Universidade de Évora - Instituto de Investigação e Formação Avançada

Programa de Doutoramento em Biologia

Tese de Doutoramento

"*Terfezia* diversity in southern Portugal and their mycorrhizal associations with *Cistus* L.: a study towards the viable production of desert truffles on acid soils"

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“To expect the world to receive a new truth, or even an old truth, without challenging it, is to look for one of those miracles which do not occur.”

Alfred Russel Wallace

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TERFEZIA DIVERSITY IN SOUTHERN PORTUGAL AND THEIR MYCORRHIZAL ASSOCIATIONS WITH *CISTUS* L.: A STUDY TOWARDS THE VIABLE PRODUCTION OF DESERT TRUFFLES ON ACID SOILS

ABSTRACT

Desert truffles are edible hypogenous ascocarps produced by *Ascomycota* (*Pezizaceae*) fungi characteristic of arid and semi-arid zones. Many of these desert truffle species are nowadays considered as valued Non-Wood Forest Products (NWFPs) endemic to the Mediterranean basin where their ascocarps, rich in proteins and poor in carbohydrates and lipids, constitute a potentially important food source for rural populations. They also represent a key component of the mycological flora on arid and semi-arid habitats, acting directly as symbiotic partners of diverse host plants, mainly *Cistaceae*, and indirectly as desertification and soil erosion prevention agents.

Terfezia (Tul. & Tul.) Tul. & Tul. is undoubtedly the best known and most diversified of all desert truffle genera, but its taxonomy is far from resolved. In addition, cultivation of desert truffles is not trivial and despite all research efforts in the last two decades, only two *Terfezia* species were to date successful cultivated both with perennial and annual *Helianthemum* species in basic soils. Research on how to cultivate *Terfezia* spp. with different plant hosts and over a broader array of soil types and pH values is still necessary to find most suitable "fungal symbiont – plant host" combinations for sustainable and efficient cultivation over a wider range of habitats.

With this in mind, this work aimed to: 1) expand the current knowledge on the diversity of genus *Terfezia* in southern Portugal; 2) study their putative mycorrhizal associations with *Cistus* spp.; and 3) develop methodologies to allow *Cistus* spp. to be used as host in desert truffle cultivation in acid soils.

Overall, the present research clearly demonstrates that *Cistus* spp. are indeed promising candidates for desert truffle cultivation and that different *Terfezia*-*Cistus* combinations can be used to enable desert truffle cultivation over a wide range of situations depending on the plantation purposes.

DIVERSIDADE DO GÉNERO *TERFEZIA* NO SUL DE PORTUGAL E AS SUAS ASSOCIAÇÕES MICORRÍZICAS COM *CISTUS* L.: UM ESTUDO PARA VIABILIZAR A PRODUÇÃO DE TRUFAS DO DESERTO EM SOLOS ÁCIDOS

RESUMO

O termo trufas do deserto descreve os ascocarpos hipógeos comestíveis, produzidos por fungos *Ascomycota* (*Pezizaceae*), característicos de zonas áridas e semiáridas. Estes são atualmente considerados como importantes produtos florestais não lenhosos, endémicos da bacia do Mediterrâneo, e constituem uma fonte de alimento, rica em proteínas e pobre em hidratos de carbono e lipídios, para as populações rurais. As trufas do deserto representam ainda um componente-chave da flora micológica em habitats áridos e semiáridos, atuando como parceiros simbióticos de diversas plantas hospedeiras, principalmente *Cistaceae,* e como agentes de prevenção contra a desertificação e erosão do solo.

Terfezia é indubitavelmente o género mais conhecido e diversificado de trufas do deserto, mas a sua taxonomia está longe de estar esclarecida. Também o seu cultivo não é trivial e, apesar de todos os esforços de pesquisa nas últimas duas décadas, apenas duas *Terfezia* foram, até o momento, cultivadas com sucesso, em solos básicos, com espécies perenes e anuais do género *Helianthemum* (*Cistaceae*). Falta ainda conhecer vários binómios "simbionte – planta hospedeira" que permitam o cultivo de *Terfezia* numa ampla variedade de solos e habitats.

Assim, os principais objetivos deste trabalho foram: 1) ampliar o conhecimento atual sobre a diversidade do género *Terfezia* no sul de Portugal, 2) estudar as suas potenciais associações micorrízicas com *Cistus* spp. e 3) desenvolver metodologias para permitir a sua aplicação no cultivo de trufas do deserto em solos ácidos.

No presente trabalho, é claramente demonstrado que *Cistu*s spp. são candidatos promissores para o cultivo de trufas do deserto e que diferentes combinações de *Terfezia-Cistus* podem ser usadas para o cultivo de trufas do deserto numa ampla gama de situações, dependendo dos propósitos da plantação.

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GENERAL INTRODUCTION

1. DESERT TRUFFLES

1.1. DEFINITION AND GENERAL FEATURES

Desert truffles are the ascocarps produced by several *Ascomycota* fungi included within the order *Pezizales,* characteristic of arid and semi-arid areas throughout the world (Moreno et al., 2014; Morte et al., 2009; Navarro-Ródenas et al., 2011). Although the name "desert truffle" is devoid of true phylogenetic or taxonomic meaning, it nevertheless groups several pezizalean fungi that have developed adaptations to extreme environments and xeric conditions, such as hypogeous or semi-hypogeous life-styles and the simultaneous loss of active spore dispersal (Kovács & Trappe, 2014). With the loss of active spore discharge, truffle forming fungi have evolved novel mechanisms for spore dispersal via small animals. Among other several selective advantages, their low surface area-to-volume ratio enables a large number of spores to be produced in a small packet of tissue. Furthermore, not being directly exposed to weather, desert truffles are protected against moisture and temperature fluctuations that might otherwise damage or inhibit spores' development (Bonito et al., 2013). Hence, the ascocarps of desert truffles are intrinsically closed or "sequestrate" globose fruiting bodies (**Fig. 1**), comprised by an outer layer (*peridium*) that surrounds the spore-bearing inner mass of the ascocarp (*gleba*) where the cells develop into structures that produces ascospores during sexual reproduction (*asci*) (**Fig. 2**). The *gleba* of desert truffles can vary from a single cavity to a set of intricate foldings or pockets of *asc*i spread over a firm matrix of *hyphal* tissue. The *asci* can be cylindrical with spores in one row (as most epigeous *Pezizaceae*) or be completely globose with or without a pedicel and with a variable number of spores (**Fig. 2**). The *ascus* walls can be more or less layered and amyloid or inamyloid. The ascospores vary in colour from hyaline to almost black, and from smooth and thin-walled to very thick-walled with intricate ornamentation (Læssøe & Hansen, 2007).

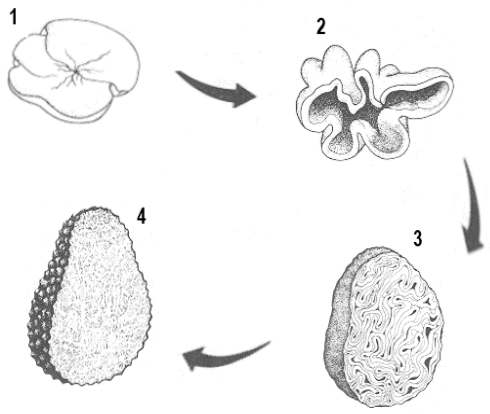

Figure 1 - Ascocarp sequestrate evolution. 1) *Peziza* ancestor, 2) intermediate *Genea*-like form, 3) intermediate *Geopora*-like form, 4) *Tuber*-like form. (Adapted from http://www.mycolog.com/CHAP4b.htm).

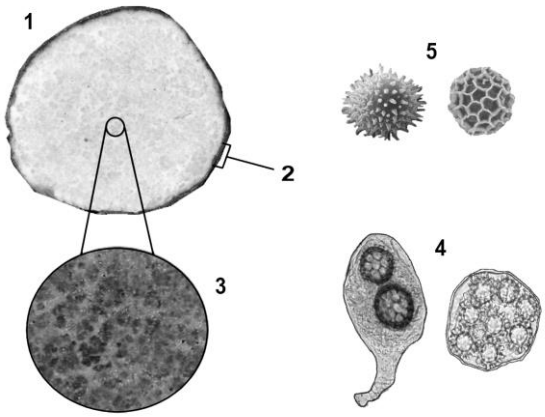

Figure 2 – Desert truffle ascocarp. 1) ascocarp in cross-section. 2) *peridium,* 3) detail of *gleba* showing pockets of *asci* (in dark), 4) *asci* with ascospores, 5) detail of spore ornamentation.

1.2. WORLD DISTRIBUTION AND PHYLOGENY

Desert truffles occur naturally in arid and semi-arid areas throughout the world, or areas with greater annual evapotranspiration potential than annual precipitation, which essentially comprise regions with the desertic climate Bw subtype, the continental steppe Bs climate, or the Mediterranean Cs climate, following Köppen's climate classification (**Fig. 3**) (Moreno et al., 2014).

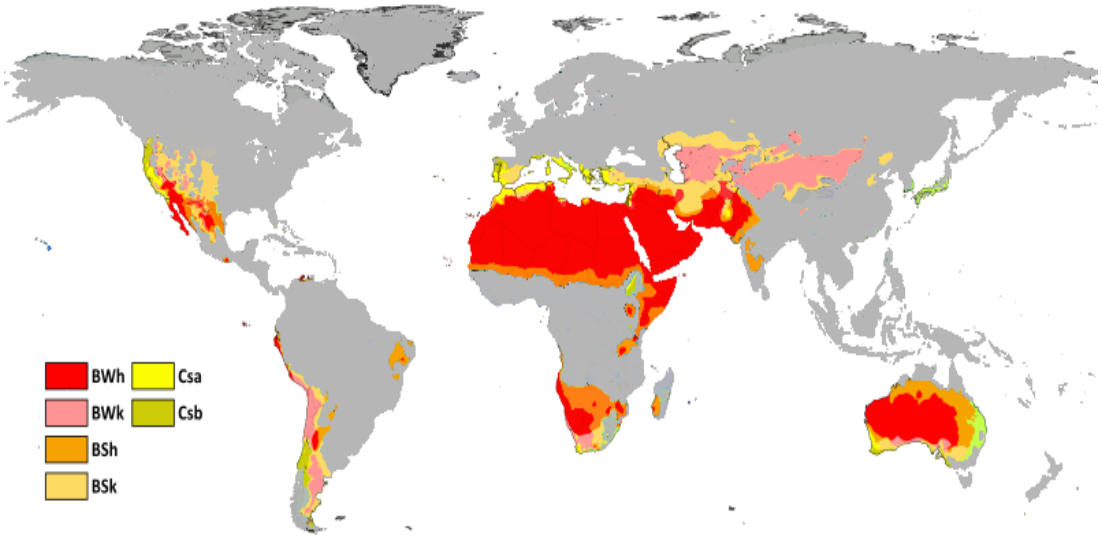

Figure 3 – World map showing the distribution of suitable climatic areas for desert truffles according to Köppen's climate classification (Peel et al. 2007)

As the world's deserts now cover about a third of the land surface and occur in every continent, these underground members of the *Pezizaceae* are well distributed around the globe (**Fig. 4**). Indeed, desert truffles have been found in the Mediterranean basin, Iraq, Kuwait, the Sahara Desert and Saudi Arabia, Hungary, Yugoslavia, China, the Kalahari Desert, Australia and North America (Kagan-Zur & Roth-Bejerano, 2008).

Figure 4 - Desert truffles world distribution according to Kagan-Zur & Roth-Bejerano (2008).

Regarding their phylogeny, desert truffles, were traditionally classified within the order *Tuberales*. Yet, the question whether *Tuberales* was monophyletic or composed by different operculates which evolved convergently into hypogeous forms was raised several times throughout the 20th century (reviewed in Laessøe & Hansen, 2007). Later, with the onset of the molecular era, repeated molecular phylogenetic studies on sequestrate fungi have undoubtedly demonstrated that certain hypogeous fungi are more closely related to epigeous members of the *Pezizales* than to other hypogeous species and that at least 15 independent incidents of epigeous fungi evolving the below-ground fruiting habit to become truffles occurred within the *Pezizales* (Kagan-Zur & Roth-Bejerano, 2008). Accordingly, the former *Tuberales,* including all desert truffle *taxa*, were distributed among six Pezizalean families: *Glaziellaceae*, *Discinaceae-Morchellaceae*, *Helvellaceae*, *Tuberaceae*, *Pezizaceae*, and *Pyronemataceae*, comprising 38 genera (Hansen & Pfister 2007). Since then, the increasing

amount of sequence data has corroborated the polyphyletic origin of this group of truffles characteristic of arid and semi-arid areas throughout the world. Nowadays, and according to Roskov et al. (2019) -Species 2000 & ITIS Catalogue of Life, 2019 Annual Checklist- all desert truffles in *Ascomycota* are included within the order *Pezizales* and distributed between several families: *Pezizaceae* (14 genera), *Tuberaceae* (6 genera), *Pyronemataceae* (6 genera), *Glaziellaceae* (1 genus), *Carbomycetaceae* (1 genus), *Morchellaceae* (1 genus) and *Helvellaceae* (2 genera). Among the most commonly referred desert truffle genera are *Terfezia*, *Balsamia*, *Delastria*, *Leucangium*, *Mattirolomyces*, *Phaeangium*, *Picoa* and *Tirmania* (Morte et al., 2009).

1.3. ECOLOGICAL AND ECONOMIC IMPORTANCE WORLDWIDE

Desert truffles are of considerable interest for ecological, agroforestry and commercial purposes (Gutierrez et al., 2003). Because of their heterotrophy for carbon, desert truffles have an obligatory symbiotic stage in their life cycle. As symbionts of many plants (mostly *Cistaceae*) that inhabit arid and semiarid zones around the world, desert truffles have a key role in maintaining the fitness and improving the resilience of those plant communities against environmental stresses, including nutrient deficiency, drought and soil disturbance (Barea et al., 2011). These mutualistic symbioses function as conduits through which energy and matter flows between the fungal symbiont and their host plants. Fungi engaged in this symbiotic life-style promotes a reduction of root biomass, while simultaneously enhances plant nutrient and water uptake capacity, by extending their mycelial networks beyond root surfaces and penetrating in soil pores that are too small for root hairs to enter. Indeed, mycelial networks of mycorrhizal fungi often connect plant root systems over broad areas and can comprise the largest portion of soil microbial biomass (Johnson & Gehring, 2007).

Desert truffles are highly appreciated by people around the Mediterranean basin for their unique musky flavor, where they contribute to the incomes of rural populations after sale or simple exchanges on rural markets (Benucci et al., 2012; Boa, 2004). Though their economic value is certainly no match for that of the “true” truffles, desert truffles are no less interesting in terms of culinary and medicinal properties (Dafri & Beddiar, 2017). Desert truffles are rich in various chemical compounds, including carbohydrates, proteins, amino acids, fibers, vitamins, minerals, sterols, terpenes and fatty acids. For instance, the protein content of some

desert truffles can reach up to 20 % of its dry weight, which is significantly higher than in most vegetables (Murcia et al., 2003). In addition, desert truffles represent an untapped source of therapeutic compounds with anti-microbial, anti-inflammatory, immunosuppressant, anti-mutagenic and anti-carcinogenic properties (Al-Qarawi & Mridha, 2012; Bradai et al., 2015; Kagan-Zur et al., 2014; Thomas et al., 2019). Thus, any newly discovered species may prove to be an important source of novel active compounds (Thomas et al., 2019).

Also, from the agroforestry point of view, there is a growing interest in introducing desert truffle's cultivation into dry environments allowing the exploitation of lands which until now have been regarded as unproductive, while simultaneously preventing soil erosion and desertification (Honrubia et al., 1992).

1.4. HISTORICAL USE AND CULTIVATION

Desert environments are typically hostile environments for most living beings except for a short period after the rainy season where they are known to offer bountiful food items, such as the desert truffles (Alsheikh, 1994). These truffles have been used from prehistoric times, as food and medicine, by the indigenous peoples of North Africa and the Middle East, by the nomadic peoples of the Kalahari, in southern Africa, and by the Aborigines of the central Australian Outback, all of which, shared the same belief that desert truffles were a God-given food, descended from the sky through thunderstorms (Alsheikh, 1994; Trappe et al., 2008a, 2008b). However, mankind's first record of desert truffles use was left by the Amorites in the Bronze Age in cuneiform's writing, inscribed on clay tablets (Sasson, 2004). Also, there are some evidences that these desert fungi were served as gourmet foods to the Egyptian pharaohs, but very few details concerning the identity of the truffles have survived (Trappe, 1990). Desert truffles were also imported by the ancient Greeks and Romans who were intrigued by their sudden appearance inside the soil: Theophrastus, one of Aristotle's students, refer to desert truffles in 500 BC as "a natural phenomenon of great complexity, one of the strangest plants, without root, stem, fiber, branch, bud, leaf, or flower" (Mandeel & Al-Laith, 2007). References of these so called "gifts of the gods" were also documented in the holy Bible as well as in the Talmud, and repeated in Arab folk tales over centuries, but the bulk of information regarding the use of desert truffles comes from the published diaries of explorers who joined Bedouin caravans that travelled along the ancient caravan routes that

crisscrossed the deserts of Asia, the Middle East, and the Sahara (Shavit, 2014). Thus, there is no doubt that these desert-adapted hypogeous fungi have been known and appreciated as a valuable commodity long before European forest truffles. However, over the centuries, the popularity of the desert truffles has faded in comparison to the later, and therefore have been rather neglected by science (Kagan-Zur & Roth-Bejerano, 2008). Even so, substantial quantities of several desert truffles species are still nowadays collected and marketed in southern Europe, North Africa, and other regions bordering the Mediterranean (Morte et al., 2008).

Desert truffle's natural production is highly erratic and quite conditioned by several factors, among which the soil type, the annual water regime, and the existence of suitable host plants (Kagan-Zur & Roth-Bejerano, 2008; Morte et al., 2008). Moreover, desert truffle's hunting does not rely on animals to scent the truffle smell, like in the case of the more fragrant European forest truffles. Therefore, it generally involves trained collectors, traditionally men, covering large areas of land searching for the prized fruitbodies (Dafri & Beddiar, 2017) which make desert truffle's hunting a particularly time-consuming activity. Furthermore, desert truffles naturally occurring areas are progressively disappearing. For instance, mining operations over large areas of the coastal desert in Egypt and Libya during World War II and, more recently, the Gulf War in Kuwait, have apparently ruined many truffle-gathering areas on the North Africa and the Middle East. Likewise, the widespread construction fever over the last decades has led to the decline of many desert truffle's natural production sites in Europe (Morte et al., 2008; Morte et al., 2012).

The raising awareness of the decline of desert truffle production sites over the Mediterranean region encouraged several countries (e.g. Kuwait) to initiate research programs aiming to recover many desert truffles natural production sites, namely, by fencing and by applying intensive irrigation on vast desert areas (Alsheikh, 1994). Though promising, these costly government-subsidized irrigation programs could not be repeated or applied routinely; new strategies had to be devised in order to face the increasing demand for desert truffles in a sustainable way. With that purpose, several researchers began a) describing the *mycorrhizae* formed between selected desert truffles and their correspondent putative plant hosts (Alsheikh, 1984; Awameh, 1981; Awameh et al., 1979; Dexheimer & Gerard, 1989; Dexheimer et al., 1985; Leduc et al. 1986); b) developing methods to promote *in-vitro* or *ex-vitro mycorrhizae* synthesis (Cano et al., 1991; Chevalier et al., 1984; Fortas & Chevalier, 1989;

Morte et al., 1994; Ravolanirina, 1986; Roth-Bejerano et al., 1990); and finally c) investigating the possibilities of desert truffle cultivation under controlled conditions (Gutiérrez, 2001; Honrubia et al., 2001; Morte et al., 2008, 2009, 2010, 2012; Navarro-Ródenas et al., 2011, Slama et al., 2010).

However, desert truffles cultivation proved to be challenging and despite the currently available valuable information on desert truffles bio/ecology, their cultivation is only now leaving its infancy, and our knowledge on their physiology, biochemistry and plant-fungus relationships, still remains fragmented (Kagan-Zur & Roth-Bejerano, 2008).

2. THE GENUS *TERFEZIA*

2.1. DEFINITION AND GENERAL FEATURES

The genus *Terfezia* (Tul. & C.Tul.) Tul. & C.Tul. (1851) is undoubtedly the best known and most frequently collected of all the desert truffle genus (Díez et al., 2002; Kagan-Zur & Roth-Bejerano, 2008). *Terfezia* taxonomy and its evolutionary relationships have been debated since long time and, in many regards, still remain insufficiently clarified (Loizides et al., 2012). The genus is typified by the species *Terfezia arenaria* (Moris) Trappe (**Fig. 5**) and comprises a heterogenic assembly of hypogeous edible *Ascomycota* species, belonging to the *Pezizaceae*. *Terfezia* species occur exclusively on the Mediterranean basin and the Middle East (Moreno et al., 2014) where they live in mycorrhizal symbiosis with the roots of various host plants, most of them *Cistaceae* (Morte et al., 2009).

Figure 5 – *Terfezia arenaria* ascocarp on a natural producing site (left) *T. arenaria* collection (right).

Terfezia ascocarps are characterized by: 1) its solid fleshy gleba without a regularly arranged *hymenium*, 2) *asci* randomly distributed throughout the gleba tissue, 3) non-amyloid asci containing 8 spores and 4) the ornamented spiny, warted or reticulated spores (Alsheikh, 1994; Hansen et al., 2001; Moreno et al., 2014; Trappe, 1971).

Many aspects of *Terfezia* life-cycle have not been completely elucidated yet. Among these, the mating system and the ploidy level of both mycorrhizal and ascogenous hyphae are the most debated aspects of *Terfezia* life-cycle. While it seems that *Terfezia* species are obligatory heterothallic[1] fungus, characterized by the maintenance of two mating types (i.e. MAT1-1 and MAT1-2) (Dyer et al., 2016; Kagan-Zur & Roth-Bejerano, 2008), regarding the ploidy level of both *Terfezia* mycelium and fruitbodies, the prevailing hypothesis is that the heterokaryotic phase is brief and is restricted to the developing ascocarps (Kagan-Zur & Roth-Bejerano, 2008). However, the number of species studied is still small, and some studies seem to support the existence of long-term heterokaryons in *Terfezia* species (Kagan-Zur et al., 1999).

2.2. PHYLOGENY AND TAXONOMY

Traditionally, all truffles and truffle-like *Ascomycetes*, including *Terfezia,* were classified in the order *Tuberales* (Alsheikh, 1994). Malençon (1938) suggested that *Terfezia* evolved from an epigeous ancestor, as all other *Tuberales*, whereas Gilkey (1939) considered *Terfezia* and *Choiromyces* to be evolved from a common hypogeous ancestor, within the *Tuberales* (Alsheikh, 1994). Trappe (1971) initially accepted *Terfezia* within the *Tuberales*, however, afterwards, he reconsidered and transferred it to the *Pezizales* (Læsøe & Hansen, 2007). Recently, molecular phylogenetic analyses have shown that *Terfezia* and other pezizalean *Ascomycetes* have actually evolved from ancestral epigeous pezizas and are now accepted within the *Pezizaceae* (Laessøe & Hansen, 2007). Likewise, the monophyly of *Terfezia* and *Tirmania* has also been accepted based on the molecular analysis of the Internal Transcribed Spacer (ITS) of nuclear ribosomal RNA region (Díez et al., 2002).

1. Heterothallic fungi are self-sterile and therefore need the presence of another individual of the opposite mating type to reproduce, whereas, homothallic fungi are self-fertile and do not need another individual of the opposite mating type to reproduce, although some homothallic species retain the ability to outcross (Rubini et al., 2011).

The first *Terfezia* species was described by Moris, as *Tuber arenarium* Moris, from Sardinia in 1829. Soon after, Tulasne and Tulasne, described two more *Terfezia* species which they named *Choiromyces olbiensis* and *C. leptodermus*. Only in 1851, the same authors proposed the creation of the genus *Terfezia*. At the time, they included five species within the genus: *T. arenaria*, *T. leptoderma*, *Terfezia olbiensis* - previously described - plus *T. berberidiodora* and *T. oligosperma* (Alsheikh, 1994; Kovács & Trappe, 2014). Meanwhile several other species were described and later, in 1869, summarized in the book "La Truffe" by Chatin. The first identification keys for the African, Asian, European and North American species were provided by Fischer, Bataille, Mattirolo and Gilkey, but it was Alsheikh who, in 1994, first monographed the genus worldwide (Alsheikh, 1994). Despite the aforementioned contributions, the nomenclatural and taxonomic history of the genus is filled with several old species names, many of them synonyms of earlier described species (Alsheikh, 1994), some lacking useful diagnostic features and most of them rarely cited after the first time (Zitouni-Haouar et al., 2018).

Since then, molecular taxonomic revisions have narrowed the genus *Terfezia* to only those species from the Mediterranean region and the Middle East, being proven to belong to the *Terfezia* s.str. (Kovács & Trappe, 2014). Furthermore, they also revealed the intraspecific diversity of *Terfezia* and multiple species complexes (Aviram et al., 2004; Bordallo et al., 2013; Díez et al., 2002; Ferdman et al., 2009; Kovács et al., 2011).

Five *Terfezia* species have been regularly reported from the Mediterranean region and the Middle East; namely, *T. arenaria* (Moris) Trappe, *T. boudieri* Chatin, *T. claveryi* Chatin, *T. leptoderma* Tul. (=*T. fanfani* Mattir.) and *T. olbiensis* Tul. & C. Tul. Additional *Terfezia* species have been described from the region, but they are now regarded as synonyms of those five species (Kovács et al., 2011; Montecchi & Sarasini, 2000). Recently, 12 new *Terfezia* species were described from the Iberian Peninsula, Canary Island, Greece and Algeria: (1) *T. albida* Ant. Rodr., Mohedano & Bordallo; (2) *T. alsheikhii* Kovács, M.P. Martín & Calonge; (3) *T. canariensis* Bordallo & Ant. Rodr; (4) *T. cistophila* Ant. Rodr., Bordallo, Kaounas & A. Morte; (5) *T. crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla; (6) *T. eliocrocae* Bordallo, A. Morte & Honrubia; (7) *T. extremadurensis* Mohedano, Ant. Rodr. & Bordallo; (8) *T. grisea* Bordallo, Kaounas & Ant. Rodr; (9) *T.* lusitanica Bordallo, Ant. Rodr., Louro, Santos-Silva & Mohedano; (10) *T. pini* Bordallo, Ant. Rodr. & Mohedano; (11) *T. pseudoleptoderma* Bordallo, Ant. Rodr. & Mohedano and (12) *Terfezia solaris-libera* Louro,

Nobre & Santos-Silva (Bordallo et al., 2012, 2013, 2015, 2018; Kovács et al., 2011; Louro et al., 2020; Zitouni-Haouar et al., 2018). Currently, the index fungorum lists 67 species records, including 11 varieties, though only 39 are considered valid names (Kirk, 2020). Finally, the present work retains 17 species and no varietal names (see section 1 for more details).

2.3. SPECIES IDENTIFICATION

Traditional taxonomy is based on external or internal morphological criteria (Jamali, 2014). However, evolution of desert truffles, such as the ones included within the genus *Terfezia*, typically involves a convergent reduction in morphological characters, otherwise useful to distinguish related epigeous *taxa* (Ferdman et al., 2005; Jamali & Banihashemi, 2012). Accordingly, given the reduced set of morphological characters within the genus and their homoplasy, assigning a name to a particular *Terfezia* specimen using only morphologic features is challenging (Díez et al., 2002). According to Kovács and co-workers (2011) only *Terfezia arenaria*, *T. claveryi* and *T. boudieri* can be easily separated by morphological characters. This situation lingered until the end of the 20th century when newly developed molecular phylogenetic studies have demonstrated beyond doubt that morphological characters of hypogeous *Ascomycota* can be unreliable (Ferdman et al., 2005). These early phylogenies revealed various misidentifications at the genus and species level (Hansen et al., 2001; O'Donnell et al., 1997). Later, molecular studies also revealed the intraspecific diversity of some *Terfezia* species and the existence of diverse species complex (including cryptic species) (Aviram et al., 2004; Bordallo et al., 2013; Díez et al., 2002; Ferdman et al., 2009; Kovács et al., 2011).

A variety of molecular techniques have been used for *Terfezia* molecular characterization. In that regard, amplification through polymerase chain reaction (PCR) followed by sequencing, and sequence comparison of target genes, became a widely useful tool in modern taxonomy and has been the most used for the *Terfezia* identification (Bordallo & Rodriguez, 2014; Bordallo et al., 2013, 2015; 2018; Ferdman et al., 2005, 2009; Kovács et al., 2011). The internal transcribed spacer (ITS) region separating the 18S and 28S nuclear ribosomal DNA (rDNA), which includes two spacers (ITS1 and ITS2) and the intervening 5.8S coding sequence (White et al., 1990), has become well characterized at the interspecific and intergeneric levels and is used as a convenient target region for the molecular identification of *Terfezia* (Sbissi et al.,

2011). Other molecular markers have also been tested for *Terfezia* identification, namely, the partial sequences of the chitin synthase, the β-tubulin genes and the Amplified Fragment Length Polymorphism (AFLP) based markers (Ferdman et al., 2009) but these approaches have been discontinued. However, recent studies have advocated that those other molecular markers (i.e. β-tubulin gene) can be used as an additional marker for *Terfezia* identification, in order to strengthen the resolution of the analysis, especially in the case of cryptic species and/ or species complexes (Radhouani et al., 2019).

2.4. GEOGRAPHIC RANGE AND DISTRIBUTION

The geographic range of the genus is currently restricted to the Mediterranean region and the Middle East. So, *Terfezia* species occurrences are circumscribed to countries bordering the Mediterranean, such as: southern Spain, Portugal, Italy, France, Hungary, Turkey, Morocco, Algeria, Libya, Egypt, Israel, the Arabian Peninsula, Iran, Iraq, Syria and Kuwait (Morte et al., 2009). Prior to this work, the available information on desert truffles distribution in Portugal was scarce and outdated. Still, four *Terfezia* species were known to occur in the country, namely, *T. alsheikii*, *T. arenaria*, *T. fanfani* and *T. olbiensis* (Bordallo et al., 2013, Chevalier, 2014). Not surprisingly *T. arenaria* is the most common and most recollected species (Machado & Ferreira, 2006). The present work, updated the existing knowledge about *Terfezia* species diversity in Portugal, and expanded the number species occurring in the country to 10 species (i.e. *T. alsheikii, T. arenaria, T. cistophila, T. extremadurensis*, *T. fanfani*, *T. grisea*, *T. lusitanica, T. pini, T. olbiensis and T. solaris-libera* sp. nov.).

Numerous ecologic factors influence the distribution of species over their geographic range, climate, soil and vegetation are among the most important. Climate is considered the key factor shaping the distribution of *Terfezia* species. It has been noticed that some species (i.e. *T. boudieri* and *T. claveryi*) are typical of extreme arid zones and, therefore, less common in Europe, but very common in North Africa, Israel and in the Arabic peninsula, where drought and higher temperatures are more severe. On the opposite end, *T. arenaria*, *T. fanfani* and *T. olbiensis* seem to be typically species of semiarid zones, being widely distributed in the western Iberian Peninsula and other European countries, but rare in southern and eastern range of the Mediterranean basin (Chevalier, 2014). Regarding soil features, members of *Terfezia* are found in a wide array of soil types, from sandy soils to heavy clay-rich ones, at

high or relatively low pH, though carbonate contents and pH were both found to strongly influence species occurrence (Bonifacio & Morte, 2014). For instance, *T. claveryi* and *T. boudieri* are found in calcareous soils, while *T. arenaria* and *T. fanfani* prefer acidic environments (Morte et al., 2009). Vegetation is the third main factor governing the geographic distribution within the genus. All *Terfezia* species are obligate symbionts of specific host plants, mainly members of the *Cistaceae* (Alsheikh, 1994; Morte et al., 2008) including different annual and perennial species of the genus *Helianthemum* and *Cistus*, but also with members of the *Fagaceae* and *Pinaceae* (i.e. oaks and pines) (Alsheikh, 1994; Díez et al., 2002; Fortas & Chevalier, 1992; Kagan-Zur & Roth-Bejerano, 2008; Morte et al., 2008). Indeed, the distribution pattern of *Terfezia* species seems to be so strongly correlated with their putative host's distribution, and both host specialization and host edaphic tolerance have been hypothesized to have played a significant role in *Terfezia* adaptive evolution. In agreement with that hypothesis were the observations that some southern European and North African *Terfezia* species (i.e. *T. arenaria*, *T. fanfani* and *T. olbiensis*) were able to establish symbiotic relations with multiple host plants including pines and oaks, as a result of the wider host diversity available, while on the other hand, the more southern species (like *T. boudieri* and *T. claveryi*) would only form *mycorrhizae* with *Helianthemum* spp., exhibiting higher level of host specificity (Díez et al. 2002). Therefore, it has been proposed that the Iberian Peninsula could have provided a pathway for *Terfezia* migration from arid and semiarid regions of North Africa to Europe (Díez et al., 2002).

