum* most probably have been lost (Calonge et al. 1985, Alsheikh 1994), so authors made their decisions based on the original descriptions or handled these species as synonyms of *T. olbiensis* based on other authors' works. Our phylogenetic analyses showed the presence of distinct phylogenetic lineages in the group. Testing whether those distinct lineages can be connected with existing morphospecies will require a complete typological and nomenclatural revision. Such a revision will necessarily be a protracted process because of type specimens with unknown locality, in collections with limited access or in collections that do not permit loans. Indeed it might be impossible because of lost type specimens, incomparable fresh characters or type specimens from which no usable morphological or molecular information can be obtained. Because of the high genetic heterogeneity and the presence of distinct phylogenetic lineages of the group we believe that treating the above-mentioned species as synonyms is not well established and could be wrong. While *Terfezia leptoderma* can be identified, it seems reasonable to designate the other spiny spored *Terfezia* species with pseudoparenchymatic peridium as *T. olbiensis* species complex, bearing in mind that this complex most probably represents several described *Terfezia* species with valid names.

In contrast to the frequent intra-individual nrDNA ITS variation of plants (see Feliner and Roselló 2007 and references therein), relatively few intrasporocarpic nrDNA ITS studies have been done on the true fungi to examine intra-individual variability. Ganley and Kobayashi (2007) reported ''remarkably low'' variation within the nrRNA gene repeats in the genomes of five fungal species. They hypothesized that the homogeneity might be due to highly effective concerted evolution. On the other hand Rooney and Ward (2005) found evidence for birth-and-death processes and purifying selection instead of concerted evolution of rRNA genes in ascomycetous fungal species. Both intraspecific (e.g. Carriconde et al. 2008, Horton 2002, Ko and Jung 2002, Murat et al. 2004, Nilson et al. 2008, Smith et al. 2007) and intragenomic/hyphal/sporocarpic variation (Aviram et al. 2004, Avis et al. 2006, Kauserund and Schumacher 2003, O'Donnell and Cigelnik 1997, Simon and Weiss 2008, Smith et al. 2007) of nrRNA genes, especially in the ITS region, have been reported in fungi. Simon and Weiss (2008) hypothesized that intragenomic/hyphal/sporocarpic variation might be more frequent than thought but perhaps overlooked. We also suspect this to be the case. In the present study the majority of the specimens with intrasporocarpic ITS variability were found in the *T. olbiensis* species complex and six out of the eight sporocarps with variable ITS belonged to one lineage (group TLO-3). The high intrasporocarpic ITS variation suggests that either species in the *T. olbiensis* complex are too recently evolved for concerted evolution or other processes (Ganley and Kobayashi 2008, Rooney and Ward 2005) to have time to eliminate variation in the gene tandem repeats of nrRNA or an ongoing diversification/speciation process started relatively recently in the lineage. The former explanation seems more likely because the specimens originate from distant localities and synchronous start of the latter in distant populations is less probable.

Aviram et al. (2004) reported the presence of two ITS types within a hypha of *T. boudieri*. The intrasporocarpic ITS variation we found in the *T. olbiensis* species complex is higher than the variation found in *T. boudieri* and mainly characteristic of one lineage of the complex. Study of the organization (i.e. intrahyphal, intrakaryotic etc.) of the high genetic

variation we found is beyond the scope of our work. Nevertheless because seven out of the eight specimens had more than two and up to seven ITS types detected they should represent a different type of variation than the heterokaryotic hyphae detected in *T. boudieri*.

Díez et al. (2002) hypothesized an edaphic and biotic specificity of *Terfezia* species. Although many specimens we studied had had no information about the soil and the plants of their localities, our results suggest a certain host specificity of some lineages of the *T. leptoderma/T. olbiensis* group to either pine or oak and cistaceous plants. The only group where all the specimens with known localities were collected from *Cistus/Helianthemum* and *Quercus* was TLO-1. So we can assume that *T. leptoderma* represented by the group TLO-1a is associated with these neighboring plants, most probably on acidic soils. Although most specimens of groups TLO-2 and TLO-3 of *T. olbiensis* with known localities were collected under pines, specimens from both groups also were collected under *Quercus* and cistaceous plants, indicating no strict specificity of the lineages either to biotic or abiotic environments.

In conclusion a recent study of ecological strategies mapped onto the tree of life (Gómez et al. 2010) used *Terfezia* as the sole representative of mycorrhizal fungi. The authors used eight as the total number of *Terfezia* species, although this number included some species whose generic assignment has been changed. Nevertheless our results indicate this might be closer to the true number of distinct lineages/species of *Terfezia* sensu stricto, represented by desert truffles in the Mediterranean region and the Middle East, than the four or five *Terfezia* species generally accepted in the literature. We found a high intraspecific and intrasporocarpic variation of both morphological characters and nrDNA ITS sequences. Together with a lack of edaphic or biotic specificities this variation might indicate ongoing diversification within some lineages of *Terfezia*.

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