2.5. ECOLOGICAL AND ECONOMIC IMPORTANCE

Taking into consideration that *Terfezia* has the broader host range of all desert truffle genera, their ecological value is greatly derived from its position as key symbiotic partners of several xerophytic host plants, in arid and semiarid ecosystems (Pérez-Gilabert et al., 2014). Arid and semiarid ecosystems extreme ecological conditions are usually restrictive to the spontaneous survival of living beings (Chenchouni, 2012). However, through mutualistic associations (mycorrhizae) with the roots of their putative xerophytic host plants, *Terfezia* species have a fundamental role in optimizing plant fitness and soil quality, improving the resilience of xerophitic plant communities against environment stresses, including nutrient deficiency, drought and soil disturbance. This adaptive strategy provides the plant with an increased

ability for nutrient capture and cycling, in soils with low nutrient availability, and provides the fungi with essential nutrients needed to survive (Barea et al., 2011).

Beside their key ecological role in arid and semiarid ecosystems, *Terfezia* species produce edible ascocarps highly prized, in several Middle-Eastern, North-African and Mediterranean countries, where they have a prominent place in the local diet and traditional medicine of native populations (Alsheikh & Trappe, 1983; Mandeel & Al-Laith, 2007; Slama et al., 2010). In fact, Saharan Bedouins and Middle-Eastern nomads have long used *Terfezia* ascocarps as a food source, as a food delicacy, and as an emergency resource, in times of food scarcity and have marketed them in desert towns of the Arabian Peninsula and North Africa for hundreds of years (Volpato et al., 2013). Trappe (1990) mentioned that on favorable years, truck-loads of *Terfezia* were brought in big sacks like potatoes to the local markets in Kuwait. Though the exact global retail market for these truffles is still largely unknown (Perez, 2013), the approximate wholesale prices per kilogram of *Terfezia* ascocarps can reach 3OO US dollars (ca. 280 € at present rate) per kilogram (Morte et al., 2012), which can represent an important additional income for the local populations (Slama et al., 2010).

3. *TERFEZIA* MYCORRHIZAS

3.1. DEFINITION OF MYCORRHIZA

The term ‘mykorrhiza’ was first used in 1885 by Frank to describe the modified root structures of forest trees and has since been extended to cover a range of mutualistic, symbiotic associations between fungi and plant roots (Smith and Read, 2008). Mycorrhizal symbiosis is the most ancient, widespread form of fungal symbiosis with plants (Finlay, 2008). These symbiotic associations likely arose during the initial land colonization by plants and are nearly ubiquitous in terrestrial habitats, occurring in over 90 % of all known plant families (Hibbett et al., 2000). Indeed, interactions between arbuscular mycorrhizal fungi and the earliest land plants dates back to at least 400 million years, when *Glomeromycota* fungi began to form structures resembling arbuscules in the roots of early Devonian plants without true root systems, including many mosses and ferns, forming what is now known as arbuscular mycorrhizas (Humphrey et al., 2010). Nowadays, mycorrhizal symbiosis between soil fungi and land plants is one of the most widespread and classical examples of mutualistic associations on earth (Feijen et al., 2018; Smith & Read, 2008). While in the traditional view

these symbioses were described as “one fungus–one plant associations”, it is now well established that most plant roots are colonized by multiple mycorrhizal fungi and that most mycorrhizal fungi are not host-specific, colonizing various host plants at the same time (Hibbett et al., 2000). Therefore, plants are frequently interconnected by common mycelial networks (CMN), the so-called ‘wood-wide-webs’, in which individual host plants form associations with groups of fungal species, whose composition may shift over time (Van der Heijden et al., 2015).

3.2. MYCORRHIZAL TYPES

Traditionally all mycorrhizal symbiosis were classified as either ectomycorrhizas or endomycorrhizas depending on the location of the fungal hyphae in relation to the root tissues of the plant. Accordingly, the ectomycorrhizas (ECM) are those associations where the fungal cells did not enter inside root cells; and the endomycorrhizas (EM) describe those that did penetrate inside root cells. Yet, this classification is now regarded too simplistic and hence, from the structural point of view, five types of mycorrhizal symbiosis are generally accepted (**Fig. 6**); However, due to the recent discovery of arbutoid and monotropoid mycorrhizae, seven different categories of mycorrhizal symbiosis are currently distinguished on the basis of their morphological characteristics and the fungal and plant species involved (Finlay 2008).

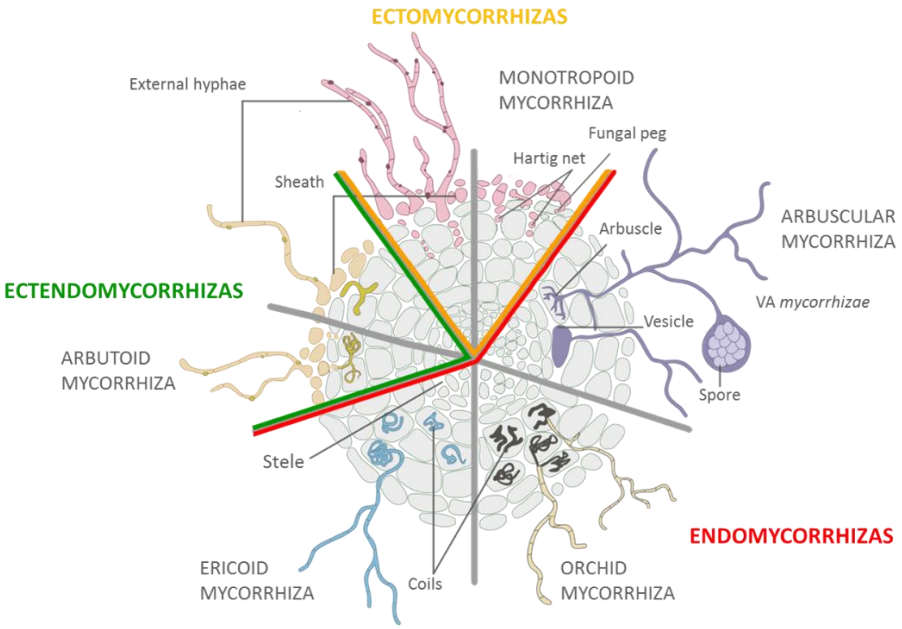

Figure 6 – Mycorrhizal types according to Finlay (2008)

3.2.1. Arbuscular mycorrhizas

Arbuscular mycorrhizas are the most widespread type of *mycorrhizae.* They are formed by members of the *Glomeromycota* and the following plant *taxa*: mosses, ferns, conifers and flowering plants They are anatomically characterized by highly branched fungal structures (i.e. arbuscules), which grow intracellularly without penetrating the host cells cytoplasmic membranes. These symbioses were previously known as vesicular-arbuscular mycorrhizas or VAM, given that some of these associations were characterized by the presence vesicles as well (Smith & Read, 2008).

3.2.2. Orchid mycorrhizas

Orchid mycorrhizas are formed by very distinct *Basidiomycota* and members of the *Orchidaceae* (Johnson & Gehring, 2007). In most types of mycorrhizal symbiosis the fungal symbionts depend upon their autotrophic plant hosts to supply carbon, however in the orchid mycorrhiza this dependency seems to be reversed, since orchids are partially or wholly achlorophyllous for some part of their life, and hence they are initially entirely dependent upon the supply of carbon and nitrogen from fungi. In orchid mycorrhizas the fungal *hyphae* penetrate into the root cells and form highly coiled hyphal mass also called pelotons. Each intracellular peloton has a short life-span, lasting only a few days before it degenerates and be digested by the orchid cell (Dearnaley et al., 2017).

3.2.3. Ericoid mycorrhizas

Ericoid mycorrhizas have been formerly regarded as the most specific of mycorrhizas because they were thought to be restricted to a few number of families within the *Ericales,* with the participation of a small group of *Ascomycota* fungi (Straker, 1996). Lately, ericoid mycorrhizas are defined has associations formed, mainly between *Ascomycota* fungi and a few *Basidomycota*, and several families of *Ericales* and mosses and liverworts (Van der Heijden et al., 2015). Ericoid mycorrhizas are characterized by loose hyphal networks around the outside of hair roots, from which they penetrate the walls of epidermal cells and cortical cells to form densely pack intracellular coils without penetrating the host cells cytoplasmic membranes (Perotto et al., 2002).

3.2.4. Ectomycorrhizas

Ectomycorrhizas are the most advanced symbiotic association between higher plants and fungi (Moore at al., 2020). They are formed between thousands of *Basidiomycota* and *Ascomycota* fungi and several *Tracheophyta* hosts (mostly trees and shrubs) (Johnson & Jansa, 2017). Anatomically, ectomycorrhizas are characterized by the presence of a fungal mantle (or sheath) that envelops host roots and a Hartig net that surrounds root epidermal and/or cortical cells (Johnson & Gehring, 2007). Hormonal interactions between plant and fungus lead to dramatically altered root architecture including the suppression of root hairs and the formation of more or less developed hyphal networks or rhizomorphs that radiate outwards from the mantle into the substrate (Van der Heijden et al., 2015).

3.2.5. Monotropoid mycorrhiza

Monotropoid mycorrhizas are formed between few non-photosynthetic genera of *Ericacea* plant family (former *Monotropaceae* family) and some *Basidiomycota* fungi, belonging to the *Russulaceae* and *Boletaceae* families (Moore at al., 2019). They are structurally similar to ectomycorrhizas as they display a well-developed fungal sheath and Hartig net. However, monotropoid mycorrhizas exhibit a highly specialized haustorium-like structure (the fungal peg) which penetrates the epidermal cells. Though the role of the fungal peg still requires experimental investigation, the assumption is that similarly to orchid mycorrhizas, in monotropoid mycorrhizas the organic carbon is transferred to the monotropoid plant by the fungal symbiont which also forms ectomycorrhizas on neighboring autotrophic plants (Smith & Read, 2008).

3.2.6. Ectendomycorrhiza

Ectendomycorrhizas are formed between a restricted group of *Ascomycota* (*Pezizales*), mostly belonging to the genus *Wilcoxina*, and a few *Pinaceae*, namely, *Pinus* (pine), *Picea* (spruce) and *Larix* (larch) (Moore at al., 2019; Smith & Read, 2008). Ectendomycorrhizas exhibit both ECM and EM characteristics, as such, a Hartig net and sheath structures are produced but intracellular penetration of living plant cells also occurs and once inside a cell the hyphae branch repeatedly. In ectendomycorrhizas the sheath may be reduced or absent whereas the Hartig net is usually well developed (Smith & Read, 2008).

3.2.7. Arbutoid mycorrhiza

Arbutoid mycorrhizas are formed between *Basidiomycota* fungi and few genera of *Ericaceae* (former *Pyrolaceae* family) and, most notably *Arbutus* and *Arctostaphylos* (Johnson & Gehring, 2007). Arbutoid mycorrhizas are very similar to ectendomycorrhizas, they display an external sheath, a Hartig net, intracellular penetration occurs and hyphal coils are produced. However, arbutoid mycorrhizas exhibit prolific extramatrical mycelium and a well-developed fungal sheath, whereas the Hartig net is normally restricted to the outer layers of root cells (Moore at al., 2019; Smith & Read, 2008).

3.3. *TERFEZIA* PUTATIVE PLANT HOSTS

Understanding the preferences or specificity for partners in *Terfezia* symbiotic relationships is of paramount importance to increase our knowledge on the dynamics of these interactions. In the past, *Terfezia* species have been putatively linked to a wide diversity of host plants, however, many of those associations have been only suggested on the basis of field observations (Alsheikh, 1994). Currently, it is consensual that *Terfezia* most often form mutualistic symbioses with members of the *Cistaceae* (particularly species of *Helianthemum* and *Cistus*), *Fagaceae* and *Pinaceae* (Alsheikh, 1994; Comandini et al., 2006; Díez et al., 2002; Kagan-Zur & Roth-Bejerano, 2008; Morte et al. 2008; Morte et al., 2009). Still, *Terfezia* species vary widely in their ability to associate with different plant hosts, demonstrating some degree of specificity not only toward particular *Cistaceae* hosts (either basophilous or acidophilous species), but also towards either pine trees or oak trees (Díez et al., 2002; Kovács et al., 2011). Despite the perceived specificity of certain *Terfezia* species towards particular hosts, the subject of *Terfezia*-plant host specificity is not easy to address. Among the most relevant factors that have contributed to obscure our knowledge on this matter is the co-existence of several putative plant-host in the majority of *Terfezia* natural production sites. For instance, in the western Iberian Peninsula, both *T. arenaria* and *T. fanfani* can be found in open Mediterranean silvo-pastoral systems dominated by holm and/or cork oaks trees, with an understory vegetation comprised by *Cistus* shrubs and/or *Tuberaria guttata* (Diez & Manjon, 2001). Likewise, *T. olbiensis* can be found in France and Italy in pine and oak forests with an understory vegetation comprised of *Cistus* spp and *Helianthemum* spp. (Chevalier, 2014). More recently, the increasing amount of sequence data flowing from fungal molecular

ecology studies, produced a high number of newly described taxa. However, many deposited sequences in available databases have little or no associated geographic and ecological information and therefore the question remains far from being resolved (Louro et al., 2019).

3.4. *TERFEZIA* MYCORRHIZAL PLASTICITY

Terfezia mycorrhizae display great structural versatility, forming different types of mycorrhizal associations depending on certain factors, such as: host species, concentration of auxins secreted by the fungi, root sensitivity to those auxins, phosphate concentrations in culture media and drought conditions (Roth-Bejerano et al., 2014; Zitouni-Haouar et al., 2014).

In conformity, *Terfezia* species are able to form: ectomycorrhizas, characterized by a Hartig net, but without a true sheath (Dexheimer et al., 1985; Gutiérrez et al., 2003; Roth-Bejerano et al., 1990); endomycorrhizas characterized by undifferentiated coil-shaped or globular intracellular hyphae penetrating the plant cells (Awameh, 1981; Gutiérrez et al., 2003; Kagan-Zur et al., 1999; Slama et al., 2010); and ectendomycorrhizas, characterized by the presence of both intercellular Hartig net and intracellular hyphae penetrating the cortex cells (Navarro-Ródenas et al., 2012, 2013). Lately, it has been observed that in some instances more than one of the above mycorrhizal types may be observed along the same root system of a single *Helianthemum* plant, a phenomenon that has been named “ectendomycorrhiza continuum” (Navarro-Ródenas et al., 2012).

3.5. *TERFEZIA* – *CISTUS* ASSOCIATIONS

The genus *Cistus* L. (*Cistaceae*) is one of the most characteristic genera of the Mediterranean flora (Carlier et al., 2008). It is comprised by a group of about 20 perennial shrub species, distributed throughout the Mediterranean region and Canary Islands. *Cistus* species are involved in many ecological processes taking place in Mediterranean ecosystems (López-Orenes et al., 2013). Furthermore, they support a vast and rich set of fungal communities, constituting mycorrhizal fungal inoculum reservoirs in the absence of host trees (Torres et al., 1995). In total, more than 200 fungal species, belonging to 40 genera, have been reported to be associated with *Cistus* among which, several edible hypogeous *Ascomycota*, mainly included in *Tuber* and *Terfezia* genera (Comandini et al., 2006). Despite the wide distribution

of *Cistus* species in the Mediterranean basin and the awareness of their relevance as putative host for many *Terfezia* species, very few studies have been undertaken to characterize and describe these mutualistic associations. For example, *Terfezia arenaria* has been documented in association with *Cistus ladanifer* L., *Cistus monspeliensis* L. and *Cistus salviifolius* L.. Similarly, *Terfezia leptoderma* s.l. has been registered in association with *Cistus albidus* L., *Cistus monspeliensis* L., *Cistus salviifolius* L. and *Cistus populifolius* L. (Alsheikh, 1994; Comandini et al., 2006). Finally, *Terfezia cistophila* has been described associated with *Cistus monspeliensis* L., *Cistus creticus* L. and *Cistus ladanifer* L.. However, to our best knowledge, no *Terfezia-Cistus* mycorrhiza was, so far, confirmed in the wild, not by morphologic nor by molecular means. Even so, the ability of *Cistus* to form mycorrhizae with *Terfezia* has been experimentally demonstrated in the past (**Table 1**).

Table 1. List of *in vivo* and *in vitro* mycorrhizal synthesis obtained so far between *Terfezia* spp. and *Cistus* spp. and its corresponding mycorrhizal structural type.

Species	Putative host	Mycorrhizal type	References
T. leptoderma s.l.	*Cistus albidus*	Ectomycorrhiza (without a true mantle)	Chevalier et al. (1984)
		Ectomycorrhiza (without a true mantle)	Leduc et al. (1986)
		Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
	Cistus incanus	Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
	Cistus monspeliensis	Ectomycorrhiza (without a true mantle)	Chevalier et al. (1984)
		Ectomycorrhiza (without a true mantle)	Chevalier et al. (1984)
	Cistus salviifolius	Ectomycorrhiza (without a true mantle)	Leduc et al. (1986)
		Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
T. boudieri	*Cistus albidus*	Ectomycorrhiza (without a true mantle)	Alsheikh (1994)
		Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
	Cistus incanus	Ecto- or Ectendomycorrhiza (without a true mantle)	Zaretsky et al. (2006)
	Cistus salviifolius	Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
T. claveryi	*Cistus albidus*	Ectomycorrhiza (without a true mantle)	Alsheikh (1994)
		Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
	Cistus incanus	Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)
	Cistus salviifolius	Ectomycorrhiza (with a thin less developed mantle)	Zitouni-Haouar et al. (2014)

For instance, *T. leptoderma* isolates were used to synthesize mycorrhizae with *C. albidus*, *C. monspeliensis* and *C. salviifolius* (Chevalier, 1984; Leduc et al., 1986). Twenty years later, Zaretsky et al. (2006) obtained mycorrhizae from transformed root clones of *C. incanus*, inoculated with *T. boudieri* collected in Israel, and more recently Zitouni-Haouar et al. (2014) reported mycorrhizae formation on *C. albidus*, *C. incanus* and *C. salviifolius* inoculated with *T.*

leptoderma, *T. boudieri*, and *T. claveryi*. Except for Zaretsky et al. (2006), that reported the formation of an ectendomycorrhiza on transformed root clone of *C*. *incanus* inoculated with *T*. *boudieri*, all other morphologic characterizations of the mycorrhizae formed between *Cistus* and *Terfezia* depict ectomycorrhizas, with well-developed Hartig net and a thin mantle. From the above, there seems to exist some plasticity of *Terfezia-Cistus* mycorrhizae. Yet, various *Terfezia-Cistus* associations were not experimentally verified so far. Furthermore, with the increase of new described *Terfezia* species in recent years, some of them *Cistus* specific (i.e. *Terfezia cistophila*), it is nowadays more crucial than ever to expand our current knowledge on these associations. In view of the above mentioned, the present work provides experimental evidences regarding the association of 4 different *Terfezia* species (i.e *T. arenaria*; *T. extremadurensis*; *T. fanfani*, *T. cistophila*) with the most widespread and common *Cistus* species in acid soils, namely, *C. ladanifer* and *C. salviifolius* (see section 2 for more details).

4. *TERFEZIA* CULTIVATION

4.1. INTRODUCTION TO TRUFFLE CULTIVATION

Truffle cultivation started long before the scientific knowledge related with the symbiotic associations between fungi and plant roots was developed. In fact, the first truffle cultivation trials were carried out by Pierre Mauléon and Joseph Talon, between 1790 and 1808, in central western and southern France. These early pioneers had the extraordinary insight of recognizing that seedlings or acorns planted under trees producing truffles were able to produce truffles when transplanted into new areas. This initial truffle cultivation system is currently known as the Talon method and is still currently used (Pérez-Moreno & Martínez-Reyes, 2014). Since then several volumes have sought to summarize the current knowledge concerning truffles or provide an overview on truffle cultivation and, nowadays, truffle cultivation in man-made orchards is feasible for many truffle species (Zambonelli et al. 2002). Yet, over the last century the global truffle cultivation industry has become highly centred on the more fragrant forest truffles (e.g. *Tuber melanosporum*, *T. borchii* and *T. aestivum*) and, as a result, desert truffle cultivation lagged several decades behind forest truffle cultivation. In fact, research on the cultivation of desert truffles started only in the late 1970s in Kuwait, when Awameh and colleagues (1979) carried out the first mycorrhizal synthess between *T.*

boudieri and *H. salicifolium*. Since then, many mycorrhizal synthesis between *Terfezia* species and various *Cistaceae* (mostly species from *Helianthemum* genus) have been obtained under controlled conditions (Morte & Andrino 2014). Yet, only after the first plantation of *Terfezia* mycorrhized plants was established in 1999, in Murcia, the increasing demand for this crop prompted more research, aiming to achieve new strategies to enable its medium-large-scale cultivation. One of the major developments in desert truffle cultivation technology was the medium to large scale introduction of inoculated seedling in field plantations. However, production of high-quality inoculated seedlings is not easy to attain, as it depends on the successful co-culture of both fungal symbiont and plant host in sterile or semi-sterile conditions. In fact, for decades, desert truffle cultivation was hindered by difficulties in obtaining good inoculum sources and due erratic seed germination and low plant survival rates in nursery conditions (Morte & Honrubia, 2009; Morte et al., 2008). Recently, new biotechnological tools have enabled mass production of high-quality *Terfezia* inoculated seedlings, but so far. only two *Terfezia* species -*T. claveryi* in Spain and *T. boudieri* in Tunisia- were successfully cultivated, both with perennial and annual *Helianthemum* species in basic soils (Morte et al., 2008, 2009, 2010, 2012; Slama et al., 2010).

4.2. FUNGAL SYMBIONT ISOLATION AND SUBCULTURING

Several types of fungal inocula have been used in the past to colonize plant roots in sterile, semi-sterile or non-sterile conditions. Among the most extensively used fungal inoculum sources are spores (gamic inoculum), mycelial pure cultures (vegetative inoculum) and colonized roots (symbiotic inoculum) (Zambonelli & Bonito, 2012). Both *Terfezia* spores and mycelial pure cultures have been used before as inoculum source, although mature spores have been used more frequently due to its faster growth (Morte et al. 2008). While there are many advantages to spore-based inoculations (i.e. inoculum is relatively cheap, easy to prepare and less time consuming) it is nevertheless difficult to obtain sterile and viable spore inocula due to associated contaminants, such as bacteria or fungal parasites, which readily colonize most conventional culture media. Therefore, *Terfezia* spore-suspensions are largely used for mycorrhizal synthesis in greenhouses (semi-sterile conditions). Regarding mycelial pure cultures (vegetative inoculum), the main limitation has to do with difficulty in maintaining pure cultures and in producing adequate amounts of biomass for large-scale mycorrhization programs. This is particularly challenging for truffle species. Indeed, isolation

and maintenance of *Terfezia* pure cultures can be challenging as many strains are unable to be sub-cultured. Furthermore, the few species actually able of being subcultured were found to produce insufficient amounts of mycelial inoculum on conventional culture media and conditions (Louro et al., 2019). The present work incorporates a European patent application for a new culture media and process for improved isolation and maintenance of *Terfezia* mycelium cultures (see Chapter V for detailed information).

4.3. HOST PLANT SELECTION AND PROPAGATION METHODS

Selecting a suitable host plant is one of the most important pre-requisites in the production of mycorrhizal plants. Suitable host plant species should be chosen taking into account edaphic and bioclimatic conditions (Morte et al., 2012). Also, it makes more sense to use perennial plant species than annual ones to maintain the cultures for more than a year (Morte et al., 2008). Yet, the only host plants tested in experimental desert truffle cultivation so far are perennial and annual species of *Helianthemum* from basic soils (Morte & Andrino, 2014). By contrast, other genera known to encompass numerous *Terfezia* host plants (e.g. *Cistus*, *Tuberaria*, *Quercus* and *Pinus*) have been rather neglected by most researchers and therefore their potential application on desert truffle cultivation is still largely unknown. As a result, selection of other suitable host plant species is still needed to enable *Terfezia* production in planned orchards in different environmental situations and over a broader range of habitats (Zitouni-Haouar et al., 2014).

Generally, conventional plant propagation methods follow to basic approaches: sexual and asexual. In sexual propagation the germinated seeds or spores formed by fusion of parental male and female gametes are used in the production of new plants. However, reproduction by seed is expected to cause considerable seedling variation given that the propagated offspring will have a genotype resulting from the recombination of those from the parental sources and therefore, exhibiting different combinations of parental characteristics (Hartmann & Kester, 1975). On the other hand, asexual propagation, also called vegetative propagation, rely on the pluripotency of some vegetative parts, found in the root and shoot apices (i.e. meristems) to give rise to a whole new plant or groups of plants. As a result, the new plants, called clones, are genotypic duplicates of the mother plants. Main methods of asexual propagation include, cuttings, layering, and division, budding and grafting (Relf, 2019). Tissue culture also known as micropropagation, is also a process of vegetative growth

and multiplication from plants tissues or seeds, in aseptic conditions on artificial growth media. Conventional vegetative multiplication is still widely used for cloned propagation of desirable plants of specific genotypes. However, certain plant species do not multiply well by vegetative multiplication because the propagated plantlets are recalcitrant to rooting (Srivastava et al., 2002). Accordingly, in recent years, tissue culture technologies have increased in industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites (Hussain et al., 2011). Tissue culture is based on concept of totipotency, or the ability of plant cells and tissues to develop into whole new plant (Chandana et al., 2018). The most used techniques developed for in vitro plant propagation, includes the induction of axillary and adventitious shoots, the culture of isolated meristems and plant regeneration by organogenesis and/or somatic embryogenesis (Iliev et al., 2010). Both conventional propagation and tissue culture methods can be used for desert truffle mycorrhizal plant production, however, it is advisable to use micropropagation whenever possible (Morte et al., 2008).

4.4. *IN VITRO* VS. *EX VITRO* PRODUCTION OF MYCORRHIZAL PLANTS

The production of mycorrhizal plants under controlled conditions is the key step of any mycorrhization program. Furthermore, it is of great importance for verification of the symbiotic compatibility between the intended fungi and their putative host plants, for physiological and biochemical studies and, also, for studies of the genetic control of the symbiosis (Repáč, 2011). Typically, mycorrhizae synthesis can be achieved by exposing the selected host plants to a given inoculum source, in a favorable growth environment. Depending on the goal, different approaches (i.e. *in vitro* and *in vivo*) can be used produce desert truffle mycorrhizal plants. However, the time required per approach is variable and depends on the type of plant propagation system and inoculum source (**Fig. 7**).

In the *in vitro* system, desert truffle mycelium, isolated in solid agar media or from liquid fermentation, is used for inoculation of micropropagated seedlings and maintained in specific grow media, especially designed to support both the fungal symbiont and the plant host, under sterile conditions (**Fig. 8**). This method has several advantages over other, semi-sterile, or non- sterile (*in vivo*) techniques. It is the only method that strictly guarantee the

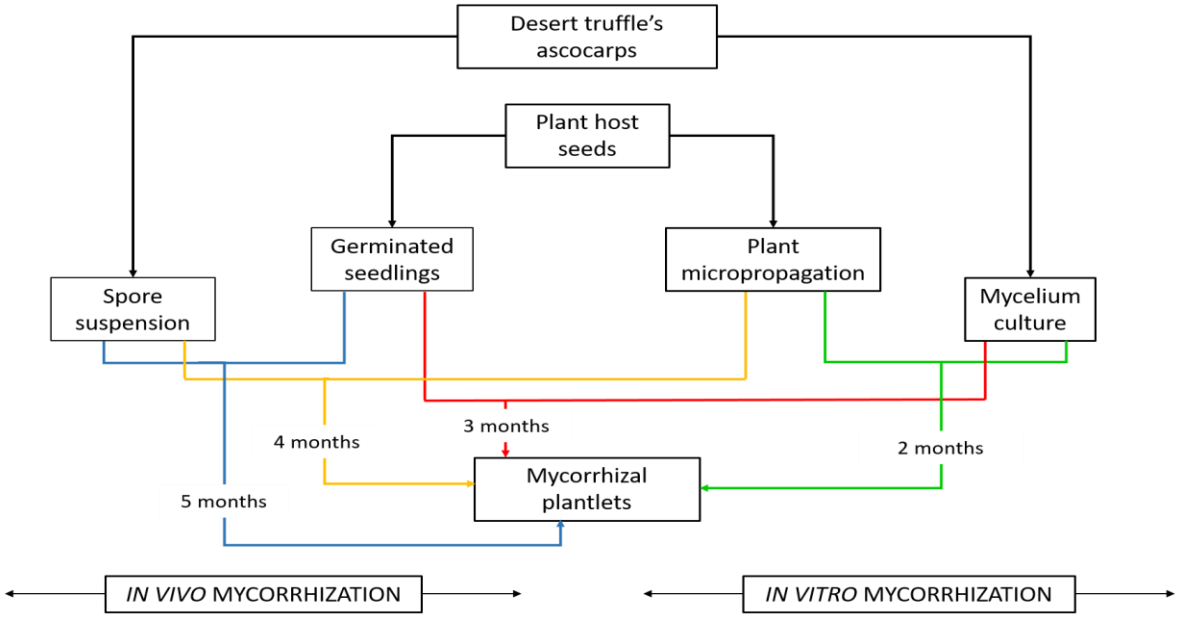

Figure 7 – *In vivo* and *in vitro* techniques of producing desert truffle mycorrhizal plants and the time required for each of them.

absence of undesirable contaminants and therefore the production of high quality and contaminants-free mycorrhized seedlings. Moreover, it has the advantage of being faster than any of the *in vivo* options and can be used all year long, since both the mycelial inoculum and the micropropagated seedlings can be produced whenever required. However, the *in vitro* system is costly because a specific equipped laboratory and specialized personnel are required to maintain a sterilized environment throughout the whole process and the scaling-up is complex (Morte et al., 2008).

Figure 8 – Illustrative example of *in vitro* mycorrhization using micropropagated *Helianthemum almeriense* Pau plantlets.

In respect to *in vivo* systems, three semi-sterile, or non- sterile techniques have also been successfully tested to produce desert truffle's mycorrhizal plants in nursery conditions (Morte et al., 2008). The first technique involves the use of previously prepared desert truffle's spore suspensions to inoculate germinated seedlings. It consists on the most low-tech and low-cost option, but quality control is problematic since it is virtually impossible to guarantee the absence of undesirable contaminants. Furthermore, desert truffle's spores may present a lag phase before germination that can reach in some cases up to 3 months; consequently, this is also the slower *in vivo* approach. Furthermore, the use of germinated seedlings has other disadvantages, for instance, most *Cistaceae* species display low and erratic seed germination rates due to hard-seededness (Thanos & Georghiou, 1988) and, hence, obtaining consistent and standardized plant material can be challenging. The second and third *in vivo* techniques are intermediary solutions which basically allow for a reduction on time throughout the whole process. For instance, mycelial forms of inoculum can be more effective than spores in colonizing roots; therefore, the inoculation of germinated seedlings with a mycelial culture allows saving time due to the rapid root system colonization by the mycelium. Similarly, using micropropagated plants allows a 2-month reduction in time due to the rapid shoot proliferation and rooting of micropropagated plantlets, when compared to the time necessary for any seedling to develop a suitable root system for mycorrhizal establishment.

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OBJECTIVES AND THESIS OUTLINE

Desert truffles have since long be reagarded as an important natural resource in the Mediterranean area, representing an important additional income for the local populations. They are also key symbiotic partners of several xerophytic host plants, in arid and semiarid ecosystems and play an important role in the maintenance of vegetation that prevents erosion and desertification. Lately, with the threat of global warming and the increasing severity of desertification in arid and semiarid areas worldwide, desert truffle cultivation is becoming an interesting alternative agricultural crop. Yet, desert truffle cultivation is still in its infancy and despite all research efforts in the last two decades, only two desert truffle species (i.e. *T. claveryi* and *T. boudieri*) were to date successful cultivated, both on *Helianthemum* species in basic soils. Research on how to cultivate *Terfezia* spp. on different plant hosts and over a broader array of soil types and pH values is still necessary in order to find most suitable "fungal symbiont – plant host" combinations for sustainable and efficient desert truffle cultivation over a wider range of habitats. With this in mind, this thesis aims to: 1) expand the current knowledge on the diversity of genus *Terfezia* in southern Portugal; 2) study their putative mycorrhizal associations with *Cistus* spp.; and 3) develop methodologies to allow *Cistus* spp. to be used as host in desert truffle cultivation in acid soils. To appropriately meet these research goals the below overall approach was designed **(Fig.9)**.

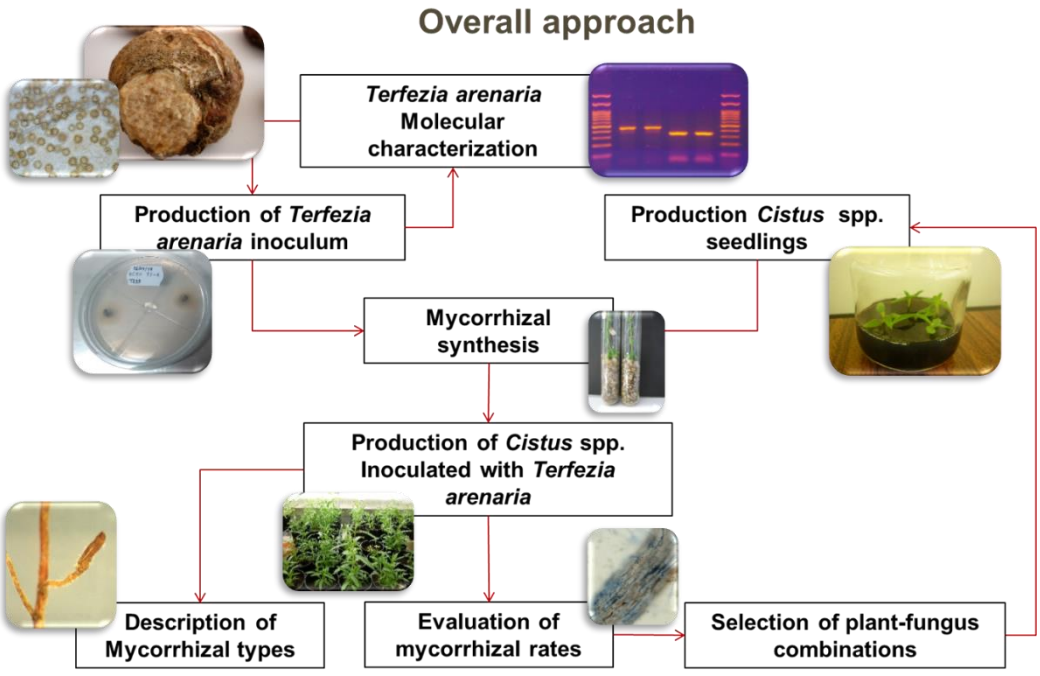

Figure 9 – Schematic representation of the research approach taken

Due to the complex nature of the research, this thesis is divided into two sections.

Section I, addresses the topic of the diversity of genus *Terfezia* and therefore, in:

Chapter I, presents a reconstructed phylogeny to the genus *Terfezia* whereby some of the more pressing taxonomic issues within the genus *Terfezia* are solved;

Chapter II and Chapter III, which resulted from extensive sampling and molecular characterization of *Terfezia* specimens collected in southern Portugal throughout this study, describes two previously unknown *Terfezia* species.

Section II, focus on the study of the mycorrhizal associations between *Terfezia* and *Cistus* and its application on desert truffle cultivation in acid soils. Thus, in:

Chapter IV an improved micropropagation protocol for mass production of *Cistus salviifolius* plantlets is presented, which has been also applied successfully by the authors for the propagation of other *Cistus* species (e.g. *C. ladanifer*);

Chapter V features the development of a new culture media and process for improved isolation and maintenance of *Terfezia* spp. mycelium cultures which has been object of a patent (European Patent Application n. 19204730.6 – 1118 (10.01.2020)).

Both preceding chapters were pivotal to enable the comparative study of the *in vitro mycorrhizae* formed by *Terfezia* spp. with both *Cistus salviifolius* L. and *Cistus ladanifer* L. that is presented in,

Chapter VI which allows to 1) provide the first anatomic descriptions of the *in vitro mycorrhizae* formed by four *Terfezia* species (i.e *T. arenaria*; *T. extremadurensis*; *T. fanfan*i, *T. cistophila*) with *C. ladanifer* and *C. salviifolius*; 2) compare the respective colonization rates; and 3) make some considerations regarding their potential application on desert truffle cultivation.

SECTION I

DIVERSITY OF GENUS *TERFEZIA* IN SOUTHERN PORTUGAL

Highlights:

- *Terfezia* classification is updated and the pressing taxonomic issues were solved.
- We proved incorrect the synonymy between *Terfezia trappei* and *Terfezia cistophila*.
- The *T. leptoderma/olbiensis* complex comprises several hidden *Terfezia* lineages.
- An identification key to *Terfezia* genus was developed and made available.
- Two new *Terfezia* species were described.

This section includes the following publications:

Louro R., Santos-Silva C., Nobre T. 2019. What is in a name? *Terfezia* classification revisited. ***Fungal Biology***, 123(4): 267-273.

Bordalo JJ., Rodríguez A., Santos-Silva C., **Louro R.**, Muñoz-Mohedano J., Morte A. 2018. *Terfezia lusitanica*, a new mycorrhizal species associated to *Tuberaria guttata* (*Cistaceae*). ***Phytotaxa***, 357(2): 141– 147.

Louro R., Nobre T., Santos-Silva C., 2020. *Terfezia solaris-libera* sp. Nov., A new mycorrhizal species within the spiny-Spored Lineages. **J Mycol Mycological Sc**i, 3(2): 000121.

CHAPTER I

What is in a name? *Terfezia* classification revisited

Data published in:

Rogério Louro, Celeste Santos-Silva, Tânia Nobre

Fungal Biology, 2019, 123(4): 267-273DOI

ABSTRACT

Desert truffles (mycorrhizal hypogeous *Ascomycota*) are found in arid and semi-arid areas of the globe and have great ecological and economic importance. *Terfezia* is undoubtedly the most diversified of all desert truffle genera, but its taxonomy is far from resolved. Specifically, the large number of newly described species plus the high intraspecific morphological variability observed within some *Terfezia* lineages as rendered the use of molecular techniques mandatory for specimen's discrimination. But the subsequent increasing amount of sequence data produced also a huge number of undescribed taxa that required determination. We compiled and used the public available ITS data on *Terfezia* spp. on the custom-curated UNITE database to reconstruct the genus phylogeny. We found at least 17 distinct lineages within the genus and successfully resolved some of the more pressing taxonomic issues, namely the *T. leptoderma*/*olbiensis* complex and some misapplied synonymy. Based on this resolved phylogeny, and motivated by the recent new described species, we proposed an identification key to *Terfezia* genus highlighting the importance of morphological and ecological characterization.

Keywords: Desert truffles, Ecology, Identification key, ITS Taxonomy, UNITE database

INTRODUCTION

Desert truffles are hypogeous Ascomycota that have evolved in several lineages within the *Pezizaceae*, and are typically found in arid and semi-arid areas throughout the world (Moreno et al., 2014; Morte et al., 2009; Navarro-Rodenas et al., 2011). They represent a key component of the mycological flora around the Mediterranean basin, establishing important mycorrhizal symbioses with diverse host plants, most often members of the *Cistaceae* (Díez et al., 2002; Kagan-Zur and Roth-Bejerano, 2008). Many of them are endemic and overall play an essential role in soil conservation - preventing erosion and desertification - of Mediterranean shrublands and xerophytic grasslands (Honrubia et al., 1992).

Terfezia Tul. & Tul. is undoubtedly the most diversified of all desert truffle genus (Kovacs and Trappe, 2014). The first *Terfezia* species was described by Moris, as *Tuber arenarium* Moris, from Sardinia in 1829. Soon after, Tulasne and Tulasne, described two more *Terfezia* species which they named *Choiromyces olbiensis* and *C. leptodermus*. Only in 1851, the same authors

proposed the creation of the genus *Terfezia*. At the time, they included five within the genus: *T. arenaria*, *T. leptoderma*, *Terfezia olbiensis* - previously described - plus *T. berberidiodora* and *T. oligosperma* (Alsheikh, 1994; Kovacs and Trappe, 2014). Meanwhile several other species were described and later, in 1869, summarized in the book "La Truffe" by Chatin. The first identification keys for the African, Asian, European and North American species were provided by Fischer, Bataille, Mattirolo and Gilkey but it was Alsheikh who, in 1994, first monographed the genus worldwide (Alsheikh, 1994).

Despite the aforementioned contributions, the nomenclatural and taxonomic history of the genus is filled with several old species names, many of them synonyms of earlier described species (Alsheikh, 1994), some lacking useful diagnostic features and most of them rarely cited after the first time (Zitouni-Haouar et al., 2018). The situation lingered in the pre-molecular era because the criteria for separating and/or identifying groups of species were limited largely to morphological, anatomic and chemical features (Bordallo and Rodríguez, 2014) which were not always unambiguous. As pointed out by Díez et al. (2002), the use of this type of features alone for classifying desert truffles is challenging, due to the reduced set of morphological characters and their homoplasy. The observed morphological convergence is likely to be environmental conditioned, but the possibility that in some cases speciation has occurred with hardly detectable morphological changes should also be acknowledged (Bordallo and Rodríguez, 2014; Díez et al., 2002). Intra-specific plasticity of phenotypes also contributes to the challenge of using some of these morphological characters for taxonomic purposes, and one has to consider that this ability of a genotype to produce different phenotypes might also be induced by the plant host or by other interacting microorganisms.

In the dawn of the molecular era, early phylogenies revealed various misidentifications at the genus and species level. Also, re-examination of some herbarium specimens and personal collections around the world, using molecular methods, exposed their inaccurate generic assignments and removed the ambiguity around their taxonomic status (Zitouni-Haouar et al., 2018). These early molecular approaches decreased considerably Terfezia richness and increased the geographic uniformity of the genus to encompass only those species regularly collected, at the time, from the Mediterranean region and the Middle East: *T. arenaria* (Moris) Trappe, *Terfezia boudieri* Chatin, *T. claveryi* Chatin, *T. leptoderma* Tul. and *T. olbiensis* Tul. & C. Tul (Kovacs and Trappe, 2014). Later, molecular studies also revealed the intraspecific diversity of some Terfezia species and the existence of diverse species complex (including

cryptic species) (Aviram et al., 2004; Bordallo et al., 2013; Díez et al., 2002; Ferdman et al., 2009; Kovacs et al., 2011). Specifically, a high intraspecific and/or intrasporocarpic rDNA internal transcribed spacer (ITS) variability was detected among collections of *T. leptoderma* (for some authors synonym of *T. fanfani*) and *T. olbiensis*, revealing at least four well supported lineages of *Terfezia* with spiny spores in addition to *T. leptoderma* (TLO-1a) (Kovacs et al., 2011). Also, considerable genetic variation was reported in *T. boudieri* (Aviram et al., 2004; Ferdman et al., 2009; Sbissi et al., 2011), and *T. claveryi* (Sbissi et al., 2011). In relation to the synonymy between *T. leptoderma* and *T. fanfani* considered as separated species in the pre-molecular era, recent phylogenetic studies do not show a clear distinction between the sequences assigned to each of those species names. Instead, most sequences previously identified either as *T. leptoderma* or *T. fanfani* are phylogenetically always nesting together in a well-supported monophyletic group - see for instance in Bordallo et al. (2013) and Bordallo et al. (2015) - or placed together with other spiny spored *Terfezia* (e.g. *T. leptoderma*/ *T. fanfani*/*T. cistophila*) - see the work of Zitouni-Haouar et al. (2018). A similar problem is poised between *T. olbiensis* and *T. lepdoderma*, since the former was considered by many authors as synonym or an immature form of *T. leptoderma* based on their morphological similarities (Díez et al., 2002). However, newly molecular and morphological studies seem nowadays to support that *T. olbiensis* is in fact a distinct and valid species (Bordallo et al., 2013). Recently, 12 new *Terfezia* species were described from the Iberian Peninsula, Canary Island, Greece and Algeria: (1) *T. alsheikhii* Kovacs, M.P. Martín & Calonge (Kovacs et al., 2011); (2) *Terfezia canariensis* Bordallo & Ant. Rodr (Bordallo et al., 2012); (3) *T. albida* Ant. Rodr., Mohedano & Bordallo; (4) *T. eliocrocae* Bordallo, A. Morte & Honrubia; (5) *T. extremadurensis* Mohedano, Ant. Rodr. & Bordallo; (6) *Terfezia pini* Bordallo, Ant. Rodr. & Mohedano; (7) *T. pseudoleptoderma* Bordallo, Ant. Rodr. & Mohedano (Bordallo et al., 2013); (8) *T. grisea* Bordallo, Kaounas & Ant. Rodr.; (9) *T. cistophila* Ant. Rodr., Bordallo, Kaounas & A. Morte (Bordallo et al., 2015); (10) *T. trappei* (R. Galan & G. Moreno) A. Paz & Lavoise (Paz et al., 2017); (11) *T. crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjon, Fortas, & Carlavilla (Zitouni-Haouar et al., 2018); and (12) *Terfezia lusitanica* Bordallo, Ant. Rodr., Louro, Santos-Silva & Mohedano (Bordallo et al., 2018). This granted *Terfezia* the title of the most speciated desert truffle genus, totalling 17 species.

What is in a name? Accurate *Terfezia* species determination is important for our understanding of the ecological functioning of the system (e.g. essential role in soil

conservation), and is crucial if we consider their economic significance for the rural populations on the Mediterranean basin. Desert truffles fruit bodies are a potentially important food source for animals and humans, rich in proteins and poor in lipids (Chevalier, 2014; Kovacs et al., 2011). Plus, given the considerable prices they may reach in local markets, their cultivation has the potential to enhance the socio-economic development of rural and/or local populations around the Mediterranean basin.

Aiming the establishment of a consensual *Terfezia* classification, we revised the public available data on this genus and constructed an identification key to the known *Terfezia* species. Based on data deposited at the custom-curated UNITE database (https://unite.ut. ee/), we have reconstructed the genus phylogeny and we con-fronted the results with putative plant host and soil parameters associated with the different specimens, whenever available. We discuss the results integrating them with meaningful morphologic and ecologic characters towards a simple to use identification key of the several Terfezia species.

METHODS

Data collection

Sequence data was obtained from the Unified system for the DNA based fungal species linked to the classification (UNITE, https://unite.ut.ee/). UNITE is the product of a consortium of fungal ecologists, taxonomists, and bioinformaticians. The custom-curated UNITE database includes many sequences from specimens that were collected and deposited by taxonomic specialists. A total of 220 Terfezia spp. genomic DNA sequences - containing a full (or partial) region comprising 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene - were retrieved [by February 2018, considering only sequences of 500 bp or more, plus the available se-quences of the recently described species *T. crassiverrucosa* (Zitouni-Haouar et al., 2018) and *T. lusitanica* (Bordallo et al., 2018)] together with information on ectomycorrhizal lineage, UNITE taxon name, geographic location, putative host, source of DNA. Whenever specimen's ecological and geographical information was missing, the European Nucleotide Archive was consulted for additional in-formation (**Supplemental Table 1**).

Phylogenetic analysis

Three known non-*Terfezia* sequences were added to the dataset as putative outgroups (*Tirmania* JF908769.1, *Cazia* AY830852.1 and *Peziza* JX414200.1). Sequences were aligned with online MAFFT version 7 using the E-INS-i strategy (Katoh et al., 2017). The phylogenetic reconstruction analysis based on the above ITS sequences was performed in BEAST v.4.2.8 software (Drummond and Rambaut, 2007), allowing the software to estimate the evolutionary model. All other settings were left as default. The output of BEAST was analysed in the software Tracer v.1.6 to determine chain convergence and burnin. The single tree that best represents the posterior distribution was summarized using the program TreeAnnotator v.2.4.8, considering a burn-in of 10 % (first 1000 trees were removed).

First we tested the hypothesis that all the database retrieved samples belong to *Terfezia* genus. This implied a comparison be-tween a first analysis of the full data set with no priors and one with all the samples putatively belonging to *Terfezia* species constraint to monophyly. Bayes factors were used to test if the topological con-strains were significantly different than the optimal topology, and were measured using twice the difference of ln likelihood (2lnBF) with 2lnBF ¼ 0e2 meaning not worth a mention, 2lnBF ¼ 2e6 meaning positive support, 2lnBF ¼ 6e10 meaning strong support, and 2lnBF > 10 meaning decisive support (Grummer et al., 2014). The non-*Terfezia* samples were removed.

With the final dataset of 202 *Terfezia* samples (after removal of non-*Terfezia* sequences) the same approach was used to estimate *Terfezia* phylogeny. Additionally, and for comparison purposes, phylogenetic relationships were also estimated using two methods: approximate maximum-likelihood (ML) and Neighbour-joining (NJ) using the software MEGA7 (Kumar et al., 2016). Branch support in the ML and NJ trees was tested by means of 1000 bootstrap replicates. This data is shown as supplementary material (**Supplemental Fig. S1 and S2**).

RESULTS

The reconstructed optimal phylogeny of all our dataset identified 18 sequences falling outside the clade of the *Terfezia* genus (**Fig. 1**). This model is significantly better (2·lnBF = 287.85; with BI ln [optimal model] = -6856.27 and BI ln [alternative model] = -7000.19). The final alignment consists of 783 bp including gaps, of which 382 have full coverage by all 202 Terfezia sequences. Considering the complete alignment, 314 positions are variable of which 236 are

parsimonious informative. Given the available Terfezia sequences, the reconstructed phylogeny ample supports the existence of 17 distinct clades representing well supported monophyletic groups (**Fig. 2**). Only one sequence (Gen-Bank accession no. AF396864), did not cluster in any of the 17 identified clades.

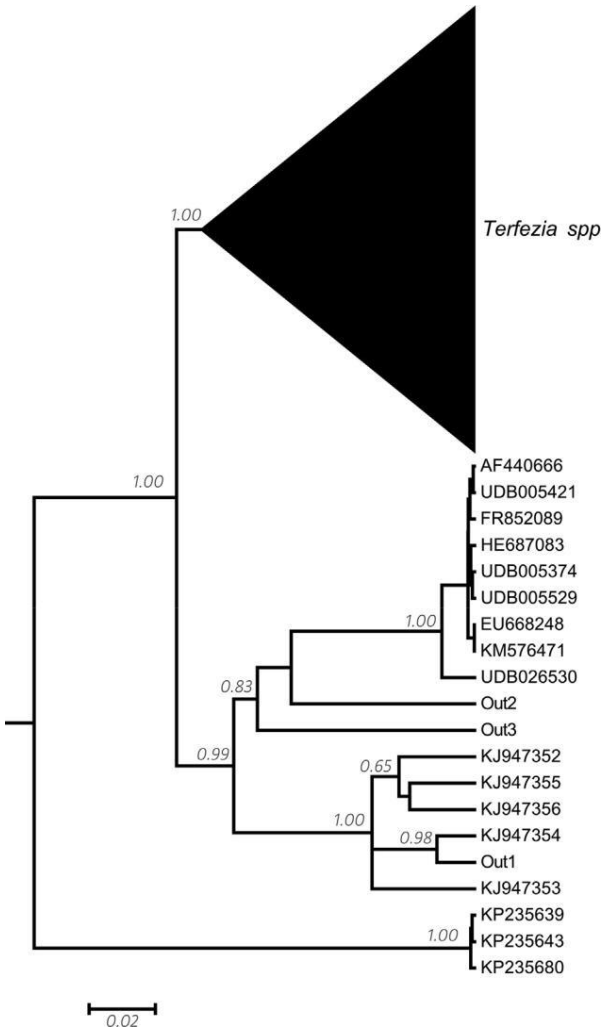

Fig. 1. Phylogenetic relationship between putative *Terfezia* species retrieved from the databases (see methods). The phylogeny corresponds to the majority rule consensus tree of trees sampled in a Bayesian analysis, and the posterior probability values are shown for main nodes. Three known non-*Terfezia* sequences were added to the dataset as putative outgroups (Out1: *Tirmania* **JF908769**; Out2: *Cazia* **AY830852** and Out3: *Peziza* **JX414200**). Qatar samples (**KJ947352**; **KJ947353**; **KJ947354**; **KJ947355**; **KJ947356**); Soil isolates unspecified geographical region (**KP235639**; **KP235643**; **KP235680**); Australia (**DQ061109**); all other sequences are from several different sources.

In line with our reassembled phylogenetic tree, all sequences previously determined by UNITE database as *T. alsheikhii*, and only these, clustered together producing a strongly supported homogenous group. The same was also true for the following clades: *T. arenaria*, *T. boudieri*, *T. claveryi*, *T. eliocrocae* and *T. grisea*. These species seem robust in terms of identification,

as all sequence-s identified by UNITE database are correct and no other available sequences (e.g. *Terfezia* sp.) match the aforementioned species. As to Clade A, we verified that it was fundamentally composed by numerous sequences identified at UNITE database only to genus level, namely, 1 isolate retrieved from a mycorrhizal root tip and 34 sequences formerly classified by Kovacs et al. (2011) as *Terfezia* aff. *olbiensis* belonging to the TLO-3 group. However, the only 2 confirmed *T. pini* sequences also nested within this clade. From the above, it seems fairly likely that the well supported Clade A, might indeed represent the species *T. pini*. This claim seems to be further corroborated by the ecology of the specimens within the group, which as far as we could retrieve the information, share the same putative plant hosts (*Pinus* spp. and *Quercus* spp.) (see **Supplemental Table 1**). The clade named *T. albida* (**Fig. 2**) included the only 2 samples formerly determined as such, plus 2 more sequences identified as *T. olbiensis* in the UNITE database. These later sequences had been suggested to represent *Terfezia* aff. *olbiensis* by Kovacs et al. (2011), which at the time included them in their proposed group TLO-2. The remaining *T. olbiensis* recognized in the UNITE database (10 sequences) clustered together in our phylogenetic analysis forming the well supported clade named *T. olbiensis*. Thus, our results corroborate the notion that *T. olbiensis* represents a true valid species. With respect to the *T. extremadurensis* clade, besides 6 sequences previously identified as such, 7 other sequences nested within the clade: one sequence previously named *T. leptoderma* and six labelled *Terfezia* sp., these latter having previously been considered as *Terfezia* aff. *olbiensis* (TLO-4) (Kovacs et al., 2011). As for the clade *T. cistophila*, the majority of the sequences (9) were correctly assigned to *T. cistophila* by UNITE. Still, 2 more sequences named *Terfezia* sp. also nested inside this clade, one being an isolate from a mycorrhizal root tip and another previously identified by Kovacs et al. (2011) as *Terfezia* aff. *olbiensis* (TLO-1). The clade *T. pseudoleptoderma* clustered together 3 sequences previously identified as such, plus 1 sequence coming from a strain isolated from a mycorrhizal root tip, which was, to date, deter-mined only to genus level (see Supplemental **Fig. S1** and **Fig. S2**). The clade *T. fanfani* grouped 5 sequences identified as such, and 12 *T. leptoderma* sequences. These latter were identified as belonging to TLO-1a and named as *T. leptoderma* by Kovacs et al. (2011). In light of this result, our analysis supports the viewpoint advocated by Venturella et al. (2004) and Chevalier (2014) that the two names are in fact synonyms, denominating specimens belonging to a same species; therefore, the correct name should be assigned according to the rules of the International Code of Nomenclature for

algae, fungi, and plants. To the only sequence identified in UNITE as *T. trappei*, our analyses suggest the inclusion of 4 more sequences, one previously identified as *T. fanfani* and three as *Terfezia* sp., reported by Kovacs et al. (2011) as *Terfezia* aff. *olbiensis* (TLO-1) and three as *Terfezia* sp., reported by Kovacs et al. (2011) as *Terfezia* aff. *olbiensis* (TLO-1).

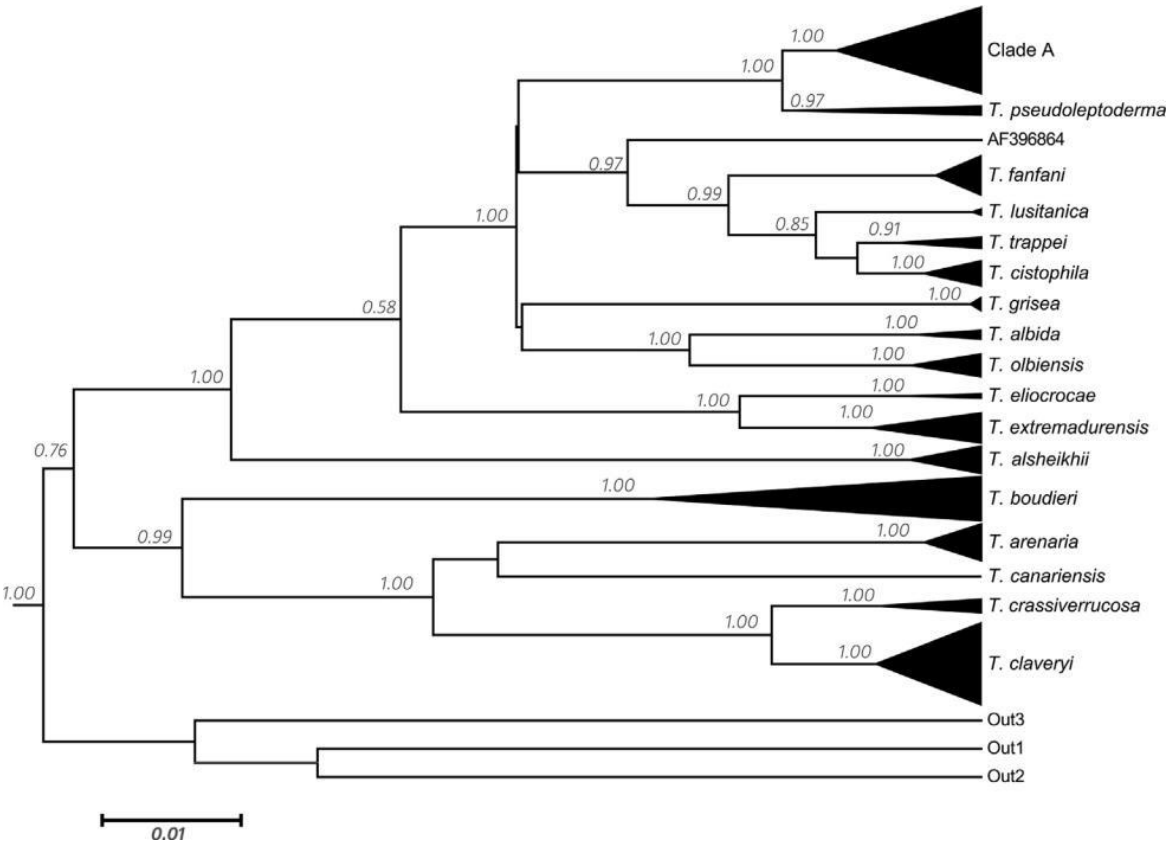

Fig. 2. Phylogenetic relationship between Terfezia species. The phylogeny corresponds to the majority rule consensus tree of trees sampled in a Bayesian analysis, and the posterior probability values are shown for main nodes. (ML and NJ analyses presented in **Supplemental Fig. S1 and Fig. S2** respectively).

Yet, the name *Terfezia trappei* was only considered taxonomically valid in 2017, after the publication of Paz and co-workers (Paz et al., 2017) which, in their monograph of the genus *Elaphomyces* in Europe, suggest that the isotype of *Elaphomyces trappei*, described by Galan and Moreno (1991), is indeed *T. trappei*.

T. crassiverrucosa well supported clade comprises the sequence from the holotype and 5 more sequences previously identified as *Terfezia* sp. in the UNITE database. *T. crassiverrucosa* is one of the most recently described *Terfezia* species and is a striking example of a cryptic species hidden under the name *T. claveryi* throughout the pre-molecular era: their morphologic similarity and sharing of known ecology, made them non-distinguishable. Finally, *T. lusitanica*, a recently described *Terfezia* of which only the recently published sequences are

known (Bordallo et al., 2018), also formed a distinct well-supported clade with none of the remaining *Terfezia* sequences analysed being included.

DISCUSSION

Traditional *Terfezia* classification has largely relied on morphological, chemical and organoleptic characters, and later, also on host plant and soil features. However, the large number of newly described species (Bordallo et al., 2012, 2013; 2015, 2018; Kovacs et al., 2011; Zitouni-Haouar et al., 2018), plus the high intraspecific morphological variability observed within some T*erfezia* lineages (Aviram et al., 2004; Bordallo et al., 2013; Díez et al., 2002; Ferdman et al., 2009; Kovacs et al., 2011) as rendered the use of molecular techniques mandatory for specimen's discrimination. Subsequently, the increasing amount of sequence data, flowing from fungal molecular ecology studies, using either classic Sanger sequencing or high-throughput sequencing technologies, produced also a huge number of undescribed taxa that needed determination. Yet, *Terfezia* species identification has not undergone the needed adjustment and updating towards minimization of data base errors.

Regardless of how the sequences are obtained, taxa determination is mainly inferred by homology, which means that the outcome of the inference is never better than the reference(s) itself. The most popular current nucleotide search tool, the National Centre for Biological Information (NCBI) Basic Local Alignment Search Tool, or BLAST, has numerous errors, poor-quality sequences, and many deposited sequences with little or no associated taxonomic nor ecological information (Nilsson et al., 2006). The downstream impact of it goes beyond naming, as it affects evolutionary studies and the biological understanding of an organism and its ecology, pathways analyses, systems, and metabolic processes as well (Klimke et al., 2011). Researcher's awareness on the shortcomings of the databases references can certainly minimize error propagation but one needs to keep in mind that this is a dynamic process that needs to be revised and updated in frame of the continuous new flow of data (Nobre et al., 2016).Through our reconstructed phylogenetic analysis, based exclusively on the custom-curated UNITE database, which includes many sequences from specimens that were collected and deposited by taxonomic specialists, we have reconstructed the genus phylogeny and were able to assign a name to almost all *Terfezia* sequences therein, that were identified only to genus level (52 sequences) or that were misinterpreted (17 sequences).

A relevant topic that also required resolution is *T. trappei*, which was described for the first time as *E. trappei*, in a period where molecular biology was not the widely disseminated taxonomical tool it is today, but that recently has undergone a reclassification as *T. trappei*, comb. nov. by Paz et al. (2017). The former authors also suggested that *T. cistophila* was a later synonym of *T. trappei* (Paz et al., 2017). However, we strongly disagree with the applied synonymy and propose that these are two independent taxa. To support this claim we highlight that despite both species share the same ecology (acid soils and *Cistus* spp. as putative plant host) and some morphological similarities, specifically in sporocarp size and colour, there are also marked differences between them, namely, in the peridium thickness (thicker in *T. trappei*), in spore dimension and ornamentation size (bigger in *T. trappei*). Our phylogenetic analysis further corroborates the existence of two distinct well-supported clades for *T. trappei* and for *T. cistophila*.

Another pressing issue that needs to be addressed is that little or no associated taxonomic, geographic and ecological information is available for many of the deposited sequences in most popular nucleotide databases. And even when that information exists, there is always the possibility of being incorrect. This seems to be the case of *T. cistophila* sequence (GenBank accession no. FJ013087) referred as associated with *Pinus pinaster* Aiton (see **Supplemental Table 1**); in our reconstructed phylogenetic tree it nests inside the clade of *T. cistophila*. If plant host is really an important feature in *Terfezia* specimen's determination (Díez et al., 2002), as we believe it is, then the information regarding the putative plant host must be given with care. As far as we know, *Terfezia cistophila* lives exclusively with *Cistus* spp (Bordallo et al., 2015). Still on misidentifications, two sequences (GenBank accession no. **HQ698145** and **HQ698146**) previously identified as *T. olbiensis* in the UNITE, in our analysis clustered inside *T. albida* clade. Given these specimens ecology, found associated with *Helianthemum* (see **Supplemental Table 1**), it is fairly clear that they are not *T. olbiensis*, which by all accounts lives in association with *Pinus* spp. and *Quercus* spp (Bordallo et al., 2013).

T. pini has only two UNITE identified sequences which clustered inside Clade A (**Fig. 2**) of our reconstructed phylogeny. Considering that most other sequences in this well supported clade are identified only to the genus level (all the *Terfezia* aff. *olbiensis* belonging to TLO-3) and given that the majority of them seem to be associated with *Pinus* and *Quercus*, it is most likely that this clade represents *T. pini*.

One of the most discussed issues over *Terfezia* classification, and one which persist to date despite the efforts to resolve it, is the taxonomic placement of *T. leptoderma* and *T. olbiensis*. While some authors tend to agree that *T. olbiensis* represent an immature form and a synonym of *T. leptoderma* (Bordallo et al., 2013; Díez et al., 2002; Moreno et al., 1986), others have chosen to consider these two as separated species (Montecchi & Sarasini, 2000) and more recently Kovacs et al. (2011) proposed it to represent a species complex hiding several lineages within (TLO-1, TLO-2, TLO-3 and TLO-4). We believe that our reconstructed phylogeny has for the first time successfully disentangled this species complex and allowed the assignment of a species name linked to each former TLO designations. Accordingly, TLO-3 sequences belong to *T. pini*, TLO-4 to *T. extremadurensis*, TLO-2 mainly to *T. olbiensis* and 2 sequences to the newly described *T. albida*, a very close species to *T. olbiensis*. And finally, TLO-1 mainly *T. fanfani* (former *T. leptoderma*), *T. cistophila*, *T. trappei*, *T. lusitanica* and seq. **AF396864** (which remains a puzzle). Regarding the only sequence which did not clustered in any of the above described clades, Díez et al. (2002) determined that the specimen which originated the sequence was morphologically similar to T. *leptoderma* - though they also noticed that it had slightly smaller spores with shorter spines - and it was associated with *Pinus halepensis* in acid soils. The combination of all the above mentioned features and taxonomic placement of this sequence in our phylogenetic reconstruction makes us hypothesize the possibility that this might indeed be a new taxon. Nevertheless, more specimens and sequences are still needed in order to confirm this hypothesis.

Direct sampling of sequences from environment or even from fungal tissue, with no further characterization, does facilitates large-scale mechanical production of *Terfezia* taxa names, based on minor sequence divergence, without taking in account the direct observation and characterization of individual organisms. Although molecular techniques are valuable tools to discriminate species, they should be always complemented with specimen's morphological and ecological description. Despite the information available on different websites, to the best of our knowledge, no complete identification key for Terfezia genus was produced so far. In this context, we propose a dichotomous identification key to aid in the identification of mature *Terfezia* specimens, based on morphological and ecological characters.

Key to *Terfezia* species

(Ascospore measurements do not include ornamentations)

1 Spiny spores 2
Warty or warty reticulated spores 11

2(1) In alkaline soils 3
In acid soils 5

3(2) With *Cistaceae*; spores ≥14 µm with blunt and/or truncated spines 4
With *Pinus* and/or *Quercus*; spores <14 µm with pointed spines ..... ***T. olbiensis***

4(3) Peridium light colour; gleba with green colours ..... ***T. albida***
Peridium dark colour; gleba without green colours ..... ***T. grisea***

5(2) Ascocarp diameter >4.5 cm 6
Ascocarp diameter <4.5 cm 7

6(5) Ascocarp brown-reddish; spores with straight pointed spines ..... ***T. fanfani***
Ascocarp brown; spores with conical (≈3-4 µm at base) blunt and truncated spines ..... ***T. extremadurensis***

7(5) Gleba without green colours; with *Cistaceae* 8
Gleba with green colours; with *Cistaceae*, *Pinus* or *Quercus* 9

8(7) Spores ≥15 µm with flexuous blunt spines; with *Cistaceae* ..... ***T. pseudoleptoderma***
Spores <15 µm with straight spines; with *Cistus* exclusively ..... ***T. cistophila***

9(7) Peridium <1 mm 10
Peridium ≈1 mm ..... ***T. trappei***

10(9) With *Pinus* and/or *Quercus*; spores with spines >4 µm long ..... ***T. pini***
With *Tuberaria guttata*; spores with spines <4 µm long ..... ***T. lusitanica***

11(1) In alkaline 12
In acid soils 16

12(11) Spores with warts making a complete and clear reticulum 13
Spores with warts sometimes forming an incomplete reticulum 14

13(12) Gleba strong pink; spores ≥18 µm ..... ***T. canariensis***
Gleba whitish; spores <18 µm ..... ***T. eliocrocae***

14(12) Spores <22 µm 15
Spores ≥22 µm ..... ***T. boudieri***

15(14) Asci with 6-8 spores; spores with spines >1.5 µm long ..... ***T. claveryi***
Asci with 4-6 spores; spores with spines <1.5 µm long ..... ***T. crassiverrucosa***

16(11) Ascocarps >2 cm; spores warty without reticulum ..... ***T. arenaria***
Ascocarps <2 cm; spores warty with a complete reticulum ..... ***T. alsheikhii***

ADDITIONAL NOTE

The increasing amount of sequence data, and the time lapse between manuscript preparation, submission and acceptance (and sometimes even later availability of the data) makes this type of work never complete. Already in the final stages of manuscript publication, we were aware of a new *Terfezia* species description (Crous et al., 2018) - *Terfezia morenoi*. We have re-run the main analysis (**Supplemental file 1**) and the newly described *T. morenoi* nests inside the monophyletic group by us designated (based on UNITE curated taxonomy and the majority rule) *T. olbiensis*. This implies that, the inclusion of these sequences does not alter the phylogenetic relationships observed and that all well supported clades remain unchanged. The morphological description of *T. morenoi* coincides on the diagnostic characters with the one of *T. olbiensis* Tul. & C. Tul., G. Bot. et al. (1845) as transcribed in Bordallo et al. (2013) (**Supplemental file 1**). Hence the above identification key to *Terfezia* genus remains valid. The name of the clade in question (in our manuscript, as *T. olbiensis* species) should be assigned - as stated before for the *T. leptoderma*/*T. fanfani* issue - according to the rules of the International Code of Nomenclature for algae, fungi, and plants.

CONCLUSIONS

The present analysis on the *Terfezia* ITS sequences accessible through the custom-curated UNITE database revealed 17 well supported independent lineages within the genus. Overall, the ITS region performed well in discriminating almost all analysed sequences, with the exception of seq. (GenBank accession no. **AF396864**), which we hypothesize that may represent an undescribed taxa given its unique set of morphological characters and its placement in our phylogeny. Further sampling is necessary to test this hypothesis. Our results show beyond doubt that the applied synonymy between *T. trappei* and *T. cistophila* is incorrect and we propose that they should be considered as two independent taxa. Regarding the sizzling debate around some lineages of *Terfezia* with spiny spores, our results highlighted several lineages hidden within the *T. leptoderma*/*olbiensis* complex (first proposed by Kovacs et al., 2011). Furthermore, our reconstructed phylogeny allowed the assignment of a species name linked to each former TLO designations (TLO-3 sequences are *T. pini*; TLO-4 are *T. extremadurensis*; TLO-2 are mainly *T. olbiensis* and 2 sequences are the newly described *T. albida*; TLO-1 comprises species of *T. fanfani*, *T. cistophila*, *T. trappei*, *T. lusitanica* and seq.

AF396864). The next step is to search the remaining sequences deposited in databases as *Terfezia* and access the extent of misidentifications and whenever possible, confirm or assign a species name based on the established taxonomy.

CONFLICTS OF INTEREST

The authors declare they have no conflict of interest in this work.

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APPENDIX A. SUPPLEMENTARY DATA

Supplemental Table 1. Final *Terfezia* ITS sequence dataset, including the respective accession numbers and sequence length plus the corresponding references. Information on DNA source; geographic location, putative host and soil type is also shown whenever available.

Terfezia species	Accession Nº	Seq. Lenght (bp)	Reference	DNA source	Collection Site	Host plant	Soil type
AF396864	AF396864	600	Diez et al. 2002	Isolate/strain	Spain:Valencia	*Pinus halepensis*	Acid
T. albida	HM056220	631	Bordallo et al. 2013	Fruitbody	Spain:Albacete	*Helianthemum sp.*	Alkaline
	HM056221	641	Bordallo et al. 2013	Fruitbody	Spain: Albacete	*Helianthemum sp.*	Alkaline
	HQ698145	660	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria guttata*	-
	HQ698146	662	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria guttata*	-
T. alsheikhii	HM056207	638	Bordallo et al. 2013	Fruitbody	Portugal:Trás-os-Montes	*Cistaceae*	Acid
	HM056208	589	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	-	-
	HQ625472	618	Buscardo et al. 2012	Root tip	Portugal:Beira Baixa	*Pinus pinaster*	-
	HQ698098	656	Kovacs et al. 2011	Fruitbody (Paratype)	Spain:Leon	*Helianthemum salicifolium*	-
	HQ698099	652	Kovacs et al. 2011	Fruitbody	Spain:Soria	*Pinus sylvestris*	-
	HQ698100	653	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria lignosa*	Acid
	HQ698113	653	Kovacs et al. 2011	Fruitbody (Holotype)	Spain:Salamanca	*Pinus pinaster, Tuberaria lignosa*	Acid
	KF007243	653	Franco et al. 2014	Root tip	Portugal:Minho	*Pinus pinaster*	-
	KF007244	658	Franco et al. 2014	Root tip	Portugal:Minho	*Pinus pinaster*	-
	KF007245	651	Franco et al. 2014	Root tip	Portugal:Minho	*Pinus pinaster*	-
	KF007255	658	Franco et al. 2014	Root tip	Portugal:Minho	*Pinus pinaster*	-
	KF007256	649	Franco et al. 2014	Root tip	Portugal:Minho	*Pinus pinaster*	-
T. arenaria	AF276674	598	Diez et al. 2002	Isolate/strain	Spain	*Tuberaria guttata*	Acid
	AF276675	598	Diez et al. 2002	Isolate/strain	Spain	*Tuberaria guttata*	Acid
	HQ698065	531	Kovacs et al. 2011	Fruitbody	Spain: Madrid	*Cistaceae*	Acid
	HQ698066	655	Kovacs et al. 2011	Fruitbody	Spain:Badajoz	*Quercus ilex*	Acid
	HQ698067	656	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria guttata*	Acid
	HQ698068	654	Kovacs et al. 2011	Fruitbody	Spain:Toledo	*Tuberaria guttata*	-
	HQ698069	659	Kovacs et al. 2011	Fruitbody	Spain: Caceres	-	-
	KF281114	633	Azul, unpublished	Isolate/strain	-	-	-
	KF281115	631	Azul, unpublished	Isolate/strain	-	-	-
	KP217812	612	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217813	612	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217814	602	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217815	602	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217816	603	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217817	604	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	KP217818	602	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
T. boudieri	AF092096	616	Ferdman et al. 2005	Fruitbody	Israel:Negev	-	-
	AF092097	612	Ferdman et al. 2005	Fruitbody	Israel:Negev	-	-
	AF092098	596	Ferdman et al. 2005	Fruitbody	Israel:Negev	-	-
	AF276672	597	Diez et al. 2002	Isolate/strain	Kuwait	*Helianthemum salicifolium*	Alkaline

	AF276673	595	Diez et al. 2002	Isolate/strain	Algeria	-	Alkaline
	AF301418	593	Ferdman et al. 2005	Isolate/strain	Israel	-	-
	AF301419	613	Aviram et al. 2004	Isolate/strain	Israel	-	-
	FN395016	594	Bouzadi et al., unpublished	Fruitbody	Libya:Hammad Al Hamra	-	-
	GU474783	617	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474789	610	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474792	615	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474793	617	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474796	614	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474797	612	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474806	581	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474807	578	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	GU474808	579	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	JN882304	659	Houseknecht et al., unpublished	Isolate/strain	Israel	-	-
	JX174047	598	Houseknecht et al., unpublished	Isolate/strain	Israel	-	-
T. canariensis	JQ858196	504	Bordallo et al. 2012	Fruitbody	Spain:Canary Island	*Helianthemum canariense*	Alkaline
T. cistophila	FJ013087	644	Rincón & Pueyo 2010	Root tip	Spain:Guadalajara	*Pinus pinaster*	-
	HQ698113	653	Kovacs et al. 2011	Fruitbody	Spain:Cordoba	*Cistus albidus*	-
	KP728821	656	Bordallo et al. 2015	Fruitbody	Spain:Caceres	*Cistus ladanifer*	Acid
	KP728822	532	Bordallo et al. 2015	Fruitbody	Spain:Badajoz	*Cistus ladanifer*	Acid
	KP728823	655	Bordallo et al. 2015	Fruitbody	Spain:Caceres	*Cistus ladanifer*	Acid
	KP728824	611	Bordallo et al. 2015	Fruitbody	Spain: Badajoz	*Cistus ladanifer*	Acid
	KP728825	610	Bordallo et al. 2015	Fruitbody	Greece:Artemida Attica	*Cistus monspeliensis, C. creticus*	Acid
	KP728826	596	Bordallo et al. 2015	Fruitbody	Greece:Rafina Attica	*Cistus monspeliensis, C. creticus*	Acid
	KP728827	585	Bordallo et al. 2015	Fruitbody	Greece:Zagora Magnesia	*Cistus monspeliensis, C. creticus*	Acid
	KP728828	594	Bordallo et al. 2015	Fruitbody	Greece:Nea Makri Attica	*Cistus monspeliensis, C. creticus*	Acid
	KP728829	595	Bordallo et al. 2015	Fruitbody	Greece:Zagora Magnesia	*Cistus monspeliensis, C. creticus*	Acid
T. claveryi	AF276670	593	Diez et al. 2002	Isolate/strain	Morocco	*Helianthemum ledifolium*	Alkaline
	AF276671	591	Diez et al. 2002	Isolate/strain	Spain	*Helianthemum salicifolium*	Alkaline
	AF301421	591	Ferdman et al. 2005	Isolate/strain	Morocco	-	-
	AF387645	578	Kovacs et al. 2011	Isolate/strain	Spain	-	-
	EU519461	591	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	FJ197819	633	Banihashemi et al. 2011	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ228093	593	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ888690	634	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ888691	634	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ888692	634	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ888693	637	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GQ888694	634	Banihashemi et al. 2010	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	GU474801	608	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum kahiricum*	Alkaline
	HM352540	634	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HM352541	633	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HM352542	634	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HM352543	634	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline

	HM352544	637	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HM352545	634	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HM352546	591	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
	HQ698070	651	Kovacs et al. 2011	Fruitbody	Spain:Burgos	-	-
	HQ698072	650	Kovacs et al. 2011	Fruitbody	Spain:Guadalajara	-	-
	HQ698073	650	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	-	-
	HQ698075	650	Kovacs et al. 2011	Fruitbody	Spain:Andalucia	-	-
	HQ698076	651	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	-	-
	HQ698077	653	Kovacs et al. 2011	Fruitbody	Spain:Jaen	-	-
	HQ698078	651	Kovacs et al. 2011	Fruitbody	Spain:Zaragoza	*Helianthemum salicicola*	-
	HQ698079	657	Kovacs et al. 2011	Fruitbody	Spain:Granada	-	-
	HQ698080	658	Kovacs et al. 2011	Fruitbody	Spain:Granada	*Helianthemum salicifolium*	-
	HQ698081	667	Kovacs et al. 2011	Fruitbody	Spain:Burgos	-	-
	HQ698082	651	Kovacs et al. 2011	Fruitbody	Spain:Ciudad Real	-	-
	HQ698083	551	Kovacs et al. 2011	Fruitbody	Spain:Almeria	*Helianthemum leptophilum*	-
	HQ698084	646	Kovacs et al. 2011	Fruitbody	Spain:Jaen	-	-
	HQ698085	653	Kovacs et al. 2011	Fruitbody	Spain:Zaragoza	-	-
	HQ698086	650	Kovacs et al. 2011	Fruitbody	Spain:Murcia	*Halimium viscosum*	-
T. crassiverrucosa	AF387646	583	Kovacs et al. 2011	Isolate/strain	Spain	-	-
	AF387647	580	Kovacs et al. 2011	Isolate/strain	Spain:Murcia	-	-
	AF387648	583	Kovacs et al. 2011	Isolate/strain	Spain	-	-
	HQ698071	655	Kovacs et al. 2011	Fruitbody	Spain:Ciudad Real	-	-
	HQ698074	658	Kovacs et al. 2011	Fruitbody	Spain:Murcia	-	-
	MF940203	513	Zitouni-Haouar et al. 2018	Fruitbody (Holotype)	Algeria	*Helianthemum sp.*	Alkaline
T. eliocrocae	HM056205	627	Bordallo et al. 2013	Fruitbody	Spain: Murcia	*Helianthemum sp.*	Alkaline
	HM056206	534	Bordallo et al. 2013	Fruitbody	Spain: Murcia	*Helianthemum sp.*	Alkaline
T. extremadurensis	AF276678	584	Diez et al. 2002	Isolate/strain	Spain:Caceres	*Tuberaria guttata*	Acid
	HM056199	544	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HM056200	508	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HM056201	600	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HM056202	596	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HM056204	591	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HQ698103	640	Kovacs et al. 2011	Fruitbody	Spain:Badajoz	-	-
	HQ698111	646	Kovacs et al. 2011	Fruitbody	Spain:Madrid	-	-
	HQ698112	660	Kovacs et al. 2011	Fruitbody	Spain:Madrid	-	-
	HQ698114	660	Kovacs et al. 2011	Fruitbody	Spain:Segovia	-	-
	HQ698115	661	Kovacs et al. 2011	Fruitbody	Spain:Segovia	-	-
	HQ698116	660	Kovacs et al. 2011	Fruitbody	Spain:Segovia	-	-
	HQ698134	534	Kovacs et al. 2011	Fruitbody	Spain:Jaen	*Cistus albidus*	-
T. fanfani	AF396862	590	Diez et al. 2002	Isolate/strain	Spain - Huelva	*Cistus ladanifer*	Acid
	HM056214	544	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	Acid
	HM056216	635	Bordallo et al. 2013	Fruitbody	Spain: Caceres	*Tuberaria guttata*	Acid
	HM056217	645	Bordallo et al. 2013	Fruitbody	Spain: Caceres	*Tuberaria guttata*	Acid
	HM056218	633	Bordallo et al. 2013	Fruitbody	Spain:Valladolid	*Cistaceae plants*	Acid

	HM056219	596	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	*Tuberaria guttata*	Acid
	HQ698087	645	Kovacs et al. 2011	Fruitbody	Spain:Caceres	-	-
	HQ698088	647	Kovacs et al. 2011	Fruitbody	Spain:Caceres	-	-
	HQ698089	647	Kovacs et al. 2011	Fruitbody	Spain:Avila	-	-
	HQ698090	656	Kovacs et al. 2011	Fruitbody	Spain:Segovia	-	-
	HQ698091	647	Kovacs et al. 2011	Fruitbody	Spain:Leon	*Helianthemum asperum*	-
	HQ698092	655	Kovacs et al. 2011	Fruitbody	Spain:Madrid	*Quercus rotundifolia, Q. pyrenaica*	-
	HQ698093	650	Kovacs et al. 2011	Fruitbody	Spain:Caceres	-	-
	HQ698094	655	Kovacs et al. 2011	Fruitbody	Spain:Zamora	*Quercus ilex, Genista sp.*	-
	HQ698095	644	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Cistus laurifolius*	-
	HQ698096	649	Kovacs et al. 2011	Fruitbody	Spain:Badajoz	*Quercus suber*	-
	HQ698097	646	Kovacs et al. 2011	Fruitbody	Spain:Toledo	*Quercus sp.*	-
T. grisea	KP189328	655	Bordallo et al. 2015	Fruitbody	Spain:Burgos	*Helianthemum sp.*	Alkaline
	KP189329	588	Bordallo et al. 2015	Fruitbody	Greece:Schinias_Attica	*Pinus spp.*	Alkaline
	KP189330	590	Bordallo et al. 2015	Fruitbody	Greece:Schinias_Attica	*Pinus spp.*	Alkaline
	KP189331	592	Bordallo et al. 2015	Fruitbody	Greece:Schinias_Attica	*Pinus spp.*	Alkaline
	KP189332	583	Bordallo et al. 2015	Fruitbody	Greece:Schinias_Attica	*Pinus spp.*	Alkaline
	KP189333	591	Bordallo et al. 2015	Fruitbody	Spain:Burgos	*Helianthemum sp.*	Alkaline
T. lusitanica	MG818752	591	Bordallo et al. 2018	Fruitbody	Portugal:Alentejo	*Tuberaria guttata*	Acid
	MG818753	656	Bordallo et al. 2018	Fruitbody (Holotype)	Spain:Caceres	*Tuberaria guttata*	Acid
	MG818754	588	Bordallo et al. 2018	Fruitbody	Portugal:Alentejo	*Tuberaria guttata*	Acid
T. olbiensis	AF276677	584	Diez et al. 2002	Isolate/strain	-	-	-
	AF387656	568	Kovacs et al. 2011	Isolate/strain	Spain	-	-
	AF387657	569	Kovacs et al. 2011	Isolate/strain	Spain	-	-
	AF396863	584	Diez et al. 2002	Isolate/strain	France	*Quercus ilex*	Alkaline
	HM056222	539	Bordallo et al. 2013	Fruitbody	Spain:Albacete	*Quercus sp.*	-
	HM056223	539	Bordallo et al. 2013	Fruitbody	Spain:Albacete	*Quercus sp.*	-
	HM056224	644	Bordallo et al. 2013	Fruitbody	Spain: Valladolid	*Pinus sp.*	-
	HM056225	628	Bordallo et al. 2013	Fruitbody	Spain:Valencia	*Pinus sp.*	-
	HQ698102	641	Kovacs et al. 2011	Fruitbody	Spain:Madrid	-	-
	HQ698147	640	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Pinus halapensis*	-
T. pseudoleptoderma	FJ013064	638	Rincón & Pueyo 2010	Root tip	Spain:Guadalajara	*Pinus pinaster*	-
	HM056211	588	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Cistaceae*	-
	HM056212	647	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Cistaceae*	-
	HM056213	631	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Cistaceae*	-
T. trappei	AF276676	583	Bordallo et al. 2013	strain	-	-	-
	HM056215	628	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	*Cistaceae*	-
	HQ698119	649	Kovacs et al. 2011	Fruitbody	Spain:Ciudad Real	*Quercus sp., Tuberaria guttata*	-
	HQ698132	643	Kovacs et al. 2011	Fruitbody	Spain:Zamora	-	-
	HQ698149	641	Kovacs et al. 2011	Fruitbody	Spain: Caceres	*Cistaceae*	-
Clade A	DQ386140	618	Barriuso et al. 2008	Isolate/strain	Spain:Huelva	*Pinus sp.*	-
	HM056209	633	Bordallo et al. 2013	Fruitbody	Spain:Valladolid	*Pinus sp.*	-
	HM056210	631	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Pinus sp.*	-
	HQ698101	647	Kovacs et al. 2011	Fruitbody	Spain:Burgos	-	-

HQ698104	634	Kovacs et al. 2011	Fruitbody	Spain:Madrid	*Pinus pinaster*	-
HQ698105	663	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	Acid
HQ698106	664	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	-
HQ698107	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	Acid
HQ698108	664	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	Acid
HQ698109	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	Acid
HQ698110	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus sylvestris*	Acid
HQ698117	654	Kovacs et al. 2011	Fruitbody	Portugal:Minho	*Pinus pinaster*	-
HQ698118	664	Kovacs et al. 2011	Fruitbody	Portugal:Minho	*Pinus pinaster*	-
HQ698120	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698121	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698122	661	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698123	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698124	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698125	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698126	663	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Quercus sp.*	-
HQ698127	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus pinaster*	-
HQ698128	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus pinaster*	-
HQ698129	664	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus pinaster*	-
HQ698130	665	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus pinaster*	-
HQ698131	664	Kovacs et al. 2011	Fruitbody	Spain:Segovia	*Pinus pinaster*	-
HQ698133	643	Kovacs et al. 2011	Fruitbody	Spain:Avila	-	-
HQ698135	663	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698136	663	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698137	663	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698138	662	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698139	663	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698140	663	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
HQ698141	663	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Pinus sp.*	-
HQ698142	665	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Pinus sp.*	-
HQ698143	663	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	*Pinus sp.*	-
HQ698144	638	Kovacs et al. 2011	Fruitbody	Spain:Caceres	-	-
HQ698148	643	Kovacs et al. 2011	Fruitbody	Spain:Valladolid	-	-

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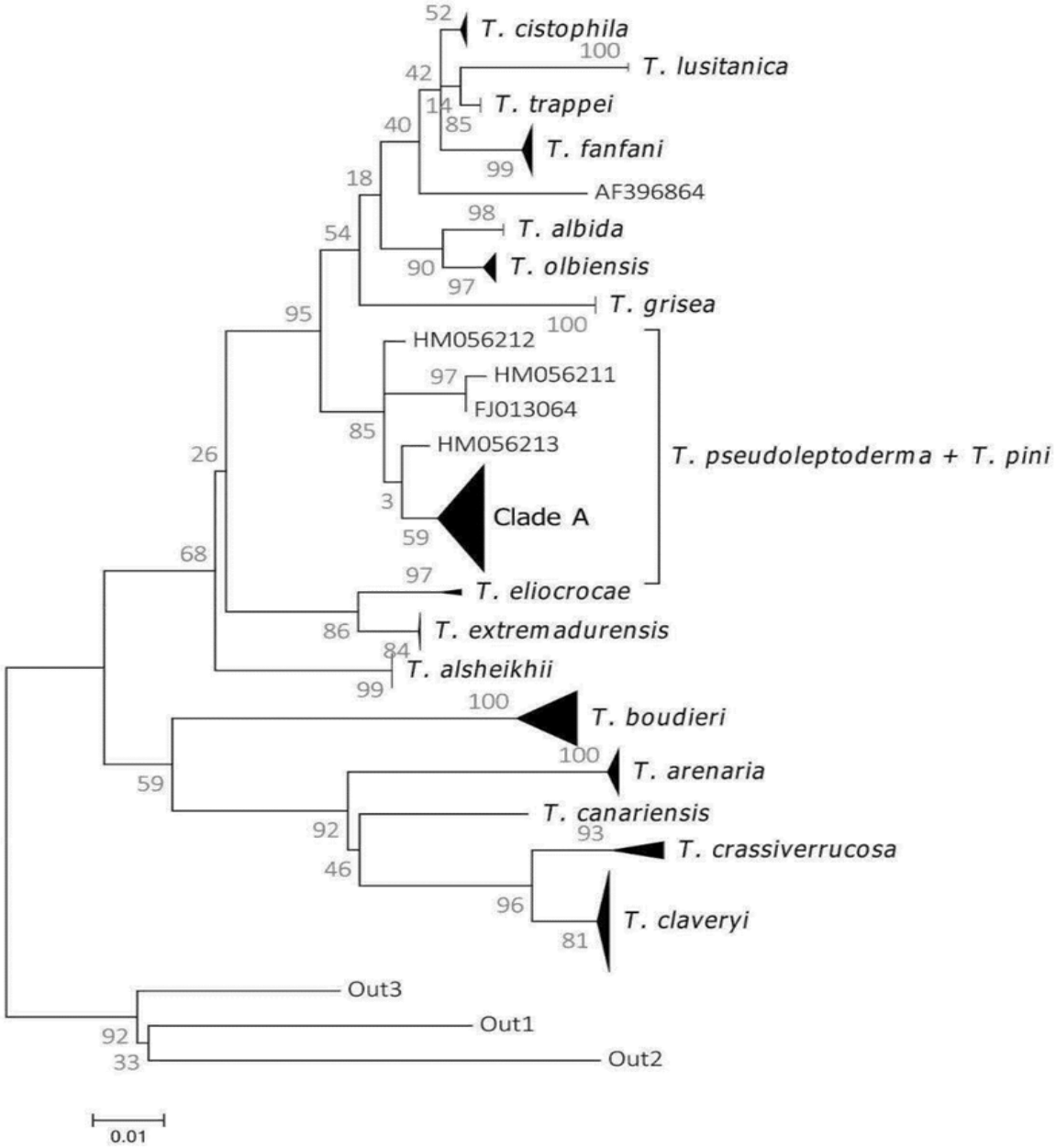

Figure S1: Molecular Phylogenetic analysis by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [1]. The tree with the highest log likelihood (-2376.37) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 205 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 380 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].
Please see main text for further explanations.

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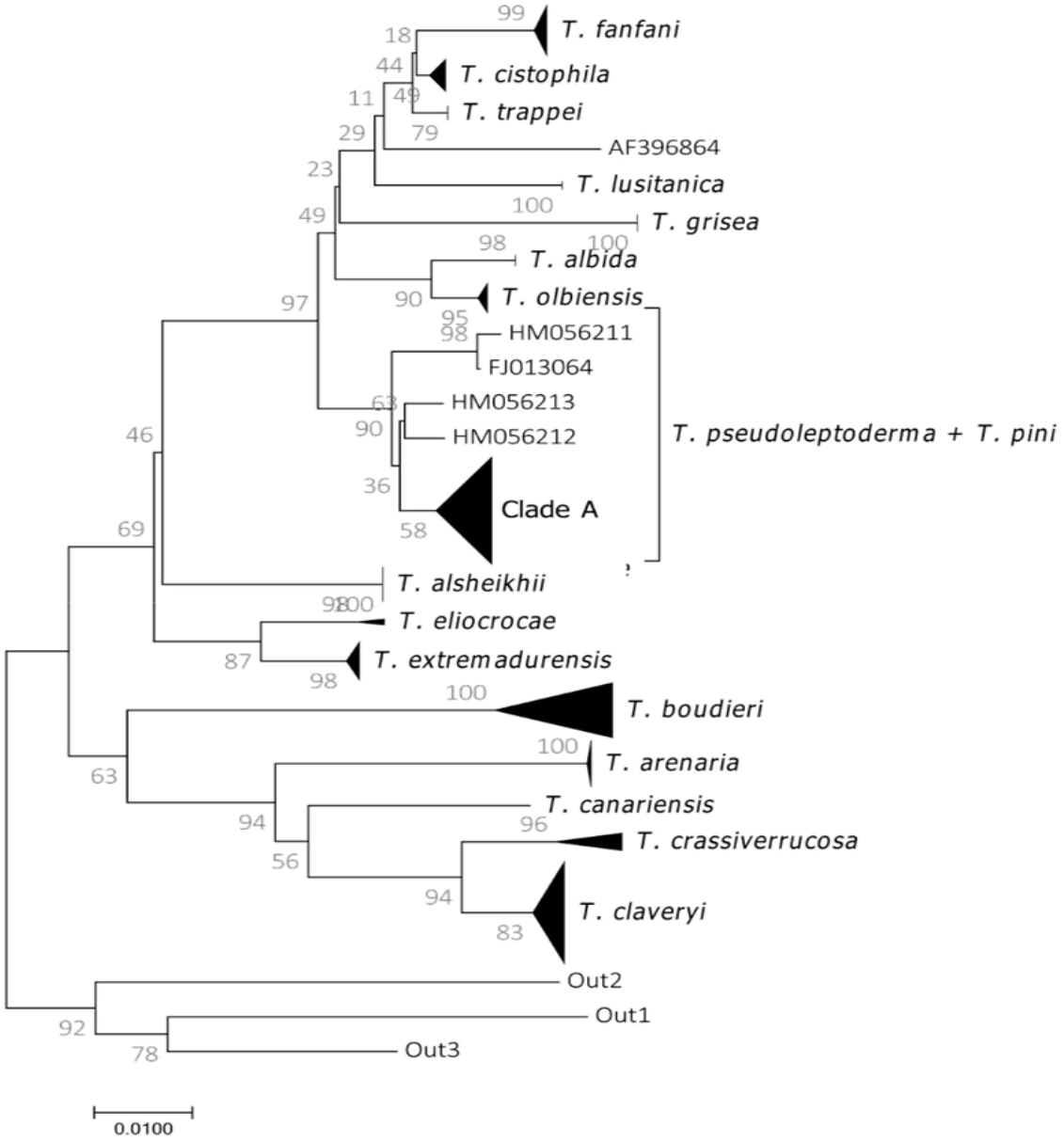

Figure S2. Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.76597399 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The 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 [3]. The analysis involved 205 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 380 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4]. Please see the main text for more information.

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SUPPLEMENTAL FILE 1

The increasing amount of sequence data, and the time lapse between manuscript preparation, submission and acceptance (and sometimes even later availability of the data) makes this type of work never complete. Already in the final stages of manuscript publication, we were aware of a new *Terfezia* species description - *Terfezia morenoi* (Crous, PWF *et al.*, 2018. Fungal Planet description sheets: 751. *Persoonia* 40: 325).

We have re-run the main analysis and added the eight sequences of the newly described *Terfezia morenoi*: **MG640478**, **MG640479**, **MG640480**, **MG640481**, **MG640482**, **MG640483**, **MG640484** and **MG640485**.

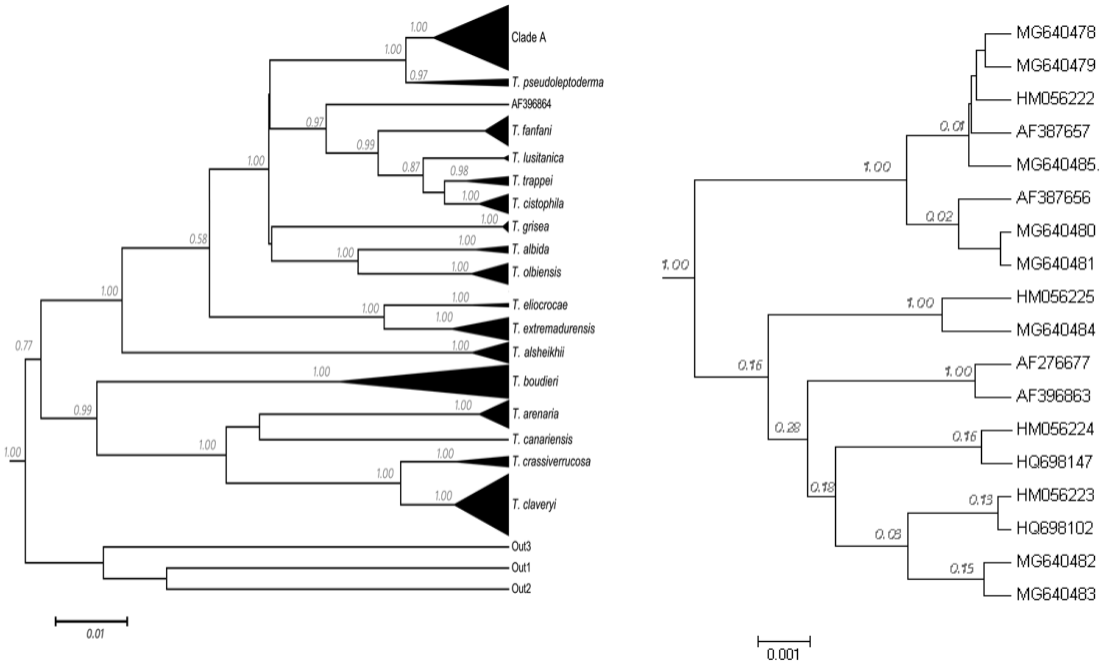

Fig. S3. Phylogenetic relationship between *Terfezia* species. a) The phylogeny corresponds to the majority rule consensus tree of trees sampled in a Bayesian analysis, and the posterior probability values are shown for main nodes. a) overall aspect highlighting relations between clades; b) detail of the clade that concerns *T. olbiensis* and *T. morenoi* sequences (**Table S1**)

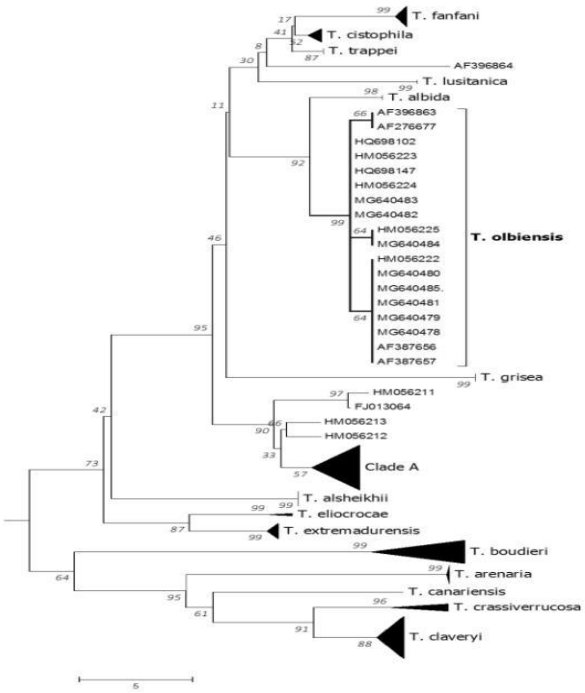

Fig. S4. Molecular Phylogenetic analysis by Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

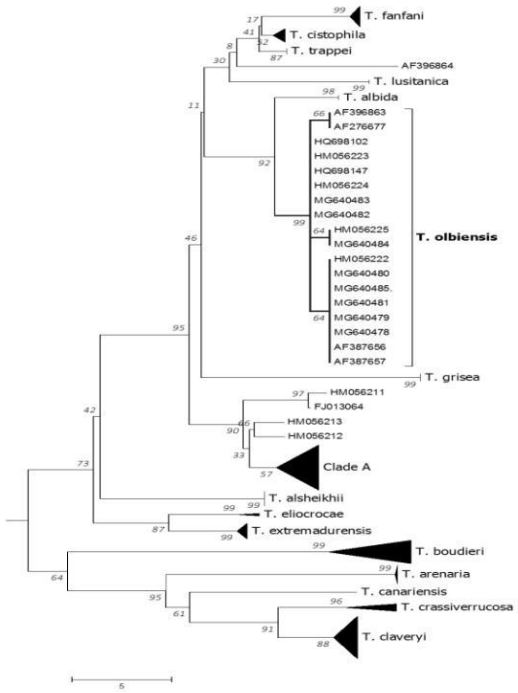

Fig. S5. Evolutionary relationships of taxa inferred by Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Table S1. Sequences nesting within the monophyletic group designated *Terfezia olbiensis* based on Fig.S3, S4 and S5 (all sequences except the ones from Crous et al., 2018, were referred in UNITE database as *Terfezia olbiensis*).

			GenBank	GenBank
Acc.	**Ref.**	**Location**	**Taxonomy***	**Taxonomy****
AF276677	Diez et al. 2002	-	*Terfezia trappei*	*Terfezia trappei*
AF387656	Kovacs et al. 2011	Spain	*Terfezia olbiensis*	*Terfezia olbiensis*
AF387657	Kovacs et al. 2011	Spain	*Terfezia olbiensis*	*Terfezia olbiensis*
AF396863	Diez et al. 2002	France	*Terfezia leptoderma*	*Terfezia leptoderma*
HM056222	Bordallo et al. 2013	Spain:Albacete	*Terfezia olbiensis*	*Terfezia morenoi*
HM056223	Bordallo et al. 2013	Spain:Albacete	*Terfezia olbiensis*	*Terfezia morenoi*
HM056224	Bordallo et al. 2013	Spain: Valladolid	*Terfezia olbiensis*	*Terfezia morenoi*
HM056225	Bordallo et al. 2013	Spain:Valencia	*Terfezia olbiensis*	*Terfezia morenoi*
HQ698102	Kovacs et al. 2011	Spain:Madrid	*Terfezia aff. olbiensis*	*Terfezia aff. olbiensis*
HQ698147	Kovacs et al. 2011	Spain:Valladolid	*Terfezia aff. olbiensis*	*Terfezia aff. olbiensis*
MG640478	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*
MG640479	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*
MG640480	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*
MG640481	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*
MG640482	Crous, et al., 2018	Spain: La Rioja	-	*Terfezia morenoi*
MG640483	Crous, et al., 2018	Spain: La Rioja	-	*Terfezia morenoi*
MG640484	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*
MG640485	Crous, et al., 2018	Spain:Albacete	-	*Terfezia morenoi*

*before September 2018; ** after September 2018

Table S2. The diagnostic characters of *Terfezia olbiensis* Tul. & C. Tul., G. Bot. et al. (1845) as transcribed in Bordallo et al. (2013) and of *Terfezia morenoi* as in Crous et al. (2018).

Diagnostic features	*Terfezia olbiensis*	*Terfezia morenoi*
Ascomata	Globose, smooth, 2-5 cm Ø, initially cream, becoming brown, frequently with black maculae where exposed to the sun or bruised.	Subglobose, smooth, 2-5 cm Ø, cream colour at first, becoming brown, black spots on the sun-exposed parts or when manipulated.
Peridium	300-500 µm thick, white in cross section, pseudoparenchymatous structure formed by ± rounded thin-walled hyaline cells that become yellow and prismatic towards the periphery.	300-500 µm thick, whitish in cross section, pseudoparenchymatous, composed of subglobose cells, 20-50 µm Ø, thin-walled, hyaline, yellowish and angular to oblong in the outermost layers.
Gleba	Initially white, then fertile tissue forming small grey (later greenish grey) islets surrounded by salmon-tinged white sterile tissue.	Whitish with small pale grey pockets at first, maturing to greyish green pockets of fertile tissue separated by whitish (sometimes with salmon pink spots) sterile veins.
Odour	-	Strong, more remarkable in mature specimens becoming unpleasant.
Taste	-	Mild
Asci	Dextrinoid when immature, sessile to occasional on a short thick peduncle, ellipsoidal to ovoid, citrus-shaped, 8-spored, 60-90 × 50-60 µm with 1-2 µm thick walls.	Nonamyloid, sessile or short-stipitate, ellipsoid to ovate, citriform, 6-8 irregularly disposed spores, 60-90 × 50-60 µm, walls 1-2 µm thick.
Ascospores	Spherical, 15–19 µm (including ornament) Ø, 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, not joined through the base.	Globose, (16–)16.5-19(–19.5) µm Ø (median = 18 µm) including ornamentation, hyaline, smooth and uni-guttulate at first, by maturity yellow ochre and ornamented with conical spines, pointed, straight, separate, 1–2(–2.5) µm long, 1 µm wide at the base.
Habitat	In limestone and clayey pine and oak woodlands without *Helianthemum* spp. from mid-March to mid-April.	In calcareous, clayey, alkaline soils, associated with *Pinus* spp. and *Quercus ilex*, with no presence of *Cistaceae*, it fructifies from March to April.

CHAPTER II

Terfezia lusitanica, a new mycorrhizal species associated to *Tuberaria guttata* (*Cistaceae*)

Data published in:

Juan-Julián Bordallo, Antonio Rodríguez, Celeste Santos-Silva, Rogério Louro,

Justo Muñoz-Mohedano, Asunción Morte

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ABSTRACT

A new *Terfezia* species associated with *Tuberaria guttata*, *Terfezia lusitanica* sp. nov., is described from Spain and Portugal. This claim it's based on the specimen's distinct morphology and unique ITS-rDNA sequence. Macro and micro descriptions and phylogenetic analyses of ITS data are provided for *T. lusitanica* and discussed in relation to similar spiny-spored *Terfezia* species. *T. lusitanica* differs morphologically from other spiny-spored *Terfezia*, that share the same habitat, by the combination of its ochre peridium colour and spores size, and in its ITS nrDNA sequence from all other ITS sequenced *Terfezia* species. Among the morphologically similar species, *T. fanfani* has a reddish peridium, *T. extremadurensis* has distinctly larger spores and tuber-like gleba, and *T. cistophila* has smaller spores, a spermatic odour, and is never found in association with *T. guttata*.

Keywords: desert truffle, hypogeous, mycorrhizal fungi, *Pezizaceae, Cistaceae*

INTRODUCTION

The genus *Terfezia* (Tul. & C.Tul.) Tul. & C.Tul. can be ranged classically from those species with spiny spores and those with reticulated-warty spores. Although sporal morphology is a good taxonomic character, it is not enough to separate spiny-spored *Terfezia* species. Other features are essential to properly identify these species, such as: macroscopic characteristics, host plants, soil pH and ITS data (Bordallo *et al.* 2013, 2015). Most *Terfezia* species establish mycorrhizal symbiosis with perennial and annual plants belonging to *Cistaceae*, mainly with *Helianthemum* species in alkaline soils (Dexheimer *et al*. 1985, Fortas & Chevalier 1992, Gutiérrez *et al*. 2003, Morte & Andrino 2014, Zitouni-Haouar *et al.* 2014) and with subspecies and varieties of *Tuberaria guttata* (L.) Fourr. in acid soils (Bordallo *et al*. 2013, 2015, Dafri & Beddiar 2017). So far, the described *Terfezia* species known to live in association to *T. guttata* in acid soils are: *T. arenaria* (Moris) Trappe (Trappe 1971), *T. fanfani* Mattir. (Mattirolo 1900) and *T. extremadurensis* Muñoz-Mohedano, Ant. Rodr. & Bordallo (Bordallo *et al.* 2013). These three species share the same ecology and are mainly found in xerophytic grasslands, without trees, on a wide range of sandy soils. The objective of the present study was to describe a new *Terfezia* species associated with *T. guttata*. For this aim, we conducted morphological studies on *T. lusitanica* specimens, collected throughout western Spain and southern Portugal, complemented with phylogenetic analyses, based on ITS-rDNA sequences from the

specimens collected and from GenBank sequences.

MATERIAL AND METHODS

Fungal collections and collecting sites

Ascomata were collected in different years and from different locations in western Spain (Extremadura, Valdehúncar) and in southern Portugal (Alentejo, Montemor-o-Novo). Throughout the collection period (from February to May), fresh specimens were photographed in the field, including the nearby plants, and brought to the laboratory for morphological study. Fragments of each specimen were frozen at −20° C for DNA amplification and the remaining was dried at 40°C and stored in sealed plastic bags, labeled with collection details. The samples are deposited at the Herbarium of the University of Murcia (MUB), Spain and at the Évora University Herbarium (UEVH-FUNGI), Portugal.

Morphological study

External ascocarp characteristics (shape, colour, appearance) were recorded from fresh specimens 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 were based on measurements of at least 100 randomly selected mature spores, outside asci, in distilled water mount. Peridium of dried specimens were rehydrated and examined in KOH 5%. Melzer's reagent was used to test the amyloidity of asci, spores and tissues. Asci and ascospores were examined using an Olympus BX51 microscope equipped with a digital camera (Olympus DP73-1-51). For species-level determination, ascomata features were compared with descriptions from Bordallo *et al.* (2013, 2015).

Molecular study

DNA amplification and sequencing

The Internal Transcribed Spacer (ITS) region of the rDNA, including the 5.8S ribosomal gene, was amplified using the ITS1F and ITS4 primers (White *et al.* 1990, Gardes & Bruns 1993). Direct PCR amplifications were carried out according to a protocol described by Bonito (2009), modified by using 150–200 mg of the ascocarps outer gleba in a final volume of 25 µL containing 1µl 20 mM of dNTPs, 1 µl 10 µM of each primer, 2 µl 50 mM $MgCl_2$, 2.5 µl 10X PCR buffer, 1 µl de BSA 1% 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. Clean PCR products were sequenced in both directions at the Molecular Biology Service (University of Murcia). Sequences generated in this study were deposited in GenBank under accession numbers MG818752, MG818753 and MG818754.

Sequence alignment and phylogenetic analysis

Terfezia lusitanica ITS sequences, and closely similar sequences from GenBank, were assembled by Clustal X followed by manual adjustment to improve alignments. ITS sequences were first compared with sequences deposited in GenBank using the BLAST algorithm (Altschul *et al*. 1997) to determine the closest relatives. The phylogenetic analysis was carried out using MEGA4 software (Tamura *et al.* 2007). The phylogenetic relationships were inferred using the Neighbour-Joining method (NJ; Saitou & Nei 1987) and Maximum Parsimony method (MP; Eck & Dayhoff 1966), using a total of 57 sequences. 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) is 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 409 positions in the final dataset, out of which 121 were parsimony informative. 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 409 positions in the final dataset, out of which 121 were parsimony informative.

The sequences from *Eremiomyces echinulatus* (Trappe & Marasas) Trappe & Kagan-Zur were chosen as outgroup. All alignments were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S22247?x-access-code=8d95afd4eaa69faff8c599da77138f3f&format=html)

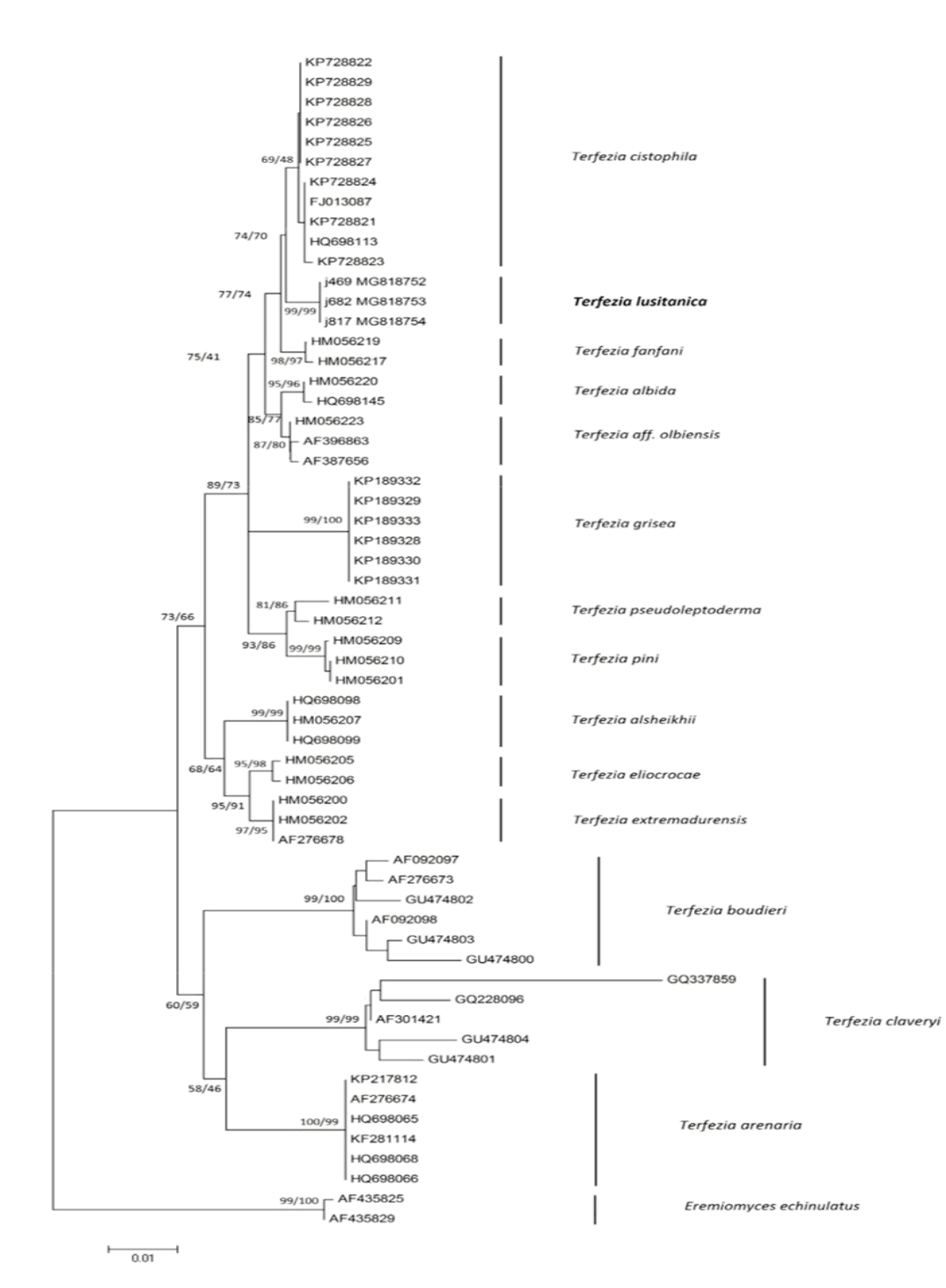

Figure 1. Neighbor-Joining (NJ) and Maximum Parsimony (MP) consensus phylogenetic tree of the ITS sequences. The first values on the branches are the NJ bootstrap proportions (≥50%) and the values after the slash represent the MP bootstrap proportions (≥50%) of 500 bootstrapping replicates.

RESULTS

Phylogenetic analysis

Sequence analyses of the ITS-rDNA from the examined samples and from GenBank produced two phylogenetic trees based on Neighbor-Joining (NJ) and the Maximal Parsimony (MP) methods, both with a virtual sampling or bootstrap of 500 replicas (**Fig. 1**). The three sequences of the new *Terfezia lusitanica* clustered independently of their harvest location, Spain or Portugal (**Fig. 1**). *T. lusitanica* is supported by the supposed event of speciation of the common ancestor with *T. cistophila*, represented by the bootstrap node 74%.

Taxonomy

Terfezia lusitanica Bordallo, Ant. Rodr., Louro, Santos-Silva, Muñoz-Mohedano sp. nov.

MycoBank: MB 824055

Type:—Spain, Extremadura, Valdehúncar, 29 April 2016, leg Ant. Rod. (**Holotype**, MUB Fung-j682, GenBank accession MG818753).

Diagnosis:—*Ascomata* hypogeous to partially emergent at maturity, subglobose to ellipsoid or partially flattened, sometimes with tapered sterile base, 2–3.5 cm in size, light ochre colour at first, becoming yellowish brown with black spots, smooth to slightly rough (**Fig. 2 a–c**). *Peridium* 200–500 µm thick, not separable from gleba, poorly delimited, whitish in cross section, pseudoparenchymatous, composed of subglobose cells, hyaline and thin-walled in the innermost layers, yellowish and with thicker walls in the outermost layers (**Fig. 2d**). *Gleba* solid, fleshy, succulent, whitish at first (**Fig. 2c**), darkening with age, becoming greenish black at maturity; with blackish grey pockets of fertile tissue surrounded by whitish, sterile, veins (**Fig. 2b**). Faint odour, not distinctive. Mild taste. *Asci* nonamyloid, subglobose to ellipsoid, sessile, 60–80 x 50–65 µm, walls 1 µm thick, with 6–8 irregularly disposed spores, randomly arranged in the gleba. *Ascospores* globose, (20–)21–23(–24) µm diam (median = 22 µm) including ornamentation; (14–)15–17(–18) µm (median = 16 µm) without ornamentation; hyaline, smooth and uniguttulate at first, by maturity dark yellow to light brown and ornamented with conical, blunt spines, sometimes cylindrical, mostly straight, but sometimes curved, separated, 3–3.5(–4) µm long, 1–2 µm wide at the base (**Fig. 2e–f**).

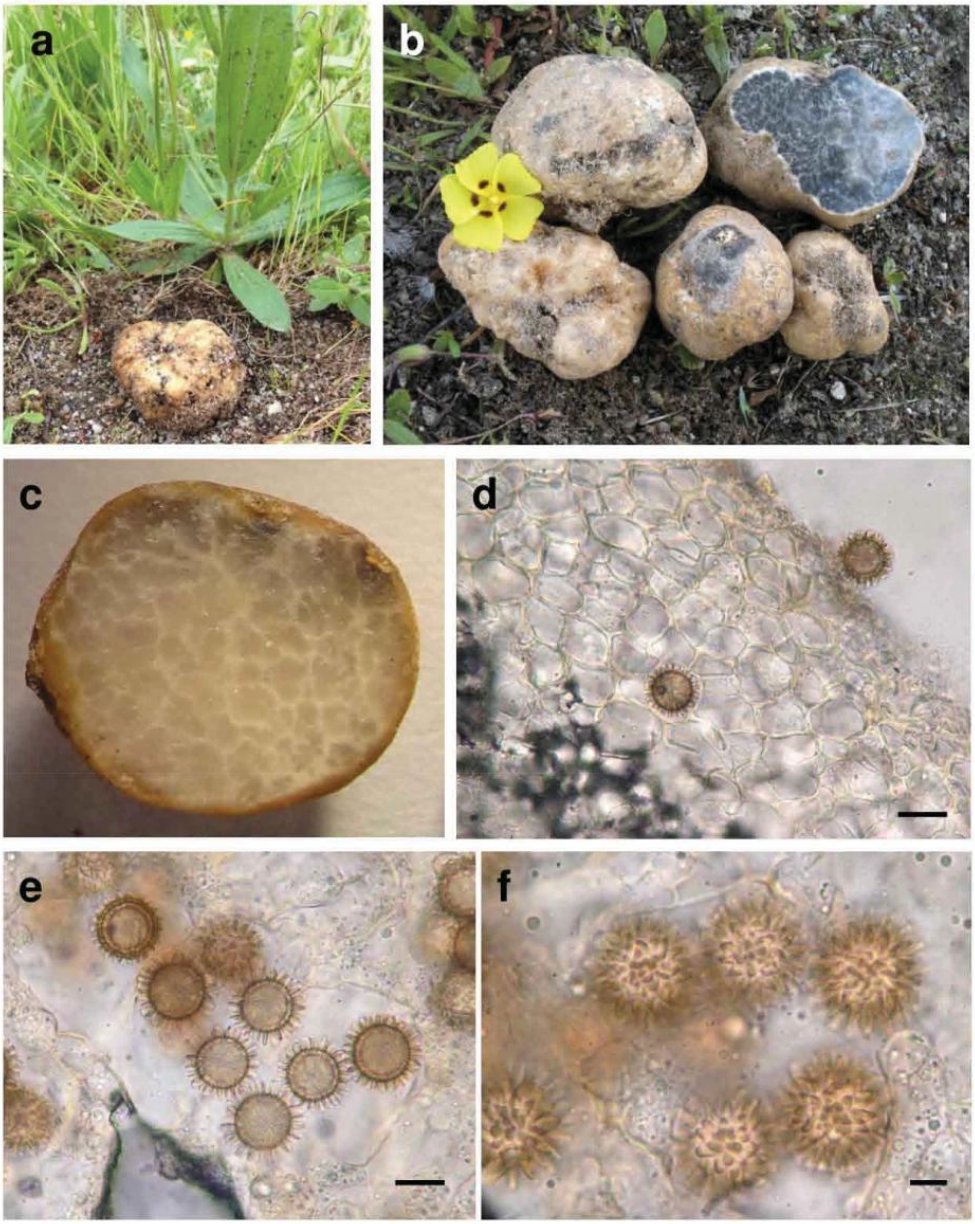

Figure 2. Macro and micro features of *Terfezia lusitanica*: (a) ascocarp collected under *Tuberaria guttata*, (b) mature ascocarps with *T. guttata* flower, (c) whitish gleba of an inmature ascocarp, (d) pseudoparenchymatous *peridium*, (e, f) ascospores. Bars: d) 20 µm; e) 13 µm; f) 7.5 µm.

Ecology/Distribution:—Extremadura (Spain) and Alentejo (Portugal), in sandy, acid soils, in grassland areas without trees, associated exclusively with *Tuberaria guttata*, in April.

Etymology:—Referring to Lusitania, the name gave by the Romans to the western region of the Iberian Peninsula, which now covers the Portuguese area below Douro River and the neighboring regions of Spanish Extremadura.

Additional collections examined:—PORTUGAL: Alentejo, Montemor-o-Novo, 03 April 2012, C. Santos-Silva (MUB Fung-j469, UEVH-FUNGI 2003065, MG818752). Same locality, 20 April 2017, C. Santos-Silva (MUB Fung-j817, UEVH-FUNGI 2003876, MG818754).

Notes:—*T. lusitanica* differs morphologically from other spiny-spored *Terfezia*, that share the same habitat, by the combination of its ochre peridium colour and spores size, and from all other *Terfezia* in its ITS nrDNA sequence. For instance, *T. fanfani* differs from *T. lusitanica* showing a reddish peridium; *T. extremadurensis* has ochre peridium, but exhibits distinctly larger spores than *T. lusitanica*; *T. extremadurensis* presents a tuber-like gleba, with meandering veins not completely surrounding the fertile tissue, and does not form pockets; *T. cistophila*, has smaller spores than *T. lusitanica*, possesses a distinctive spermatic odour, and different host plants, never found associated with *T. guttata.*

Key to spiny-spored *Terfezia* associated with *Tuberaria guttata*

1a. Peridium with reddish colours……*T. fanfani*
1b. Peridium without reddish colours……..2
2a. Tuber-like gleba……*T. extremadurensis*
2b. Without tuber-like gleba……..*T. lusitanica*

DISCUSSION

The morphological features of *T. lusitanica* (**Fig. 2**) and the phylogenetic analyses results (**Fig. 1**) strongly suggest we are facing a new species. *T. lusitanica* is morphologically different from other *Terfezia* species previously described (Bordallo *et al.* 2013, 2015, Moreno *et al.* 1986, Tulasne & Tulasne 1851, Mattirolo 1900). *T. lusitanica* shares soil preferences and the host plant (*T. guttata*) with *T. arenaria*, *T. fanfani* and *T. extremadurensis*, but differs in morphological characteristics and phylogenetic distances (**Fig. 1**). According to our experience, the species *T. lusitanica*, *T. extremadurensis* and *T. fanfani* have never been found associated with other host plant than *Tuberaria guttata*.

Host specialization and edaphic tolerances have been considered key aspects in the species diversity of *Terfezia* genus (Díez *et al.* 2002). The host plant *T. guttata* is a spring-annual herb, very abundant among other wild species in the forests and uncultivated areas of the western Mediterranean region (Portugal, Spain, south of France, north of Africa). This plant is

characterized by its ecological plasticity, and can be found in a large array of xerophytic grasslands, from coastal dunes to sandy mid-mountain lawns, mostly on siliceous soils. For that reason, different subspecies and varieties are recognized (Castroviejo *et al.* 1993). Detailed observations of *T. guttata* flower structure and function, revealed substantial variations in the breeding system, presenting chasmogamous flowers that can self-pollinate and therefore make the plant self-compatible (Herrera 1992). The high sub-speciation of this plant species could have an effect on the associated fungal species. Further studies, merging mycorrhizal fungi and their host plants evolution, are essential to comprehend the desert truffles diversity and biogeography (Bordallo *et al.* 2015).

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CHAPTER III

Terfezia solaris-libera sp. nov., a new mycorrhizal species within the spiny-spored lineages

Data published in:

Rogério Louro, Tânia Nobre, Celeste Santos-Silva

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ABSTRACT

A new *Terfezia* species -*Terfezia solaris-libera* sp. nov., associated with *Tuberaria guttata* (*Cistaceae*) is described from Alentejo, Portugal. *T. solaris-libera* sp. nov. distinct morphology has been corroborated by its unique ITS-rDNA sequence. Macro and micro morphologic descriptions and phylogenetic analyses of ITS data for this species, are provided and discussed in relation to similar spiny-spored species in this genus and its putative host plant *Tuberaria guttata. T. solaris-libera* sp. nov. differs from other spiny-spored *Terfezia* species by its poorly delimited and thicker peridium and distinct spore ornamentation, and from all *Terfezia* spp. in its ITS nrDNA sequence. In comparison, *T. fanfani* usually reach large ascocarp dimensions, often with prismatic peridium cells, with olive green tinges in mature gleba and a different spore ornamentation. *T. lusitanica* has a lighter yellowish and thinner peridium and a blackish gleba upon maturity, *T. extremadurensis* has a thinner well delimited peridium and *Tuber*-like gleba and *T. cistophila* has a spermatic odour and is exclusively associated with *Cistus* spp..

Keywords: desert truffle; hypogeous; *Ascomycota*; *Pezizaceae; Cistaceae*; *Tuberaria guttata;* Portugal

INTRODUCTION

Terfezia species are ectomycorrhizal desert truffles (*Ascomycota, Pezizales*), found in arid and semi-arid environments, around the Mediterranean basin. Most *Terfezia* species establish mycorrhizal symbiosis with perennial and annual plants belonging to *Cistaceae*, mainly with *Helianthemum* species in alkaline soils [1-5] and with *Tuberaria guttata* (L.) Fourr., and its subspecies and varieties, in acid soils [6-8]. Many *Terfezia* species are endemic and overall play an essential role in soil conservation -preventing erosion and desertification- in Mediterranean shrublands and xerophytic grasslands [9]. The genus *Terfezia* (Tul. and C.Tul.) Tul. and C.Tul. is undoubtedly the most specious desert truffle genus [10], with a great number of novel species being described every year [11]. Morphological, ecological and chemical features, often ambiguous, are still the main criteria for separating and/or identifying groups of species [12]. For instance, one of the most solid criteria for discriminating all known *Terfezia* species is spore morphology. Yet, and without molecular

techniques, is nowadays almost impossible to separate most spiny-spored *Terfezia* beyond doubt.

Currently, the described spiny spored *Terfezia* species exclusively associated to *T. guttata* in acid soils are *T. fanfani* Mattir. [13], *T. extremadurensis* Muñoz-Mohedano, Ant. Rodr. and Bordallo [6] and *T. lusitanica* Bordallo, Ant. Rodr., Louro, Santos-Silva, Muñoz-Mohedano [14]. The present study describes a new spiny spored *Terfezia* species in association with *T. guttata* in sandy acid soils, found in Alentejo (Portugal). Furthermore, a specimen belonging to *T. cistophila* was also found and here reported as a new record for Portugal.

METHODS

Fungal collections and collecting sites

Terfezia ascocarps were collected in different years and from different locations in Centre and Southern Portugal. Throughout the collection period (from February to April), fresh specimens were brought to the laboratory for morphological and molecular characterization. The putative plant host was registered and soil samples were collected nearby each specimen. Fragments of each specimen were frozen at -20 ºC for DNA amplification and the remaining specimen were dried at 40 °C and stored in sealed plastic bags, labeled with collection details. All samples are deposited at the Herbarium of the Évora University Herbarium (UEVH-FUNGI), Portugal. Soil samples (50 mm diam., 150 mm depth) were collected in each collection site. A compose sample of 6 soil samples replicas per site was made and analysed at the Laboratório Químico Agrícola Rebelo da Silva (INIAV/LQARS) for particle size and subsequent soil textural classification [15] and water pH measurements.

Morphological study

External ascocarp characteristics (shape, colour, appearance) were in detail recorded from fresh specimens. Ascocarps were then cut and the morphology of the peridium and gleba was described. Microscopic observations were performed in distilled water, KOH 5% and Melzer's reagent. Spores dimensions are based on a minimum of 100 randomly selected spores outside asci. Peridium of dried specimens were rehydrated and examined in KOH 5%. Melzer's reagent was used to test the amyloidity of asci, spores and tissues. Asci and ascospores were examined using a Leica DM750 microscope equipped with a digital camera (Leica ICC50 W).

For identification, ascomata were compared with the descriptions from Bordallo et al. [6, 7, 14] and Mattirolo [13].

DNA amplification and sequencing

DNA extraction from the analysed specimen was performed by CTAB method, following the protocol described in Nobre et al. [16]. All extraction products were stored at -20 ˚C and later used directly in the PCR. The Internal Transcribed Spacer (ITS) region of the rDNA, including the 5.8S ribosomal gene, was amplified using the ITS5 and ITS4 primers [17]. PCR reactions were conducted using 1 µl of the extracted DNA in a standard 25 µl reaction, with 0.5 pmol/µl of each primer, 1.5 mM MgCl2, 0.5 mM dNTPs and 0.04 U/ml Taq DNA polymerase. PCR reactions were performed using a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: an initial denaturalization step for 3 min at 95 °C, followed by 35 cycles consisting of: 30 s at 95 °C, 30 s at 95 °C (annealing temp.), 1 min at 72 °C, and a final extension at 72 °C for 10 min. All the PCR product were purified using the NZYGelpure kit (from NZYTech, Lda) and sequencing was done commercially (STAB VIDA, Lda.).

Phylogenetic analysis

The final dataset comprised 78 *Terfezia* ITS sequences (Table 1, Supplemental Table 1) and a *Tirmania nivea* specimen as outgroup (FN395015). Additional to the sequences pertaining the collected specimens, the dataset comprised 3 representative sequences from each *Terfezia* clade previously identified and described in [11]. Care was taken to always include the *Terfezia* species type sequence as deposited in the comprehensive database GenBank® (www.ncbi.nlm.nih.gov/genbank/). All sequences were assembled, edited and aligned with online MAFFT version 7, using the E-INS-i strategy [18]. The phylogenetic reconstruction analysis based on the above ITS sequences was performed in BEAST v.4.2.8 software [19], allowing the software to estimate the evolutionary model. All other settings were left as default. Three independent Markov Chain Monte Carlo (MCMC) runs, starting from randomly chosen topologies were performed as a safeguard against spurious results. The MCMC were run for 10,000,000 generations with data sampled every 1,000 generations. Log-file outputs were inspected in Tracer v.1.6 to determine chain convergence and burnin. The first 10% of

the generations were discarded as the burn-in, the tree-file outputs from individual MCMC runs were combined in LogCombiner v.2.4.8. and the maximum clade credibility tree and corresponding posterior probabilities were obtained using TreeAnnotator v.2.4.8 [19]. To test the congruence between methods, reconstructions with Maximum Likelihood (ML), Minimum Evolution (ME) and Neighbor-Joining (NJ) methods were performed in Mega 10.0.5 [20]. The best-fit nucleotide substitution model was selected with the program jModelTest [21] applying the Akike information criterion (AIC) and this information was used to calculate the distances in the ML analysis.

RESULTS

Beside the new species described thereafter, our collections included also specimens belonging to other spiny-spored *Terfezia* (Table 1). Regarding soil analyses, even though not exhaustive, revealed distinct micro-ecological preferences, namely, *T. lusitanica* prefer sandier soils and *T. solaris-libera* sp. nov. less sandy soils. As for, *T. fanfani,* it showed a larger range of edaphic preferences, all inside the major group of sandy soils. *T. cistophila*, was found only once and in loamy sand soils (**Table 1**), nevertheless, it is worth mention since is the first reference of the species in Portugal.

Table 1. *Terfezia* collections analysed.

Species	GenBank	Voucher No.	Collection Date	Collection Site (Portugal)	Plant host	Soil type
T. cistophila	MN338749	2004068	20/04/2018	Alentejo, Évora	*Cistus salviifolius*	Loamy sand, pH 5.6
T. lusitanica	MN338748	2004668	22/03/2019	Lisboa, Alcochete	*Tuberaria guttata*	Sandy soils, pH 6.1
	MG818754	2003876	20/04/2017	Alentejo, Montemor-o-Novo	*T. guttata*	Loamy sand, pH 5.5
	MN338747	2004669	22/03/2019	Lisboa, Alcochete	*T. guttata*	Sandy soils, pH 6.1
	MN338745	2003487	20/04/2016	Alentejo, Montemor-o-Novo	*T. guttata*	Loamy sand, pH 5.5
	MN338744	2003442	24/04/2016	Alentejo, Mora	*T. guttata*	Loamy sand, pH 5.7
	MN338746	2004677	22/03/2019	Lisboa, Alcochete	*T. guttata*	Sandy soils, pH 6.1
	MG818752	2003065	03/04/2012	Alentejo, Montemor-o-Novo	*T. guttata*	Loamy sand, pH 5.5

	MN338740	2004051	22/03/2018	Alentejo, Évora	*T. guttata*	Sandy loam, pH 6.2
	MN338738	2004078	20/04/2018	Alentejo, Évora	*T. guttata*	Loamy sand, pH 5.6
	MN338742	2004680	20/03/2019	Alentejo, Ponte de Sor	*T. guttata*	Loamy sand, pH 5.7
	MN338734	2003847	30/03/2017	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338741	2004664	02/03/2019	Alentejo, Évora	*T. guttata*	Sandy loam, pH 6.2
T. fanfani	MN338735	2004054	22/03/2018	Alentejo, Évora	*T. guttata*	Sandy loam, pH 6.2
	MN338736	2004058	22/03/2018	Alentejo, Évora	*T. guttata*	Sandy loam, pH 6.2
	MN338737	2004087	25/04/2018	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338739	2004088	25/04/2018	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338743	2004678	22/03/2019	Lisboa, Alcochete	*T. guttata*	Loamy sand, pH 5.2
	MN338731	2004089	25/04/2018	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338729	2003820	30/03/2017	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338730	2003821	30/03/2017	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
T. solaris-libera sp. nov.	MN338727	2003840	30/03/2017	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338728	2003846	30/03/2017	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0
	MN338733	2004593	27/02/2019	Alentejo, Évora	*T. guttata*	Sandy loam, pH 6.0
	MN338732	2004746	14/02/2019	Alentejo, Arraiolos	*T. guttata*	Sandy loam, pH 6.0

Taxonomy

Name: *Terfezia solaris-libera* Louro, Nobre, Santos-Silva, sp. nov.

Type: PORTUGAL, Alentejo, Arraiolos, in roadside verges near grasslands areas without trees, associated exclusively with *Tuberaria guttata*; 25 April 2018; leg. C. Santos-Silva, Holotype: UEVH-FUNGI 2004089.

Diagnosis: Ascomata 1.5-2.5 cm in diam., hypogeous, subglobose and light coloured to brownish. Peridium 500-700 µm thick, poorly delimited and pseudoparenchymatous, composed of subglobose cells of variable size. Gleba with brownish pockets of fertile tissue surrounded by whitish, sterile, veins, becoming uniformly brownish coloured at maturity. Ascospores globose, (20-) 21.6(-23) µm diam. (median = 22 µm) including ornamentation; (15-)15.7(-17) µm (median = 16 µm) without ornamentation; light brown and ornamented with

conical, blunt spines, sometimes cylindrical, generally straight, but sometimes slightly curved, separated, 2-3 µm long, 1-1.5 µm wide at the base. *Terfezia solaris-libera* sp. nov. differs from other spiny-spored *Terfezia* species associated with *Tuberaria guttata* by its poor delimited thicker peridium and different sporal ornamentation, and from all *Terfezia* spp. in its ITS nrDNA sequence. *T. fanfani* usually reaches larger ascocarp dimensions, has prismatic peridium cells, olive green tinges in mature gleba and different spore ornamentation constituted by sharp thin elongated conic spines (2-)3-4(-5) µm long; *T. lusitanica* has a lighter yellowish and thinner peridium and a blackish gleba upon maturity and finally *T. extremadurensis* has a thinner well delimited peridium and tuber-like gleba. Exclusively associated with *Cistus*, *Terfezia cistophila* shares a similar habitat as the aforementioned species, differing from all in its spermatic odour.

Etymology: "Solaris" refers to the sun, due to its particular spore ornamentation and ecology, and "libera" due to the collection date of the type (25 April, the Freedom day in Portugal which commemorates the Carnation Revolution).

Description: Ascomata hypogeous, subglobose, 1.5-2.5 cm in diameter, light ochre colour at first, becoming darker in maturity, smooth (Fig. 1A, 1B). Faint odour, not distinctive. Mild taste. Peridium 500-700 µm thick, not separable from gleba, poorly delimited, whitish in cross section, pseudoparenchymatous, composed of subglobose cells of variable size, hyaline and thin-walled in the innermost layers (max. 40 µm diam.), yellowish and with thicker walls in the outermost layers (max. 8 µm diam.) (Fig. 1C).

Gleba solid, fleshy, succulent, whitish at first, then with brownish pockets of fertile tissue surrounded by whitish sterile veins, finally becoming uniformly brownish coloured at maturity (Fig. 1A, 1B).

Asci nonamyloid, subglobose, sessile, 65-80 x 50-65 µm diam., walls 1 µm thick, with 6-8 irregularly disposed spores (Fig. 1F, 1G), randomly arranged in the gleba.

Ascospores. globose, (20-) 21.6(-23) µm diam. (median = 22 µm) including ornamentation; (15-)15.7(-17) µm (median = 16 µm) without ornamentation; hyaline, smooth and uniguttulated at first, by maturity light brown and ornamented with conical, blunt spines, sometimes cylindrical, generally straight, but sometimes slightly curved, separated, 2-3 µm long, 1-1.5 µm wide at the base (Fig. 1D, 1E).

Habitat and Distribution: Hypogeous mycorrhizal fungi, in sandy loam (80–83% sand material), acid soils (pH 6), appearing solitary or in small groups, from February to April, in roadside verges near grasslands areas without trees, associated exclusively with *Tuberaria guttata.* Occurring in Southwestern Iberian Peninsula.

Specimens examined: PORTUGAL: Alentejo: Arraiolos, 30 March 2017, C. Santos-Silva leg. (UEVH-FUNGI 2003820, GenBank: MN338729; UEVH-FUNGI 2003821, GenBank: MN338730; UEVH-FUNGI 2003840, GenBank: MN338727; UEVH-FUNGI 2003846, GenBank: MN338728); Idem, idem, Arraiolos, 14 February 2019, R. Louro leg. (UEVH-FUNGI 2004746; GenBank: MN338732); Idem, idem, Évora, 27 February 2019, R. Louro leg. (UEVH-FUNGI 2004593, GenBank: MN338733).

Remarks: *Terfezia solaris-libera* sp. nov. differs from other spiny-spored *Terfezia* species associated with *Tuberaria guttata* by its poor delimited thicker peridium and different sporal ornamentation, and from all *Terfezia* spp. in its ITS nrDNA sequence. *T. fanfani* usually reachs large ascocarp dimensions, often with prismatic peridium cells, with olive green tinges in mature gleba and a different sporal ornamentation. *T. lusitanica* has a lighter yellowish and thinner peridium and a blackish gleba upon maturity, *T. extremadurensis* has a thinner well delimited peridium and *Tuber*-like gleba. Exclusively associated with *Cistus*, *Terfezia cistophila* shares a similar habitat as the aforementioned species, differing from all in its spermatic odour. It can be separated from *T. solaris-libera* sp. nov. by its thinner peridium and smaller spores.

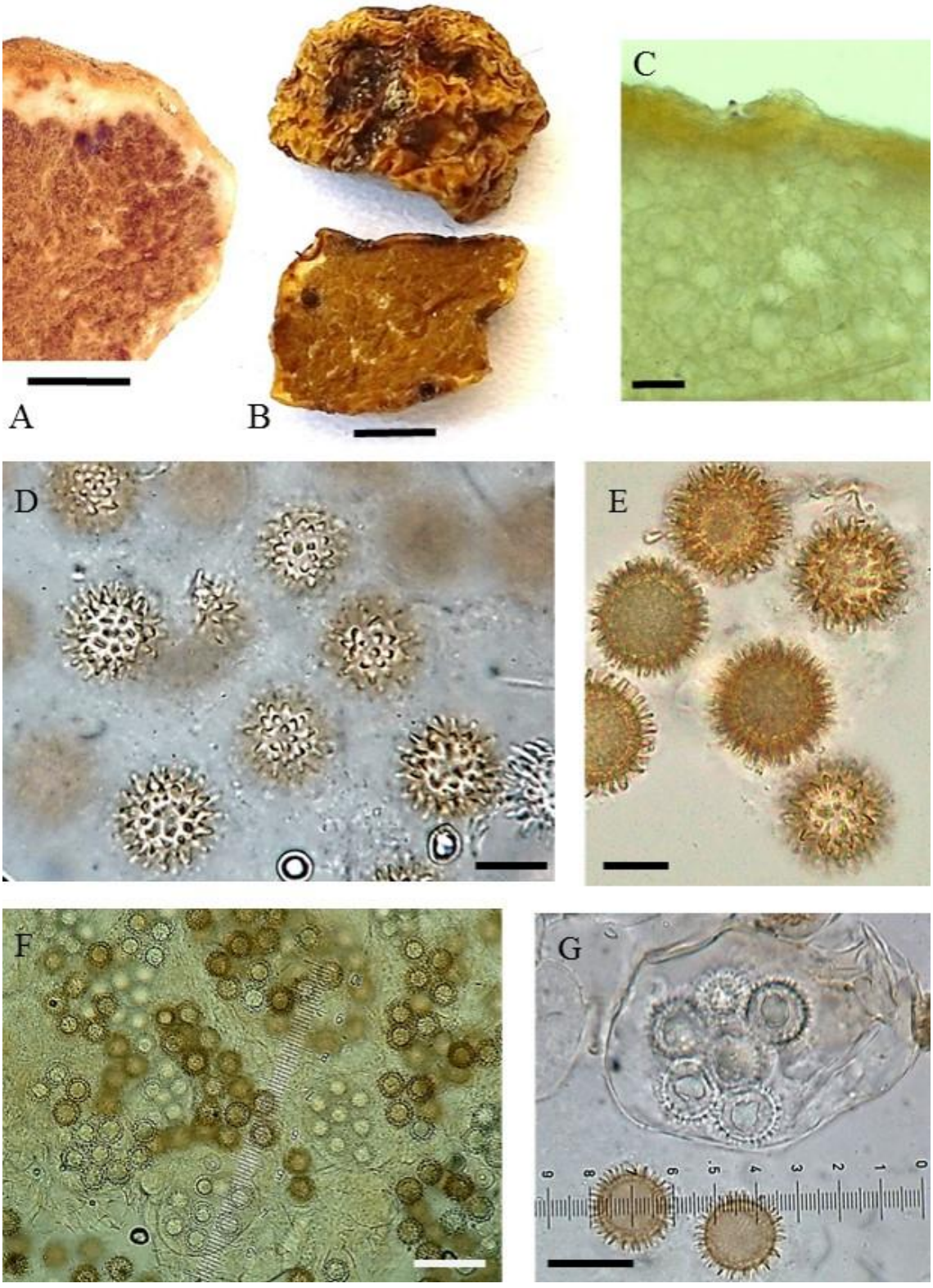

Figure 1. *Terfezia solaris-libera*, sp. nov.: **A**: fresh mature ascocarp sectioned, **B**: dry ascocarp external peridium and gleba, **C**: detail of the pseudoparenchymatous peridium; **D** and **E**: details of mature and immature spore ornamentation under different light conditions; F and **G**: asci and mature and immature ascopores. **Bars: A** 50 mm; **B** 50 mm; **C** 50 µm; **D** 15 µm; **E** 10 µm; **F** 45 µm; **G** 20 µm.

Key to examined species

1a. Associated exclusively with *Cistus* and with spermatic odor……*T. cistophila*

1b. Associated, mainly, with *Tuberaria guttata* without spermatic odor…….. 2

2a. Peridium with reddish colour and gleba with olive-green tinges.....*T. fanfani*

2b. Peridium with no reddish colours and gleba with no green tinges..... 3

3a. Gleba with blackish-grey pockets at maturity……*T. lusitanica*

3b. Gleba with brownish pockets at maturity……*T. solaris-libera* sp. nov.

Phylogenetic analysis

The reconstructed phylogeny ample supports the newly described species *T. solaris-libera* sp. nov. and corroborates the existence of 17 distinct clades representing well supported monophyletic groups (Fig. 2). The sequence originally named *T. leptoderma* (GenBank accession no. AF396864) remains isolated and does not nested inside of any clade. All reconstructed phylogenies were congruent, regardless the method used (Supplemental Material 1). The clade comprising the *T. solaris-libera* sp. nov. sequences harbors also the Genbank sequences HM056215, HQ698132 and HQ698149 (Fig. 2). The sequences referred to above belong to ascocarps collected in Spain, initially identified as *Terfezia* sp. (Badajoz, 2010), *T. aff. olbiensis* (Zamora, 2011) and *T. aff. olbiensis* (Cáceres, 2011) respectively. The other spiny-spored *Terfezia* species were also well supported in monophyletic groups, separating specimens of *T. lusitanica*, *T. cistophila* and *T. fanfani*.

DISCUSSION

The morphological characters of *T. solaris-libera* sp. nov. (Fig. 1) and the ITS based phylogenetic analyses (Fig. 2) provide strong support that it is a new species. *T. solaris-libera* sp. nov. is morphologically different from other *Terfezia* species previously described [6, 7, 13, 14, 22-26]. The images here presented serve not only the species description goal but also are intended to be used as tools for mycologists dealing with morphological identification of *Terfezia* species. Images of the diagnosing characters referring to the gleba and peridum are presented in optical microscopy under current working conditions. Likewise, and because it is well known that maturity of the specimens is crucial for its identification, and hence care

was taken to present images from both immature and mature spores (being the last ones needed for identification purposes).

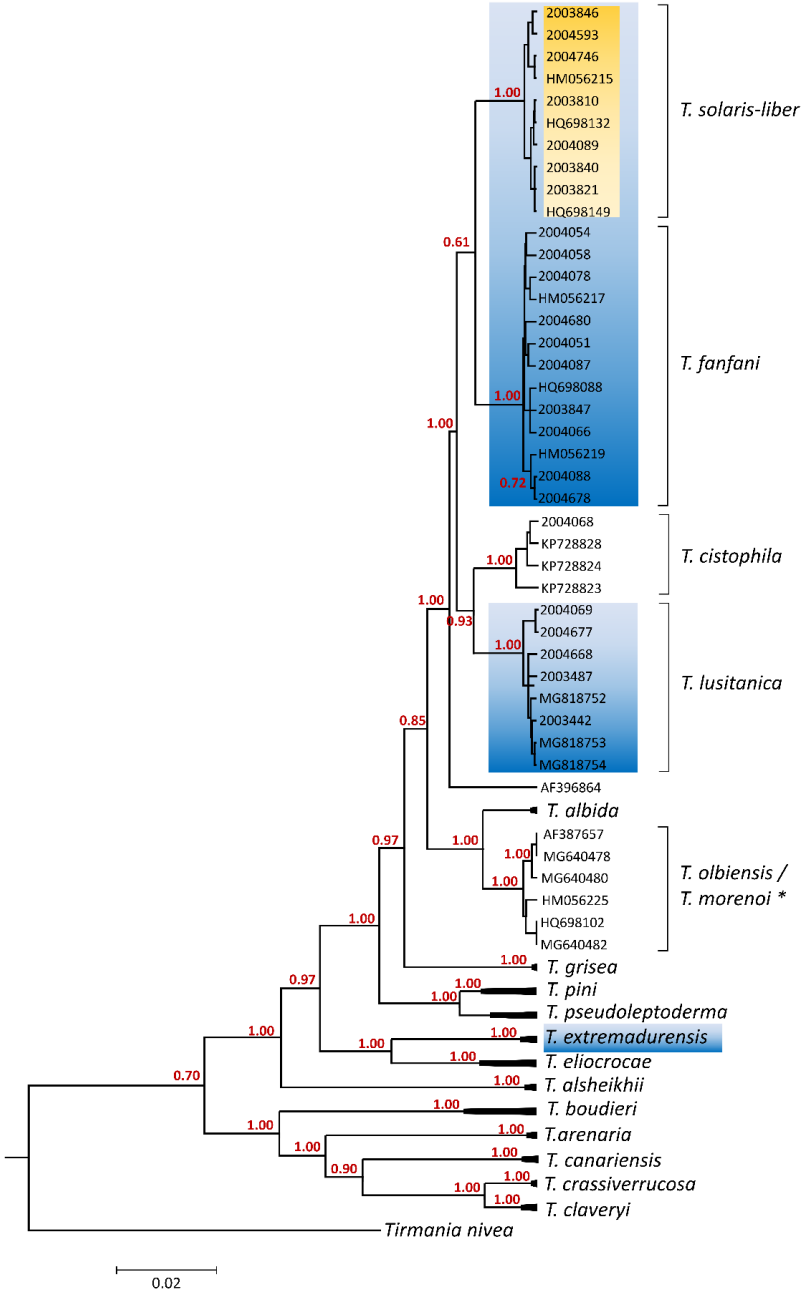

Figure 2. Phylogenetic relationship between *Terfezia* species. The phylogeny corresponds to the majority rule consensus tree of trees sampled in a Bayesian analysis, and the posterior probability values are shown for main nodes. (ML, ME and NJ analyses presented in Supplemental Material 1). Newly obtained sequences are coded with the herbarium number (**Table 1**). Other sequence codes correspond to GenBank accession numbers (**Supplemental Table 1**). The spiny spore species associated with *T. guttata* are highlighted in blue; the new described species in orange. * clade comprising three *T. olbiensis* and three *T. morenoi* sequences (see [11] and [25]).

T. solaris-libera sp. nov. shares soil preferences and the host plant species (*Tuberaria guttata*) with *T. extremadurensis, T. fanfani* and *T. lusitanica*, but differs in morphological characteristics and phylogenetic distances. According to our experience, the species *T. solaris-libera* sp. nov., *T. extremadurensis*, *T. fanfani* and *T. lusitanica*, have never been found with other host plant different from *Tuberaria guttata*. These host-symbionts specificity dynamics raises several questions, particularly due to the vast polymorphism of *T. guttata* [27]. This host plant is characterized by high ecological plasticity and by substantial variations in the breeding system [28] with different subspecies and varieties being recognized [29]. This, as recently highlighted [14], can have an impact on the specificity of the associated *Terfezia* species. The overall scarcity of information on the host plant of the different species of *Terfezia* was also recently discussed [11]and in the case of the *T. guttata* the care in collecting information should go a step-forward towards the reconstruction of a co-phylogeny of host species varieties and *Terfezia* symbionts, disentangling eventual specificity dynamics and co-evolutionary patterns.

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Supplemental Table 1

Terfezia species	Accession Nº	Seq. Lenght (bp)	Reference	DNA source	Collection Site	Host plant	Soil type
AF396864	AF396864	600	Diez et al. 2002	Isolate/strain	Spain:Valencia	*Pinus halepensis*	Acid
	HM056220*	631	Bordallo et al. 2013	Fruitbody	Spain:Albacete	*Helianthemum sp.*	Alkaline
T. albida	HM056221	641	Bordallo et al. 2013	Fruitbody	Spain: Albacete	*Helianthemum sp.*	Alkaline
	HQ698146	662	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria guttata*	-
	HM056207	638	Bordallo et al. 2013	Fruitbody	Portugal:Trás-os-Montes	*Cistaceae*	Acid
T. alsheikhii	HM056208	589	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	-	-
	HQ698100*	653	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria lignosa*	Acid
	HQ698066	655	Kovacs et al. 2011	Fruitbody	Spain:Badajoz	*Quercus ilex*	Acid
T.arenaria	HQ698067	656	Kovacs et al. 2011	Fruitbody	Spain:Salamanca	*Tuberaria guttata*	Acid
	KP217815	602	Dafri & Beddiar 2017	Fruitbody	Algeria	*Tuberaria guttata*	-
	AF092096	616	Ferdman et al. 2005	Fruitbody	Israel:Negev	-	-
T. boudieri	FN395016	594	Bouzadi et al., unpublished	Fruitbody	Libya:Hammad Al Hamra	-	-
	GU474808	579	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum sp.*	-
	JQ858196	504	Bordallo et al. 2012	Fruitbody	Spain:Canary Island	*Helianthemum canariense*	Alkaline
T. canariensis	JQ858188	351	Bordallo et al. 2012	Fruitbody	Spain:Canary Island	*Helianthemum canariense*	Alkaline
	JQ858190*	351	Bordallo et al. 2012	Fruitbody	Spain:Canary Island	*Helianthemum canariense*	Alkaline
	KP728823	655	Bordallo et al. 2015	Fruitbody	Spain:Caceres	*Cistus ladanifer*	Acid
T. cistophila	KP728824	611	Bordallo et al. 2015	Fruitbody	Spain: Badajoz	*Cistus ladanifer*	Acid
	KP728828*	594	Bordallo et al. 2015	Fruitbody	Greece:Nea Makri Attica	*Cistus monspeliensis, C. creticus*	Acid
	HM352540	634	Jamali & Banihashemi 2012	Isolate/strain	Iran	*Helianthemum sp., Carex sp.*	Alkaline
T. claveryi	GU474801	608	Sbissi et al. 2011	Isolate/strain	Tunisia	*Helianthemum kahiricum*	Alkaline
	HQ698080	658	Kovacs et al. 2011	Fruitbody	Spain:Granada	*Helianthemum salicifolium*	-
	AF387646	583	Kovacs et al. 2011	Isolate/strain	Spain	-	-
T. crassiverrucosa	AF387647	580	Kovacs et al. 2011	Isolate/strain	Spain:Murcia	-	-
	MF940203*	513	Zitouni-Haouar et al. 2018	Fruitbody (Holotype)	Algeria	*Helianthemum sp.*	Alkaline
	HM056205	627	Bordallo et al. 2013	Fruitbody	Spain: Murcia	*Helianthemum sp.*	Alkaline
T. eliocrocae	HM056206	534	Bordallo et al. 2013	Fruitbody	Spain: Murcia	*Helianthemum sp.*	Alkaline
	MF940200	547	Zitouni-Haouar et al. 2018	Fruitbody	Algeria	*Helianthemum lippii*	Alkaline
	HM056199*	544	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
T. extremadurensis	HM056202	596	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Tuberaria guttata*	-
	HQ698134	534	Kovacs et al. 2011	Fruitbody	Spain:Jaen	*Cistus albidus*	-
	HM056217*	645	Bordallo et al. 2013	Fruitbody	Spain: Caceres	*Tuberaria guttata*	Acid
T. fanfani	HM056219	596	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	*Tuberaria guttata*	Acid
	HQ698088	647	Kovacs et al. 2011	Fruitbody	Spain:Caceres	-	-
	KP189328	655	Bordallo et al. 2015	Fruitbody	Spain:Burgos	*Helianthemum sp.*	Alkaline
T. grisea	KP189330*	590	Bordallo et al. 2015	Fruitbody	Greece:Schinias_Attica	*Pinus spp.*	Alkaline
	KP189333	591	Bordallo et al. 2015	Fruitbody	Spain:Burgos	*Helianthemum sp.*	Alkaline
	MG818752	591	Bordallo et al. 2018	Fruitbody	Portugal:Alentejo	*Tuberaria guttata*	Acid
T. lusitanica	MG818753*	656	Bordallo et al. 2018	Fruitbody (Holotype)	Spain:Caceres	*Tuberaria guttata*	Acid
	MG818754	588	Bordallo et al. 2018	Fruitbody	Portugal:Alentejo	*Tuberaria guttata*	Acid
	MG640480*	539	Crous et al. 2018	Fruitbody	Spain:Albacete	*Pinus spp., Quercus spp.*	Alkaline
T. morenoi	MG640478	539	Crous et al. 2018	Fruitbody	Spain:Albacete	*Pinus spp., Quercus spp.*	Alkaline
	MG640482	542	Crous et al. 2018	Fruitbody	Spain: La Rioja	*Pinus spp., Quercus spp.*	Alkaline
	AF387657	569	Kovacs et al. 2011	Isolate/strain	Spain	-	-
T. olbiensis	HM056225	628	Bordallo et al. 2013	Fruitbody	Spain:Valencia	*Pinus sp.*	-
	HQ698102	641	Kovacs et al. 2011	Fruitbody	Spain:Madrid	-	-
	HM056209	633	Bordallo et al. 2013	Fruitbody	Spain:Valladolid	*Pinus sp.*	-
T. pini	HM056210*	631	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Pinus sp.*	-
	HQ698138	662	Kovacs et al. 2011	Fruitbody	Spain:Palencia	*Quercus spp.*	-
	HM056211*	588	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Cistaceae*	-
T. pseudoleptoderma	HM056212	647	Bordallo et al. 2013	Fruitbody	Spain:Burgos	*Cistaceae*	-
	HM056213	631	Bordallo et al. 2013	Fruitbody	Spain:Caceres	*Cistaceae*	-
T. solaris-libera	HM056215	628	Bordallo et al. 2013	Fruitbody	Spain:Badajoz	*Cistaceae*	-
	HQ698132	643	Kovacs et al. 2011	Fruitbody	Spain:Zamora	-	-
	HQ698149	641	Kovacs et al. 2011	Fruitbody	Spain: Caceres	*Cistaceae*	-

* Type material

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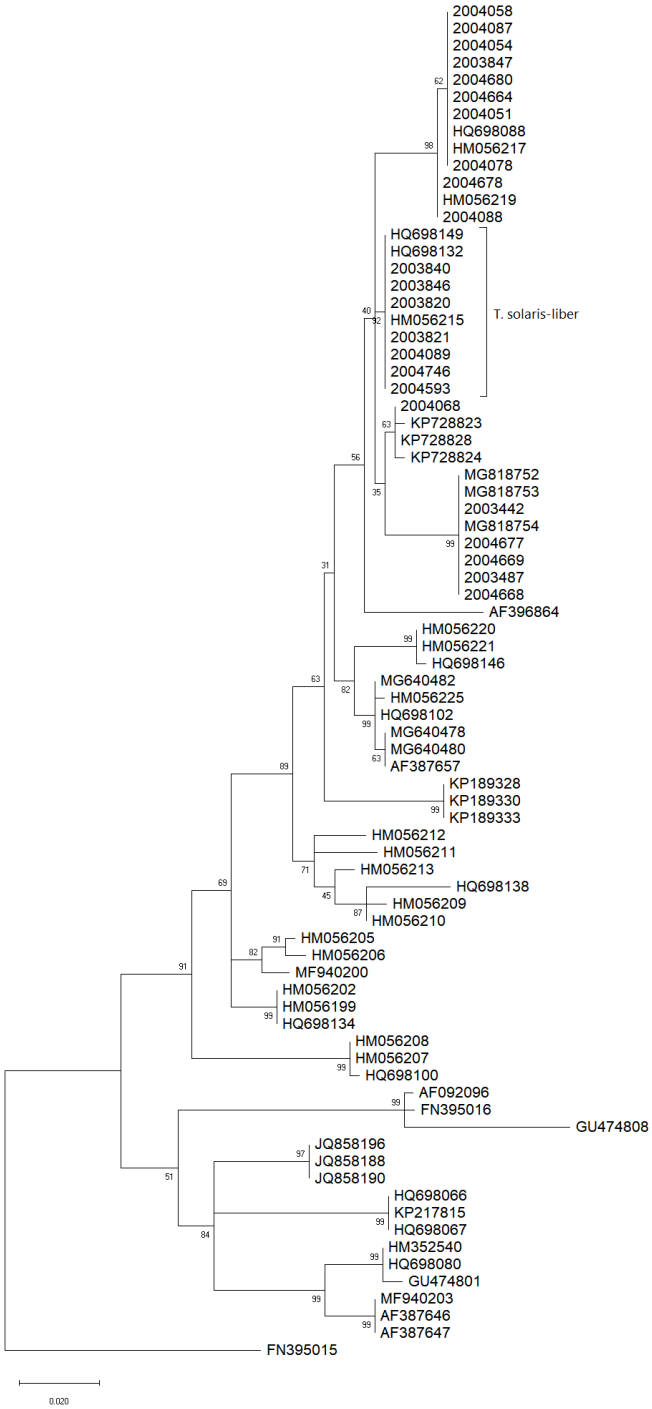

Fig.S1. The relation between specimens was inferred using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood (-1968.39) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.13% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95 % site coverage were eliminated, i.e., fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option).

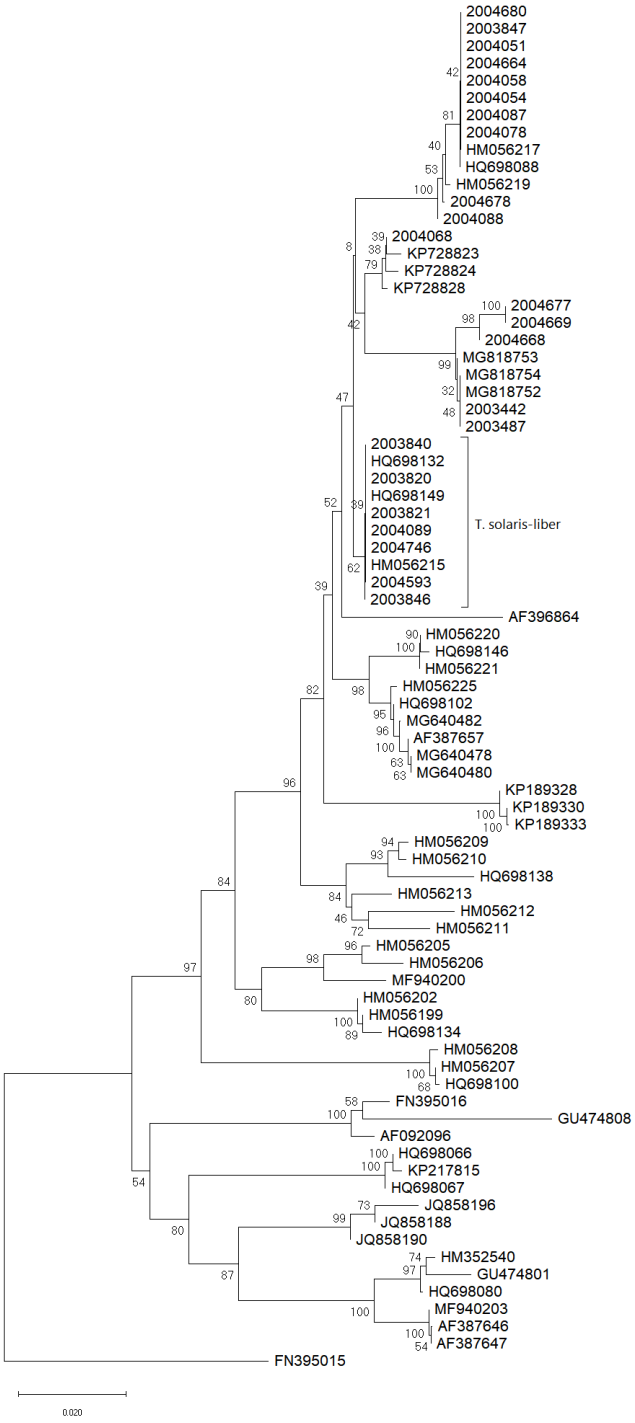

Fig.S2: The relation between specimens was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.69421768 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The 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 p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site.

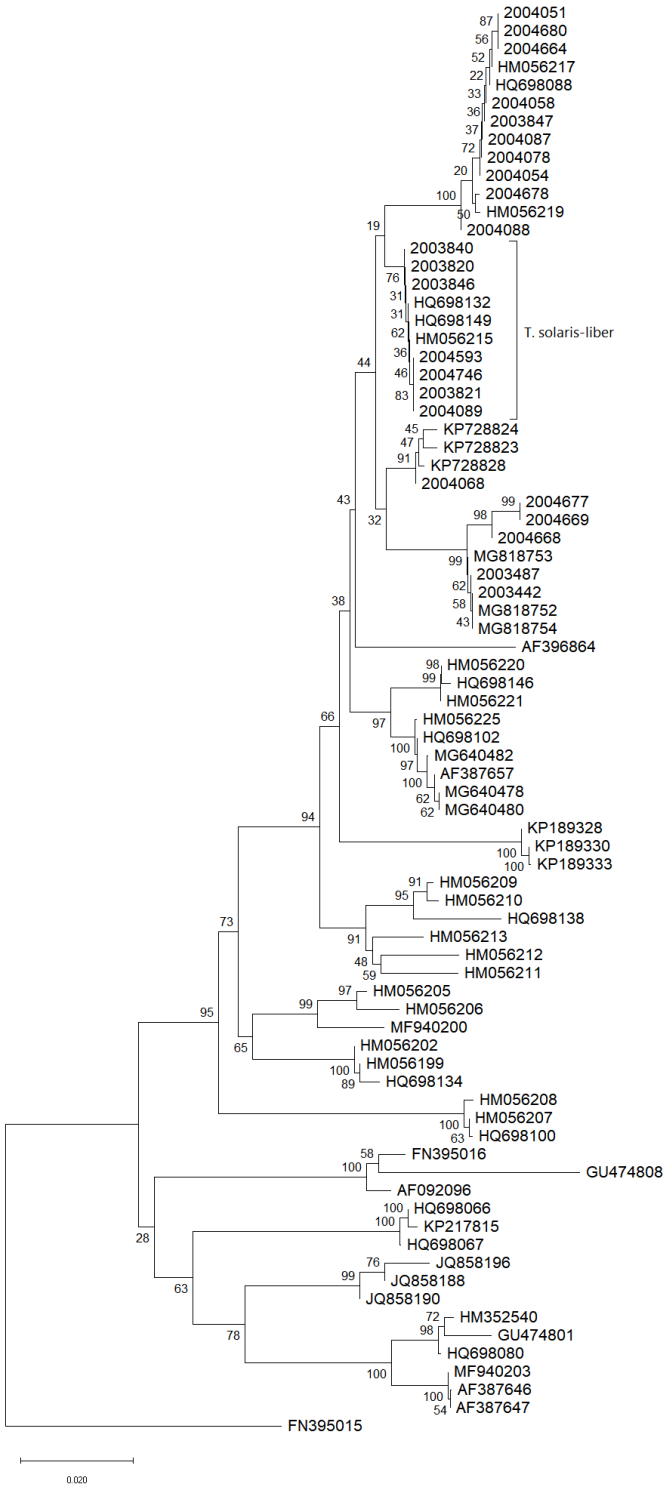

Fig.S3: The relation between specimens was inferred using the Minimum Evolution method (Rzhetsky and Nei 1992). The optimal tree with the sum of branch length = 0.69543083 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The 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 p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 1.

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SECTION II

MYCORRHIZAL ASSOCIATIONS BETWEEN *TERFEZIA* AND *CISTUS*_INFERENCES FOR DESERT TRUFFLE CULTIVATION

Highlights:

• An optimized *in vitro* micropropagation protocol for rapid multiplication of true-to-type *Cistus salviifolius* plants was developed

• A new fully-synthetic culture media and process for improved isolation and maintenance of terfezia spp. mycelium cultures was designed

• There is an extraordinary compatibility of *Cistus salviifolius* and *Cistus ladanifer* with *T. arenaria*, *T. fanfani*, *T. extremadurensis* and *T. pini*

• *Terfezia arenaria* showed significantly lower mean frequencies of infection on both *Cistus salviifolius* and *Cistus ladanifer*

• The four *Terfezia* species studied do form ectomycorrhizas with a true sheath, and with a well-developed Hartig net but with varying degrees of mantle development

This section includes the following publications:

Louro R., Peixe A., Santos-Silva C. 2017. New Insights on Cistus salviifolius L. Micropropagation. *Research & Reviews: Journal of Botanical Sciences*, 6(3): 10-14.

Louro R., Santos-Silva C. 2020. New culture media for improved isolation and growth of Terfezia spp. mycelium on agar plates. European Patent Application n. 19204730.6 – 1118 (10.01.2020)

Louro R., Natário B., Santos-Silva C. 2020. Morphologic characterization of the *in vitro mycorrhizae* formed between four *Terfezia* species (*Pezizaceae*) with *Cistus salviifolius L.* and *Cistus ladanifer L.* (to be submitted to the journal *Mycorrhiza*)

CHAPTER IV

New Insights on *Cistus salviifolius* L. Micropropagation

Data published in:

Rogério Louro, Augusto Peixe, Celeste Santos-Silva

Research & Reviews: Journal of Botanical Sciences, 2017, 6(3): 10-14DOI

ABSTRACT

One of the major concerns in the establishment of any mycorrhization program is ensuring the mass production of sterile, consistent and standardized plant material. In the present study, a successful protocol for micropropagation of *Cistus salviifolius* L. was developed. The process was initiated from nodal segments excised from mature *C. salviifolius* plant selected due to its mycorrhizal capacities. Murashige and Skoog basal medium supplement with gibberellic acid (0.5 mg/L) and of 6-Benzylaminopurine (0.5 mg/L) was the best medium for proliferation purposes and successful rooting was achieved with the same basal medium supplemented with Indole-3-butyric acid (0.5 mg/L). The proposed methodology represents a novelty because it allowed the rapid multiplication of *C. salviifolius* starting from mature explants, here reported for the first time, using lower plant growth regulators concentrations than the previously reported for this particular *Cistus* species.

Keywords: *Cistus salviifolius*, Micropropagation, Mycorrhization, GA3, BAP, IBA

INTRODUCTION

The genus *Cistus* L. (*Cistaceae*) is one of the most characteristic genera of the Mediterranean flora [1]. It encompasses a group of about 20 perennial shrub species, distributed throughout the Mediterranean region and Canary Islands, all sharing the same distinctive feature, a combination of diverse hair types on the leaf, stem, and calyx [2,3]. *Cistus* species exhibit a range of specific adaptations as well to Mediterranean environments, such as, fire-dependent seed germination, insect-dependent pollination, flower-dependent reproduction and spring-dependent phenology [4].

Cistus species are involved in many ecological processes taking place in Mediterranean ecosystems [5]. Furthermore, they support a vast and rich mycobiota, constituting reservoirs for mycorrhizal fungal inoculum in the absence of host trees [6]. In total, more than 200 fungal species, belonging to 40 genera, have been reported to be associated with *Cistus*. Among which, several edible hypogeous *Ascomycota*, mainly included in *Tuber* and *Terfezia* genera, and commonly known as truffles [7]. Truffles are highly sought-after and some species command extraordinary prices in local markets, however, due to their ectomycorrhizal

nature, truffles must be cultivated in orchards with their plant hosts [8]. As proposed by Giovannetti and Fontana, the wide variety of *Cistus* (and other *Cistaceae*) environmental and ecological requirements makes them ideal candidates to increase the range of habitats where truffles can be grown [9]. Thereby, inoculating these *Cistaceae* with truffle inoculum and planting them in a primary stage of truffle forest repopulation, has become an extremely interesting new use for *Cistus* plants and one with great economic importance and potential for forestry purposes [10].

Cistus salviifolius L. a low subshrub up to 1 m tall, with ovate to rounded leaves and white flowers is the most widely spread species of the genus around the Mediterranean basin [3]. It can occur in sandy soils over a wide range of habitats and has been regularly reported as a plant host for various *Terfezia* species, which makes it one of the best choices for planned Terfezia cultivation over a wide range of habitats [7,11,12].

Conventional propagation methods are still the main means for obtaining many ornamental *Cistus* varieties [13]. Nevertheless, vegetative propagation proved to be problematic when wild varieties were used, so in vitro micropropagation approaches begun to be tested in the nineties, to overcome the problem of clone production from selected individuals [14-18]. It is widely accepted that tissue culture techniques can represent a reliable and feasible alternative for the rapid multiplication and production of true-to-type plants in limited space and time [19]. The success and efficiency of these in vitro micropropagation techniques is influenced by many factors, such as, plant genotype, the physiological status of the explants, culture medium and plant growth regulators (PGRs) [20].

In what concerns *Cistus* micropropagation, one of the most important factors, especially in the shoot proliferation stage, is the amount of cytokinin hormone. Indeed, M'Kada et al. working with nodal segments, excised from mature plants of *Cistus* × *purpureus* Lam., observed that the in vitro establishment of the initial explants represented a limiting step, since half of them were unable to develop new shoots. Cytokinins are known to delay senescence, promote mitosis, and stimulate differentiation of the meristem into shoots and roots [20]. Thus, in early works, high concentrations of cytokinins were experimented in order to stimulate the proliferation of new shoots excised from seedlings with satisfactory results [3,13]. Despite these early experiments resulted in successful micropropagation of various *Cistus* species, among which of *C. salviifolius*, it is known that at high levels cytokinins tend to induce callusing, which can utterly compromise the clonal nature of the micropropagated

plants and inhibit the elongation of individual shoots [15,20]. In such cases, the addition of gibberellic acid (GA3) to the plant tissue culture media, has been shown to diminish or prevent the formation of somatic embryos, adventitious roots or shoots and promote inter-node extension and enhance apical dominance [21]. Recently, improved protocols for shoot regeneration using shoot tips of mature *Cistus* plants using only small amount of PGRs have been developed for *C. creticus* and *C. clusii* [13,22].

To our best knowledge, no report was published to date on the shoot regeneration of *C. salviifolius* with high ability to mycorrhize with *Terfezia*, using low concentrations of PGRs and starting from mature explants. Therefore, the aim of the present work was to establish a rapid and optimized in vitro micropropagation protocol for rapid multiplication and production of true-to-type *Cistus salviifolius* plants, thus allowing its application for mass production of mycorrhized plants and ultimately enabling *Terfezia* cultivation over a wider range of habitats.

MATERIALS AND METHODS

Plant Material

C. salvifolius plantlets growing in Herdade da Mitra, near Évora (Alentejo, Portugal) (38°32'N; 8°01'W; 220 m a.s.l.)., were collected on November 2013 in a Montado area with natural shrub undercover dominated by *Cistus* spp. The area belongs to the Mediterranean pluviseasonal-oceanic bioclimate and is located in the low mesomediterranean bioclimatic belt. It has a dry to subhumid ombrotype with a mean annual temperature ranging from 9.2°C to 21.5°C and a mean annual rainfall of 664.6 mm [23,24]. All *C. salvifolius* plantlets were washed and disinfected twice with a bleach solution (1% NaOCl (w/v)), potted in sterile substrate (sand, vermiculite, soil; 1:1:1) and placed in a growth chamber for 30 days (24°C/21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 µmol-m-2s-1). The plantlets were inoculated with *Terfezia arenaria* spores obtained from dry sporocarps stored in the UEVH Fungi Herbarium. Plant survival and mycorrhization rates were evaluated three months after inoculation, according to the protocol proposed by Giovannetti and Mosse [25]. Ninety three percent of the plantlets survived, and of those 82% were successfully mycorrhized with *T. arenaria*. All plantlets were maintained under those

artificial growth conditions, for 24 months, until become adult plants. After that period, mycorrhizae persistence was evaluated and the plant that showed higher micorrhization rate (95%) was chosen to be the source of the initial explants for the in vitro culture. Single node segments, each with two opposite buds, were excised from actively growing shoots.

Explant Sterilization

Explant - single nodal segments - were surface sterilized in a four-step procedure: 1) immersion in ethanol (70%) for 2 min; 2) one rinse in bi-distilled water; 3) immersion in CaCl2-O2 (1%) with eight drops of Tween 20 for 20 min; 4) three rinses with bi-distilled water.

Shoot Proliferation

During the culture establishment phase, the authors observed that *C. salviifolius* explants grown in MS basal medium without growth regulators did not produce new shoots. Furthermore, the explants shown hyperhydricity symptoms and stunted appearance, leading to high mortality rates and low multiplication rates. The later problems were solved with the addition of 0.5 mg/L of gibberellic acid (GA3) to the basal media (data not shown). However, shoot proliferation rate continued to be unsatisfactory to our purposes [26]. Thus, for proliferation purposes, it was necessary to test different media formulation and to ascertain if the addition of a cytokinin would improve the production of new shoots. Bearing that in mind, two basal media: MS and WPM, both supplemented with GA3 (0.5 mg/L) with or without 6- Benzylaminopurine (BAP) (0.5 mg/L) were tested, namely: MSG (MS+0.5 mg/L GA3), MSGB (MS+0.5 mg/L GA3+0.5 mg/L BAP), WPMG (WPM+0.5 mg/L GA3), WPMGB (WPM+0.5 mg/L GA3+0.5 mg/L BAP) [27].

The experiment was conducted with 50 explants per treatment, 10 explants in each of the five culture flasks, in a total of 200 explants. The explants were subcultured every 30 days to fresh medium during three months. Cultures were kept in a growth chamber with 24°C/21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 µmolm-2s-1). At the end of the experiment, the number of new shoots and the number of nodes per shoot were determined. Proliferation rate was evaluated considering the number of shoots per explant.

Rooting

Given that the explants did not form roots in the previous media formulations, a trial for rooting purposes was conducted. MS basal medium was chosen since it proved to be the best medium in the shoot proliferation stage. To induce plant rooting two approaches were taken in consideration: 1) the direct addition of auxins to the media and 2) the promotion of the natural production of auxins by the explants. For that purpose, different formulations were tested using MS basal medium, supplemented with or without activated charcoal and/or Indole-3-butyric acid (IBA), namely: MSC (MS+0.2% of activated charcoal), MS0.1 (MS+0.1 mg/L IBA), MS0.5 (MS+0.5 mg/L IBA), MS0.1C (MS+0.1 mg/L IBA+0.2% of activated charcoal), MS0.5C (MS+0.5 mg/L IBA+0.2% of activated charcoal).

The trial was conducted using 50 explants per treatment, 10 explants in each of the five culture flasks, in a total of 250 explants. The explants were subcultured every 30 days to fresh medium during three months. During that time, cultures were kept in a growth chamber with 24°C /21°C (+1°C) day/night temperature and 15 h light period, under cool white fluorescent light (36 µmolm-2s-1). At the end of the experiment, the number of roots and the tap root length of each explant were recorded. The rooting rate was evaluated considering the number of roots per explant.

Statistical Analysis

The experiments were conducted under a complete randomized block design and data behavior was evaluated by ANOVA analysis. Differences within and between treatments were estimated by mean of separation analysis, using the least significant difference.

RESULTS

Shoot Proliferation

C. salviifolius explants, cultured on media without growth regulators or only with cytokinin, did not thrived, showing hyperhydricity symptoms and stunted appearance. The addition of 0.5 mg/L GA3 to the basal media, not only improved shoot elongation but also prevented hyperhydricity in the new shoots and/or leaves (data not shown), allowing the successful establishment of *C. salviifolius in vitro* culture.

Culture establishment was overall more efficient with MS formulations than with WPM, with significant differences concerning both, the number of nodes per shoot and number of shoots per explant. Furthermore, the highest shoot proliferation rate was achieved in MS supplemented with 0.5 mg/L BAP (**Table 1**), and thus the best media for multiplication purposes was MSGB.

Table 1. Proliferation rate (shoot number per explant), shoot length and the number of nodes per shoot on the four-tested media (MSG: MS+0.5 mg/L GA3; MSGB: MS+0.5 mg/L GA3+0.5 mg/L BAP; WPMG: WPM+0.5 mg/L GA3; WPMGB: WPM+0.5 mg/L GA3+0.5 mg/L BAP). Means followed by the same letters are not significantly different at $p \leq 0.05$.

Variables	MSG	MSGB	WPMG	WPMGB
n°shoots/explant	2.96±0.22a	4.32±0.19b	2.06±0.17a	2.90±0.19a
Shoots length (cm)	3.07±0.15a	3.27±0.13a	2.98±0.12a	3.09±0.12a
n°nodes/shoot	5.32±0.63a	5.90±0.87a	4.35±0.51b	4.70±0.48b

Rooting

Root induction was successfully achieved in all tested medium. The highest rooting rate (8 roots/explant) was observed in MS basal medium supplemented with 0.5 mg/L IBA (**Table 2**),

Table 2. Rooting rate (n° roots/explant) and tap root length on the five-tested media (MSC: MS+0.2% of activated charcoal; MS0.1: MS+0.1 mg/L IBA; MS0.5: MS+0.5 mg/L IBA; MS0.1C: MS+0.1 mg/L IBA+0.2% of activated charcoal; MS0.5C: MS+0.5 mg/L IBA+0.2% of activated charcoal). Means followed by the same letters are not significantly different at $p \leq 0.05$.

Variables	MSC	MS0.1	MS0.5	MS0.1C	MS0.5C
n°roots/explant	1.43±0.10a	2.24±0.23a	8.04±0.65b	2.01±0.19a	2.09±0.18a
Tap root length (cm)	0.83±0.10a	1.16±0.08a	1.71±0.11b	1.07±0.14a	1.18±0.14a
Rooted explants (%)	77	83	85	67	73

with significantly production of more and longer roots. The addition of activated charcoal alone induced the formation of roots in more than 70% of the explants, but in a few number and length, not assuring the plant survival in the next steps. Moreover, the simultaneously addition of charcoal and IBA did not improve the rooting rates.

DISCUSSION

One of the major concerns in the establishment of any mycorrhization program is ensuring the mass production of sterile, consistent and standardized plant material. Tissue culture techniques have the potential to overcome the problem of clonal production from selected individuals, as they provide the means to rapidly multiply and produce true-to-type plants. Reports on the in vitro propagation of *Cistaceae* are still scarce and so far, only Iriondo et al. described a micropropagation system applicable to *C. salviifolius* starting from nodal segments excised from seedlings, using high concentrations of BAP [13]. In this study we tackled these issues and developed the first in vitro micropropagation protocol for shoot regeneration and rooting from mature *C. salviifolius* plants. Furthermore, our study show that is possible to obtain similar proliferation rates as those observed by Iriondo et al. [15], using smaller amounts (0.5 mg/L) of 6-Benzylaminopurine (BAP) thus reducing the risk of somaclonal variation. One noteworthy difference was the need to add 0.5 mg/L GA3 in the culture establishment and shoot proliferation stages, to improve shoot elongation and prevent hyperhydricity. One possible explanation for this fact is the genetic traits of the selected plant, whose metabolic pathways might be slightly different from the others. The selected plant showed remarkable mycorrhizal abilities and it is known that ectomycorrhizal fungi can produce and release phytohormones, among which GAs [28-30]. Therefore, it is possible that the addition of GA3 to the media aided to simulate the natural conditions, which might have favored the plant establishment. The rooting rate obtained with MS0.5 represents an improvement compared with the previous work of Iriondo et al. [15], which obtained a rooting rate of 4.4 roots/explant using IBA (≈1.0 mg/L). The addition of charcoal did not improve the root production, in fact charcoal diminish the rooting rate probably due to the inhibition of plant IBA uptake.

CONCLUSION

In summary, the present work proposes a new methodology which allow the rapid multiplication of *C. salviifolius* starting from mature explants, using lower plant growth regulators concentrations than the previously reported for this particular *Cistus* species. This *in vitro* micropropagation protocol can be useful for multiplication and production of selected *Cistus salviifolius* genotypes, particularly when the purpose is the mass production of plant material for mycorrhization assays.

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CHAPTER V

New culture media for improved isolation and growth of *Terfezia* spp. mycelium on agar plates.

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ABSTRACT

The present invention relates to a culture media and to a process for improved isolation and maintenance of mycelium cultures of “desert truffles” included within the genus *Terfezia*, the most species rich of all of desert truffle genera. The culture media and process of the present invention improve the isolation rates and enhances *Terfezia* spp. hyphal proliferation in a reliable and reproducible way. Therefore, the present invention lays in the technical domain of biochemistry and microbiology, in particular to a process and composition to produce desert truffle’s mycelium that can be used in several industries such as pharmaceutical, cosmetic, food and agriculture.

TECHNICAL DOMAIN

The present invention relates to a culture media and to a process for improving isolation and maintenance of mycelium cultures of “desert truffles” included within the genus *Terfezia*, the most species rich of all of desert truffle genera.

Cultivation of these ectomycorrhizal *Ascomycota* implies the co-culture of both fungal symbiont and plant host in sterile or semi-sterile conditions. However, isolation and maintenance of *Terfezia* spp. pure cultures can be challenging. Moreover, many strains are unable to be sub-cultured, and so far, the few successful attempts, mainly with *Terfezia* spp. from alkaline soils, were found to grow much too slowly to produce adequate amounts of mycelial inoculum in conventional culture media and conditions.

The culture media and process of the present invention improve the isolation rates and enhances *Terfezia* spp. hyphal proliferation in a reliable and reproducible way. Therefore, the present invention lays in the technical domain of biochemistry and microbiology, in particular to a process and composition to produce desert truffle’s mycelium that can be used in several industries such as pharmaceutical, cosmetic, food and agriculture.

BACKGROUND OF THE INVENTION

The term truffle usually designates the fruiting body (ascocarp) of a subterranean *Ascomycota* fungus. These underground fruitbodies are produced by ectomycorrhizal fungi that live in close association with the roots of diverse plant species. In addition to the much-valued forest truffles species, included in the genus *Tuber,* numerous others truffle-bearing species are found in arid and semi-arid areas throughout the world, these are commonly referred as

desert truffles. Many of these desert truffle fungi are of considerable interest for ecological, agroforestry and commercial purposes. Although not as flavoured as forest truffles, their ascocarps are nevertheless highly prized as food, for their unique musky flavour and high content of proteins, and as medicine, due to their anti-bacterial properties against a wide range of bacteria. Among desert truffles, the most highly valuable and frequently collected species, belong to the genus *Terfezia*.

Like most mycorrhizal fungi, desert truffles lack the ability to survive in the soil without a plant host. They depend solely on photosynthates, such as sugars, supplied by their plant hosts, and in exchange provide water and valuable nutrients, such as phosphorus, which may not be readily available in an assimilable form to the plant, thus, aiding in its establishment and survival in harsh environments. In such associations, the majority of nutrient exchange occurs in an interface, designated as mycorrhiza, comprised of intercellular and/or extracellular hyphal network and the plant root cells.

Truffle cultivation began as early 1790, when Pierre Mauléon noticed an "obvious symbiosis" between oak trees and truffles. Mauléon then began to cultivate truffles, by taking acorns from trees known to have produced truffles and sowing them in chalky soil. Later, in 1808, Joseph Talon had the idea of transplanting some seedlings that he had collected at the base of oak trees known to host truffles in their root system, a system that is known in French as “trufficulture”. Since then, several volumes, have sought to summarize the current knowledge concerning truffles or provide an overview on truffle cultivation. However, not a single one of these works deals specifically with desert truffles. In fact, the earliest reports on the successful attempts at desert truffle cultivation date back some 20 years, and only recently desert truffle cultivation began blooming. For example, the first plantation of *Terfezia* mycorrhized seedlings was established in 1999 in Murcia, but so far, only two *Terfezia* species, *T. claveryi* in Spain and *T. boudieri* in Tunisia, were successfully cultivated, both with perennial and annual Helianthemum species in basic soils.

Desert truffles cultivation is not easy to achieve. It depends on the successful co-culture of both fungal symbiont and plant host in sterile or semi-sterile conditions, and for decades it was hampered by difficulties in obtaining good inoculum sources. The first *in vitro* germination of *Terfezia* ascospores have been described in the nineteen seventies. Since then, various formulations were tested. For instance, Awameh & Alsheikh (1979a, 1979b, 1980a, 1980b) used the KISR medium, developed at the Kuwait Institute for Scientific

Research, for the isolation, conservation and multiplication of different *Terfezia*. Later, Ravolanirina (1986) used the Mma medium developed by Hewitt (1966), to successfully cultivate both Tirmania and Terfezia species. Soon after, Fortas & Chevalier (1992) studied the ability of Terfezia arenaria spores to germinate in different media, namely, KISR, Mma, Ma (Cristomalt 1%) and INRA-Morizet medium (Payan 1982). Other formulations were tested, specifically, the Fontana medium (Bonfante & Fontana 1973) and MMN medium (Marx 1969), both of which were believed to facilitate hyphal proliferation. However, isolation of *Terfezia* spp. continued to be challenging and the mycelium growth rates slow and extremely variable between species. Furthermore, most isolated strains were unable to grow after sub-culturing.

Document WO2014020215A1 describes a method for production of *Ascomycota* mycelial inoculum, in particular of *Terfezia* spp., comprising several steps, including the isolation of the mycelium and its inoculation in a modified oat solid culture medium (MOM), purification and subsequent growth in a modified Biotin-Aneurin-Folic Acid (BAF) liquid culture medium, in order to finally obtain the purified fungal mycelium.

Document WO2018114751A1 describes a method for induction and production of truffle peridia, comprising the step of inducing the formation of peridia and of multiplying the peridia in a nutritive medium, after having isolated the mycelium by an extraction process, being the said nutritive medium based on malt and/or yeast extract.

Non-synthetic culture media by definition include some sort of natural ingredients of variable chemical composition which may differ from batch to batch. On the other hand, synthetic media have a known chemical composition, so the same medium can be duplicated with a high degree of accuracy. Therefore, there is a need to develop a reliable and reproducible culture media that allows improved isolation and growth of *Terfezia* spp. mycelium on agar plates and thus the production of the related product in a large scale for mass production of inoculated seedlings. For this purpose, the present invention relates to a new fully synthetic culture medium (LS) that is advantageous for both isolation and supporting hyphal growth of these *Ascomycota* fungi which live as obligate symbionts of many *Cistaceae* plants.

DESCRIPTION OF THE INVENTION

The present invention relates to a culture media and to a process for isolation and maintenance of *Terfezia* spp. mycelium cultures.

1. LS culture medium

A fully synthetic culture media is herein described as medium LS. Given the obligate nature of the symbiotic relationship of desert truffles and their plant hosts, the basic nutrient requirements of cultured plant cells and/or tissues should be similar for cultured ectomycorrhizal fungi. Yet, it is likely that these plant tissue culture media are not optimal for improved grow of the fungal symbiont by itself. Hence, the LS culture medium encompasses an intermediate composition between the conventional culture media generally used to grow *Terfezia* spp. putative plant hosts, and the media typically used to isolate desert truffles.

Most commonly used culture media are generally composed by macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements and solidifying agents. Thus, in order to simplify comparisons between LS and other culture media, the LS medium composition henceforth described is divided into de following components: carbon sources, macronutrients, iron chelates, micronutrients, vitamins, phytohormones and others.

Desert truffles can assimilate diverse carbon sources, even highly polymerized sugars, such as, starch and pectin. However, *Terfezia* strains are more specific regarding the utilization of some sugars as carbon source, for instance, they do not assimilate certain sugars, i.e. arabinose, xylose, fructose and galactose. In fact, some of these sugars, like galactose, are considered as growth inhibitors for certain *Terfezia* species. Hence, the two best suited carbon sources for *Terfezia* are glucose and sucrose, though sucrose has been proven to perform better than glucose.

Thus, in an embodiment of the invention, the LS culture media comprises sucrose and/or glucose as carbon source. New insights on carbohydrate allocation at the plant–fungus interface of ECM fungi shows that hexoses, such as glucose, are delivered by the host plant, originating from sucrose secretion followed by invertase-dependent hydrolysis at the common apoplast of the plant–fungus interface. However, while most *Basidiomycota* ECM fungi lack the invertase activity which implies that they are dependent on the enzyme activity

(sucrose hydrolysis) of the plant partner, in contrast, the *Ascomycota* ECM fungi do have invertase genes, indicating that these fungi may obtain the majority of their carbon as sucrose. Alternatively, sucrose can also play an essential role in signalling pathways which might promote spore germination or hyphae growth in some *Ascomycota* ECM fungi. Indeed, the key role of sugars as signalling molecules is well illustrated by the variety of sugar sensing and signalling mechanisms discovered in free-living microorganisms such as fungi. Given the above, the use of sucrose as the only carbon source is highly recommended for preparation the LS culture medium, within the range of 5 to 15 (g/L), preferably 7.5 to 15 (g/L), even more preferably of 10 (g/L). Thus, in another embodiment of the invention, the LS culture media comprises only sucrose as carbon source.

Concerning macronutrients, it is known that phosphorous, calcium and magnesium are particularly important to support the growth of *Terfezia* mycelium. Also, *Terfezia* spp. shows a clear preference for nitrate salts, but in the absence of these salts they can use phosphates and sulphates salts, as substituents. Therefore, in another embodiment of the invention, the LS medium comprises as macronutrients KNO_3 at a concentration of 0.40 and 0.55 (g/L), preferably of 0.475 (g/L); NH_4NO_3 at a concentration of 0.35 and 0.48 (g/L), preferably of 0.413 (g/L); $CaCl_2.2H_2O$ at a concentration of 0.093 and 0.13 (g/L), preferably of 0.11 (g/L); $MgSO_4.7H_2O$ at a concentration of 0.093 and 0.106 (g/L), preferably of 0.079 (g/L); and KH_2PO_4 at a concentration of 0.036 and 0.049 (g/L), preferably of 0.043 (g/L).

Iron chelates and micronutrients are trace elements required by many fungi to initiate germination and sustained growth, but only in small amounts. For example, iron deficiency may result in stunted growth, and in excess can be extremely toxic. In consequence, a good adjustment of these trace elements is of paramount importance. Regarding the iron chelates, the LS medium comprises: Na_2EDTA in the range of 0.0079 to 0.0107 (g/L), preferably of 0.0093 (g/L) and $FeSO_4.7H_2O$ in the range of 0.0059 to 0.0080 (g/L), preferably of 0.0070 (g/L). Other micronutrients, such as zinc, copper, and molybdenum are also important to activate some enzymes such as e.g. catalase and superoxide dismutase (SOD), which depend on the presence of transition metals to be able to perform their redox functions, and aid the fungal species to avoid damage by reactive oxygen species (ROS). Like the iron chelates, these micronutrients are often toxic in excess, hence they are present in the LS medium in very small amounts, namely, $MnSO_4.4H_2O$ at a concentration of 0.0036 and 0.0049 (g/L), preferably of 0.0042 (g/L); $ZnSO_4.7H_2O$ at a concentration of 0.0018 and 0.0025 (g/L),

preferably of 0.0022 (g/L); $CuSO_4.5H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L); $CoCl_2.6H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L); $Na_2MoO_4.2H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L); H_3BO_3 at a concentration of 0.0013 and 0.0018 (g/L), preferably of 0.0016 (g/L), and KI at a concentration of 0.00018 and 0.00024 (g/L), preferably of 0.0016 (g/L).

Usually, only water-soluble vitamins (e.g. vitamin B_1 or thiamine, B_2 or riboflavin, B_6 or pyridoxine and vitamin H or biotin) are required by fungi. Yet, some fungi lack the biosynthetic capacity to produce vitamins, partially or completely, and thus present a limited growth in the absence of vitamins. The LS medium encompasses a selected pool of vitamins and growth factors that act as coenzymes or as constituents of coenzymes, specifically: thiamine hydrochloride at a concentration of $2.1x10^{-5}$ and $2.9x10^{-5}$ (g/L), preferably of $2.5x10^{-5}$ (g/L), nicotinic acid at a concentration of 0.00011 and 0.00014 (g/L), preferably of 0.00013 (g/L), pyridoxine hydrochloride at a concentration of 0.00011 and 0.00014 (g/L), preferably of 0.00013 (g/L), myo-Inositol at a concentration of 0.021 and 0.029 (g/L), preferably of 0.025 (g/L)and glycine at a concentration of 0.00043 and 0.00058 (g/L), preferably of 0.00050 (g/L).

The LS medium also comprises a combination of cytokinin and auxin hormones. Several mycorrhizal fungi produce compounds that are similar to plant hormones, such as auxins, cytokinins (CKs), gibberellic acids (GAs), ethylene (ET), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). The biosynthesis of such hormones is normally associated with plant host root modifications often required in these symbiotic interactions. Additionally, it has also been suggested that plant hormones, such as auxins and cytokinins, play a role in several physiological processes, for e.g. to break spore dormancy and promote spore germination, enhance hyphal development and nutrient uptake in fungi themselves.

Therefore, in the scope of the present invention adequate cytokinins are adenine-type cytokinins represented by kinetin, zeatin, and 6-benzylaminopurine (BAP), preferably BAP. Concerning auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-Naphthalene acetic acid (α-NAA) can be used in the preparation of the LS medium, preferably Indole-3-acetic acid (IAA). The effect of phytohormones on fungal physiology are greatly concentration-dependent and can differ strongly from one species to another. So, for the preparation of the LS medium, it is highly advisable that BAP and IAA do not exceed the proposed concentrations. Hence, in a preferred embodiment, the LS medium comprises a

combination of 6-benzylaminopurine (BAP) in the range of 0.0004 to 0.0006 (g/L), preferably of 0.0005 (g/L), and indole-3-acetic acid (IAA) in the range of 0.0004 to 0.0006 (g/L), preferably of 0.0005 (g/L). Finally, the LS medium can be solidified with a gelling agent, such as agar present in a concentration in the range of 7 to 15 (g/L), preferably 8.5 to 11.5 (g/L), even more preferably (10 g/L).

2. Process for isolating *Terfezia* spp. mycelium using LS medium

The process for isolation of *Terfezia* spp. comprises an initial step, whereby, a scalpel blade is used to cut the ascocarps and to expose the interior (glebal tissue) and a final step, by which a small (≈2 mm^3) piece from the central gleba, that contains a mixture of hyphae and spores, is plated (inoculation by direct transfer) onto a petri dishes (90 mm Ø) containing fresh LS medium. Afterwards, the glebal tissue may growth and can be periodically transferred (subcultured) onto new Petri dishes containing the same agar medium. If the mycelium maintains its morphological characteristics during the subculturing process, the isolation is successful, and a pure culture of that fungal species is stabilized. These stabilized mycelial pure cultures are hereafter designated isolates.

3. Process for maintaining *Terfezia* spp. mycelium isolates using LS medium

As soon as the mycelium pure cultures are stabilized, it becomes necessary to maintain their viability and purity by keeping the pure cultures free from contaminations. For maintaining *Terfezia* spp. mycelium isolates, pure cultures are transferred periodically onto fresh LS medium (subculturing) to allow the continuous growth and viability of the isolates. Inoculation of new LS medium plates should be made under a laminar air-flow cabinet, by direct transfer of 4-week-old mycelial discs cut from hyphal growth of a selected *Terfezia* spp. stabilized pure cultures. Petri dishes are then incubated in the dark at 25 ± 2°C for 90 days. The subculturing process has to be repeated periodically every three months, in order to maintain the viability of the isolates.

EXAMPLES

EXAMPLE 1. Preparation of LS culture media

LS culture media was prepared by mixing each compound listed in **Table 1** in 1 L of water or by adding predefined volumes of previously prepared stock solutions with known concentrations. However, direct weighing of some media components (e.g., micronutrients and vitamins) that are required only in milligram or microgram quantities in the final formulation may not be performed with sufficient accuracy, so, previous preparation of concentrated stock solutions and subsequent dilution into the final media is preferable to mixing each compound individually in 1 L of water. Accordingly, 1 litre of LS culture media was prepared by the following process:

1) The macronutrient stock solution [10x] concentrated was prepared by weighing 19 g of KNO3; 4.4 g of CaCL2.2H2O; 3.7 g of MgSO4.7H20; 1.7 g of KH2PO4 and 16.5 g of NH4NO3. Each component was then dissolved separately in a small amount of distilled water and added separately to an Erlenmeyer flask, while agitating without heating. Next, distilled water was continuously added to the Erlenmeyer flask, until the final volume of 1 Litre was reached. The macronutrient stock solution [10x] concentrated was then placed in an amber glass bottle to prevent photodecomposition and stored at 4 °C until further use.

2) The iron chelates stock solution [100x] concentrated was prepared by weighing 3.725 g of Na2EDTA.2H2O and 2.785 g of FeSO4.7H2O. Both components were then dissolved separately in a small amount of distilled water and added separately to an Erlenmeyer flask, while agitating without heating. Next, distilled water was continuously added to the Erlenmeyer flask, until the final volume of 1 Litre was reached. The iron chelates stock solution [100x] concentrated was then placed in an amber glass bottle to prevent photodecomposition and stored at 4 °C until further use.

3) The micronutrient stock solution [100x] concentrated was prepared by weighing 1.69 g of MnSO4.4H2O; 0.86 g of ZnSO4.7H2O; 0.0025 g of CuSO4.5H2O; 0.0025 g of CoCL2.6H2O; 0.025 g of Na2MoO4.2H2O; 0.62 g of H3BO3 and 0.083 g of KI. Each component was then dissolved separately in a small amount of distilled water and added separately to an Erlenmeyer flask, while agitating without heating. Next, distilled water was continuously added to the Erlenmeyer flask, until the final volume of 1 Litre was reached. The micronutrient

stock solution [10x] concentrated was then placed in an amber glass bottle to prevent photodecomposition and stored at 4 °C until further use.

4) The vitamins stock solution [100x] concentrated was prepared by weighing 0.01 g of thiamine hydrochloride; 0.05 g of nicotinic acid; 0.05 g of pyridoxine hydrochloride; 10 g of myo-Inositol and 0.2 g of glycine. Each component was then dissolved separately in a small amount of distilled water and added separately to an Erlenmeyer flask, while agitating without heating. Next, distilled water was continuously added to the Erlenmeyer flask, until the final volume of 1 Litre was reached. The vitamins stock solution [100x] concentrated was then divided in four 250 mL amber glass bottles and stored at 4 °C until further use.

5) The Cytokinin stock solution [1x] concentrated was prepared by weighing 1 g of 6-benzylaminopurine (BAP) and dissolving it in 1 litre of distilled water. The BAP stock solution [1x] concentrated was then divided in 500 (2 mL) Eppendorf safe-lock tubes and stored at 4 °C until further use.

6) The auxin stock solution [1x] concentrated was prepared by weighing 1 g of indole-3-acetic acid (IAA) and dissolving it in 1 litre of distilled water. The IAA stock solution [1x] concentrated was then divided in 500 (2 mL) Eppendorf safe-lock tubes and stored at 4 °C until further use.

7) After preparation of all stock solutions, 25 ml of the macronutrient stock solution [10x] was added to a measuring cup on a stirring plate. Next, 2.5 ml of the iron chelates stock solution [100x] was also added to the same measuring cup on the stirring plate. Then, 2.5 ml of the micronutrient stock solution [100x] was also transferred to the same measuring cup. Next, 2.5 ml of the vitamins stock solution [100x] was also added to the same measuring cup on the stirring plate. Afterward, 500 µL of the cytokinin stock solution [1x] and 500 µL of the auxin stock solution [1x] were also added to the same measuring cup with the aid of a micropipette. Next, 10 g of sucrose and 10 g of agar were weighted and placed into the same measuring cup. To make up the final volume of 1 litre, 966.5 ml of distilled water was measured using a graduated beaker and poured into the stirring mixture. Since the initial pH value of the stirring mixture was lower than 5.5 some drops of KOH (0.1 M) solutions were used to adjust the pH to the desired value (pH = 5.5).

Finally, the content of the measuring cup was poured into a 2 L Erlenmeyer flask, closed with aluminium foil. The LS culture media was then sterilized in an autoclave at 121 °C for 15 minutes. After cooling, to near 22 °C, the LS medium was distributed into Petri dishes (90 mm Ø; 25 mL each), under a laminar air-flow cabinet.

Table 1. Composition of LS culture medium

Class	Component	Quantity (g/L)
Carbon source	Sucrose	10.0
Macronutrients	KNO3	0.475
	CaCl2.2H2O	0.11
	MgSO4.7H2O	0.0925
	KH2PO4	0.0425
	NH4NO3	0.4125
Iron Chelates	Na2EDTA	0.00931
	FeSO4.7H2O	0.00696
Micronutrients	MnSO4.4H2O	0.004225
	ZnSO4.7H2O	0.00215
	CuSO4.5H2O	0.000006
	CoCl2.6H2O	0.000006
	Na2MoO4.2H2O	0.00006
	H3BO3	0.00155
	KI	0.00021
Vitamins	Thiamine hydrochloride	0.000025
	Nicotinic acid	0.000125
	Pyridoxine Hydrochloride	0.000125
	myo-Inositol	0.025
	Glycine	0.0005
Phytohormones	6-Benzylaminopurine (BAP)	0.0005
	Indole-3-acetic acid (IAA)	0.0005
Others	Agar	10.0
	pH	5.5

EXAMPLE 2. Effect of LS culture medium vs. conventional culture media on *Terfezia* spp. isolation

Fresh *Terfezia* ascocarps were harvested from different locations in the Centre and Southern Portugal, between February 2007 and April 2007 and brought to the laboratory. Upon arrival, all fresh ascocarps were washed in tap water, brushed free of adhering soil particles and cleaned superficially with ethanol (70%). Afterward, each ascocarp was sorted by species and maturity stage and the best-preserved specimens of each collection were selected for isolation. In total, 8 specimens of each species (*T. arenaria, T. fanfani*, *T. extremadurensis*, *and T. pini*) were used in the experiment. In a laminar air-flow cabinet, these ascocarps were then carefully broken, opened and small pieces (1–2 mm across) of glebal tissue (containing both hyphae and spores) were aseptically excised from the inner part of the fruit bodies with a

scalpel and plated (inoculated by direct transfer) onto Petri dishes (90 mm Ø) containing the LS medium or one of the semi-synthetic and synthetic conventional media listed in **Table 2**. All media pH was adjusted to pH = 5.5 and three replicate plates from each treatment (medium) were inoculated, totalling 30 petri dishes per specimen. The Petri dishes were then labelled, sealed with parafilm and placed on an incubation chamber, in the dark at 25 ± 2°C for 90 days. As for the remaining fragments of each specimen, half were frozen at -20 °C for further DNA characterization and the remaining half were dried at 40 °C and stored in sealed plastic bags, labelled with collection details and deposited at the Évora University Herbarium (UEVH-FUNGI), Portugal.

Table 2. Conventional culture media used for isolation and cultivation of *T. arenaria*

Type of media	Name	Composition per litre
Semi-synthetic	**Hagen/Modess**	Glucose 5 g, malt extract 5 g, KH_2PO_4 0.5 g, NH_4CL 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeCL_3$ (1%) 0.5 mL, Thiamine 50 g and agar 15 g
	Malt agar (1%) (MA)	Malt extract 10 g, agar 15 g
	K.I.S.R.	Malt extract 10 g, Peptone 6.5 g, KNO_3 0.13 g, $Ca(NO_3)$ 0.55 g, Agar 15 g
	Biotin-aneurin-folic acid agar (BAF)	Glucose 30 g, yeast extract 0.2 g, peptone 2 g, $CaCl_2.2H_2O$ 0.1 g, NaCl 0.025 g, KH_2P0_4 0.5 g, $MgS0_4.7H_2O$ 0.5 g, $FeCl_3.7H_2O$ 10 mg; $MnS0_4$ 5 mg, $ZnS0_4.7H_2O$ 1 mg, thiamine 0.05 mg; folic acid 0.1 mg; inositol 50 mg; biotin 0.001 mg; agar 15 g
	Nutrient agar (NA)	Meat extract 1 g, yeast extract 2 g, peptone 5 g, NaCL 5 g, agar 15 g
	Sabouraud Dex agar (SDA)	Glucose 40 g, Peptone from casein 5 g, Peptone from Meat 5 g, Histidin 1 g, Lecithine 0.70 g, Polysorbate 80 5 g, $Na_2S_2O_3$ 0.5 g, Agar 15 g
	Tryptic Soy Agar (TSA)	Peptone from casein 15 g, Soya Peptone 5 g, NaCL 5 g, agar 15 g
	Fontana	Glucose 6.67 g, Peptone 1.67 g, Casein hydrolysate 0.33 g, KH_2P0_4 0.33 g, $CaCl_2$ (1%) 1.67 ml, $MgS0_4$ (1%) 0.167 ml, $MnSO_4$ (1%) 0.167 ml, $ZnSO_4$ (1%) 0.167 ml, $FeCl_3$ (1%) 0.167 ml, Thiamine 0.033 g, Agar 15 g
Synthetic	**Modified Melin-Norkrans (MMN)**	Glucose 10 g, $CaCl_2$ 0.05 g, NaCl 0.025 g, $NH_4.2HPO_4$ 0.25 g, KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.15 g, $FeCl_3$ 0.012 g, Thiamine 0.0001 g, agar 15 g

After inoculation, all plates were checked weekly and, when needed, transferred onto new Petri dishes containing the same agar medium (subculture), until the end of the trial (90 days after). The isolation success rate of each medium was then calculated as the number of strains successfully isolated divided by the total number of strains. The data summarized in **Fig.1** show that the LS medium led to the successful isolation of 20 *Terfezia* strains from a total of 32, which represents a success rate of near 60%. Regarding the conventional media tested, only MMN medium was effective for the isolation of the tested *Terfezia* strains, but with a considerably lower, only 6%, success rate. Furthermore, while all four *Terfezia* species were able to form colonies in LS medium (**Fig.2**), only *T. fanfani* and *T. arenaria* were isolated on MMN medium, the first with an isolation percentage of 10% and the second of 6%.

EXAMPLE 3. Effect of LS culture medium vs. conventional culture medium on growth and maintenance of *Terfezia* spp. mycelium

Mycelial growth of two *Terfezia* isolates were evaluated on 10 different culture media (LS medium or one of the media listed in **Table 2**; all with pH = 5.5). For the trial, two *Terfezia* strains were randomly selected from a pool of *Terfezia* isolates: *T. arenaria* strain Ta195 (UEVH-FUNGI 2003875) and *T. fanfani* strain Tf235 (UEVH-FUNGI 2004080, both 4-week-old. The above cited strains were successfully isolated, following the process described in the **example 2**, on LS medium prepared as described in **example 1**, prior to this assay. The subsequent process was repeated for each *Terfezia* strain: under a laminar air-flow cabinet, one mycelial disc (5 mm Ø) was cut and removed from the edge of a colony of Ta195 isolate, and transferred into the centre of a Petri dish (90 mm Ø) containing one of the tested media. The process was repeated 10 times for each culture media, totalling 100 petri dishes per *Terfezia* isolate. All Petri dishes were then labelled, sealed with parafilm and placed on an incubation chamber, in the dark at 25 ± 2°C. *Terfezia* mycelial growth was measured weekly as the average of the two perpendicular diameters of the colony. Measurements were made at the bottom of each Petri dish to avoid visual distortion.

Results show that only the LS and MMN media were able to support any mycelial growth of *T. arenaria* and *T. fanfani*. Furthermore, significant statistical differences were found between mean colony diameters for both *T. arenaria* and *T fanfani*, growing in LS and MMN media. As for the *T. arenaria* strain, mycelia grew more vigorously in the LS media than in MMN, reaching a mean colony diameter at week 7 of 2.76 cm and a maximum mean colony diameter

at week 13 of 5.96 cm in the LS medium (**fig. 3**). The same pattern was observed for *T. fanfani* (**fig. 4**) which achieved a mean colony diameter at week 7 of 2.40 cm and a maximum mean colony diameter at week 13 of 3.24 cm in the LS medium. Navarro-Ródenas and collegues (2011) using MMN medium have obtained a maximum colony diameter of 1.32 cm for *T. claveryi* at week 7. Hence, our results clearly show that the new LS medium represents an improvement in relation to the standard MMN medium, the only fully synthetic media, thus far, known to support the growth of *Terfezia* spp. mycelia.

CLAIMS

1. A **synthetic culture media (LS)** *Terfezia* spp. comprising:

   - **sucrose** and/or **glucose** as carbon source at a concentration in the range of 5 to 15 (g/L), preferably of 7.5 to 15 (g/L), even more preferably of 10 (g/L);

   the following macronutrients:

   - KNO_3 at a concentration of 0.40 and 0.55 (g/L), preferably of 0.475 (g/L);
   - NH_4NO_3 at a concentration of 0.35 and 0.48 (g/L), preferably of 0.413 (g/L);
   - $CaCl_2.2H_2O$ at a concentration of 0.093 and 0.13 (g/L), preferably of 0.11 (g/L);
   - $MgSO_4.7H_2O$ at a concentration of 0.093 and 0.106 (g/L), preferably of 0.079 (g/L);
   - KH_2PO_4 at a concentration of 0.036 and 0.049 (g/L), preferably of 0.043 (g/L);

   the following micronutrients:

   - iron chelates Na_2EDTA at a concentration in the range of 0.0079 to 0.0107 (g/L), preferably of 0.0093 (g/L), and $FeSO_4.7H_2O$ in the range of 0.0059 to 0.0080 (g/L), preferably of 0.0070 (g/L);
   - $MnSO_4.4H_2O$ at a concentration of 0.0036 and 0.0049 (g/L), preferably of 0.0042 (g/L);
   - $ZnSO_4.7H_2O$ at a concentration of 0.0018 and 0.0025 (g/L), preferably of 0.0022 (g/L);
   - $CuSO_4.5H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L);
   - $CoCl_2.6H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L);

- $Na_2MoO_4.2H_2O$ at a concentration of $5.1x10^{-6}$ and $6.9x10^{-6}$ (g/L), preferably of $6.0x10^{-6}$ (g/L);
- H_3BO_3 at a concentration of 0.0013 and 0.0018 (g/L), preferably of 0.0016 (g/L), and
- KI at a concentration of 0.00018 and 0.00024 (g/L), preferably of 0.0016 (g/L),

the following vitamins and growth factors:

- thiamine hydrochloride at a concentration of $2.1x10^{-5}$ and $2.9x10^{-5}$ (g/L), preferably of $2.5x10^{-5}$ (g/L),
- nicotinic acid at a concentration of 0.00011 and 0.00014 (g/L), preferably of 0.00013 (g/L),
- pyridoxine hydrochloride at a concentration of 0.00011 and 0.00014 (g/L), preferably of 0.00013 (g/L),
- myo-Inositol at a concentration of 0.021 and 0.029 (g/L), preferably of 0.025 (g/L), and
- glycine at a concentration of 0.00043 and 0.00058 (g/L), preferably of 0.00050 (g/L),

and the following phytohormones:

- cytokinins of the adenine-type cytokinins selected from kinetin, zeatin, and 6-benzylaminopurine (BAP), preferably BAP, and
- the auxins selected from indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-Naphthalene acetic acid (α-NAA), preferably Indole-3-acetic acid (IAA).

2. A **synthetic culture media (LS)** according to claim 1 wherein the carbon source is sucrose.

3. A **synthetic culture media (LS)** according to claim 1 or 2 wherein the phytohormones are 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) and are present in the following concentration:

   - 6-benzylaminopurine (BAP) in the range of 0.0004 to 0.0006 (g/L), preferably of 0.0005 (g/L), and
   - indole-3-acetic acid (IAA) in the range of 0.0004 to 0.0006 (g/L), preferably of 0.0005 (g/L).

4. A **synthetic culture media (LS)** according to any of the claims 1 to 3 further comprising

a gelling agent, preferably agar at a concentration of 7 to 15 (g/L), preferably of 8.5 to 11.5 (g/L), more preferably of 10 (g/L).

5. A **process of isolating *Terfezia* spp. mycelium** comprising placing a part of the central gleba tissue of an ascocarp of *Terfezia* spp. onto a cultivation recipient containing LS culture medium, as described in any of the claims 1 to 4.

6. A **process of maintaining *Terfezia* spp. mycelium isolates** comprising the following steps:
   a) direct transference of 4-week-old mycelial discs cut from colonies of selected *Terfezia* spp. stabilized pure cultures onto LS medium, as described in any of the claims 1 to 4,
   b) incubation of the cultures of (a) in the dark at 25 ± 2 ºC for approximately 90 days,
   c) repeating the steps (a) and (b) every 3 months.

DESCRIPTION OF THE FIGURES

Fig.1 is a diagram showing the number of isolated *Terfezia* specimens per culture media tested, wherein it is possible to see that LS medium led to a successful isolation of 20 *Terfezia* strains from a total of 32, which represents a success rate of near 60%. Regarding the conventional media tested, only MMN medium was effective for the isolation of the tested *Terfezia* strains, but with a considerably lower, only 6%, success rate.

Fig. 2 is a diagram showing the percentages of isolated strains using the new LS medium per *Terfezia* species, wherein it is possible to see that all four *Terfezia* species were able to form colonies in LS medium, whilst only *T. fanfani* and *T. arenaria* were isolated on MMN medium, the first with an isolation percentage of 10% and the second of 6%.

Fig. 3 shows mycelial growth (cm) for *T. arenaria* (strain Ta195) over a period of 13 weeks on 10 different culture media, wherein it is possible to see that mycelia grew more vigorously in the LS media than in MMN, reaching a mean colony diameter at week 7 of 2.76 cm and a maximum mean colony diameter at week 13 of 5.96 cm in the LS medium.

Fig. 4 shows mycelial growth (cm) for *T. fanfani* (strain Tf235) over a period of 13 weeks on 10 different culture media, wherein it is possible to see that mycelia grew more vigorously in the LS media than in MMN, achieving a mean colony diameter at week 7 of 2.40 cm and a maximum mean colony diameter at week 13 of 3.24 cm in the LS medium.

Fig. 1

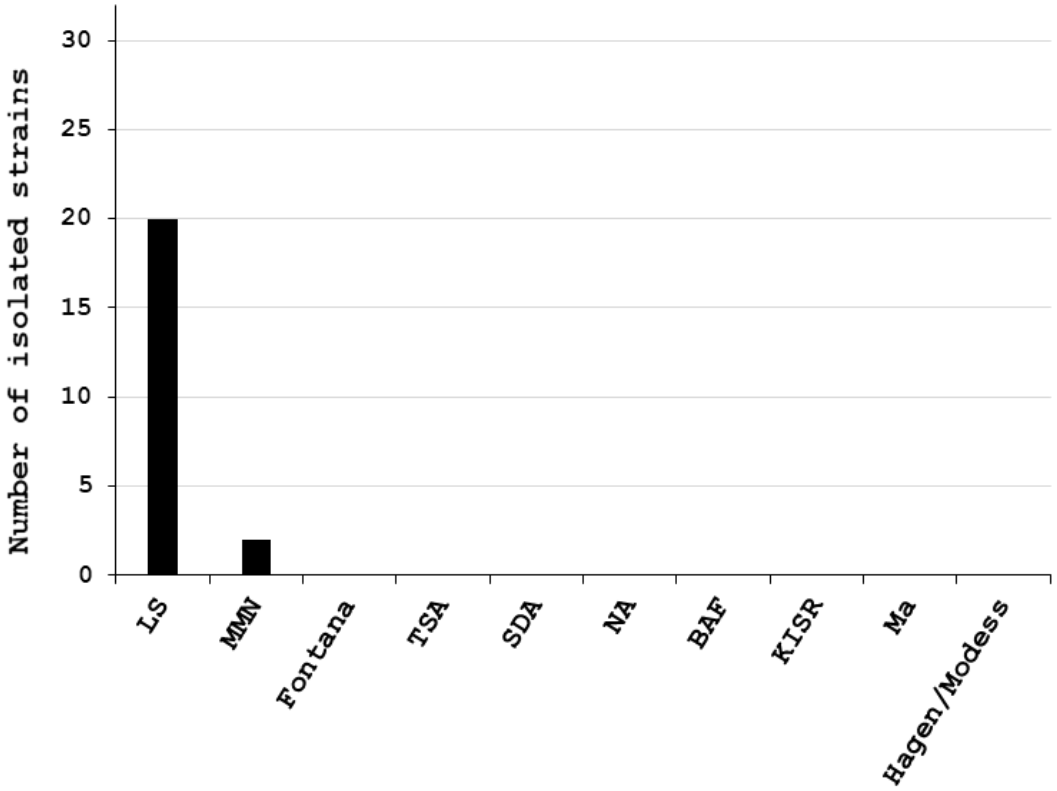

Number of isolated strains
30
25
20
15
10
5
0
LS
MMN
Fontana
TSA
SDA
NA
BAF
KISR
Ma
Hagen/Modess

Fig. 2

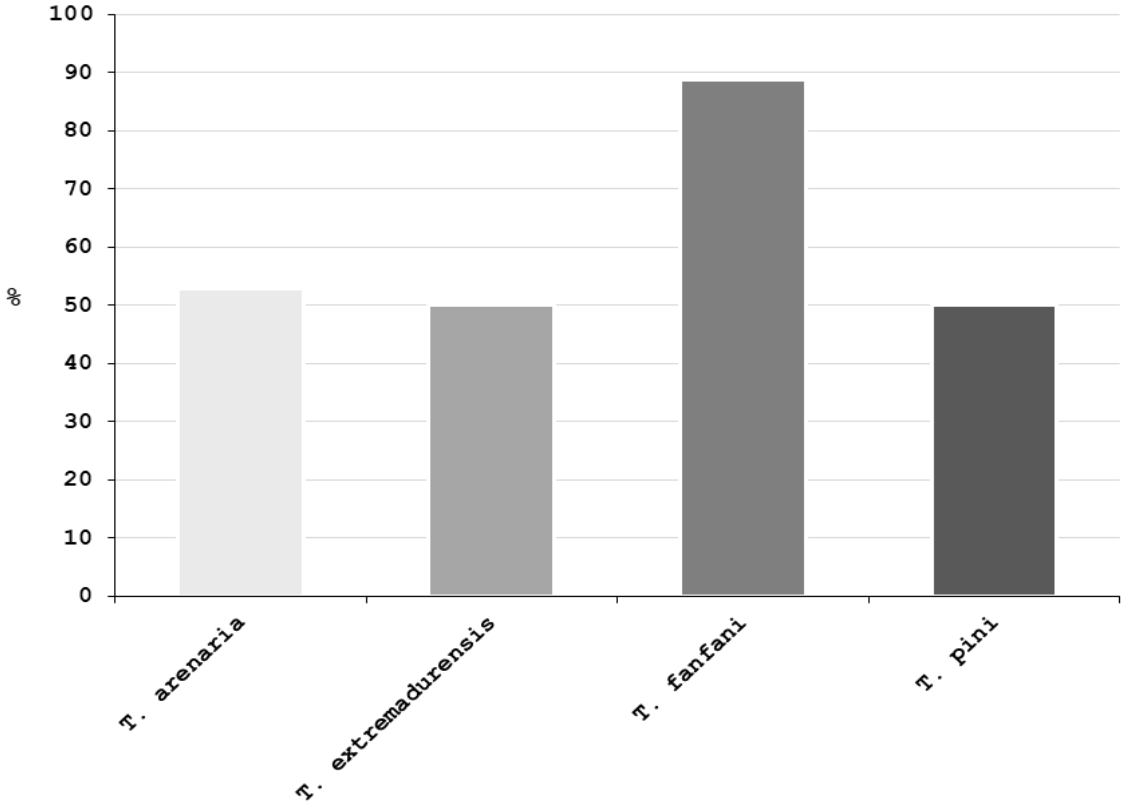

100
90
80
70
60
50
40
30
20
10
0
%
T. arenaria
T. extremadurensis
T. fanfani
T. pini

Fig.3

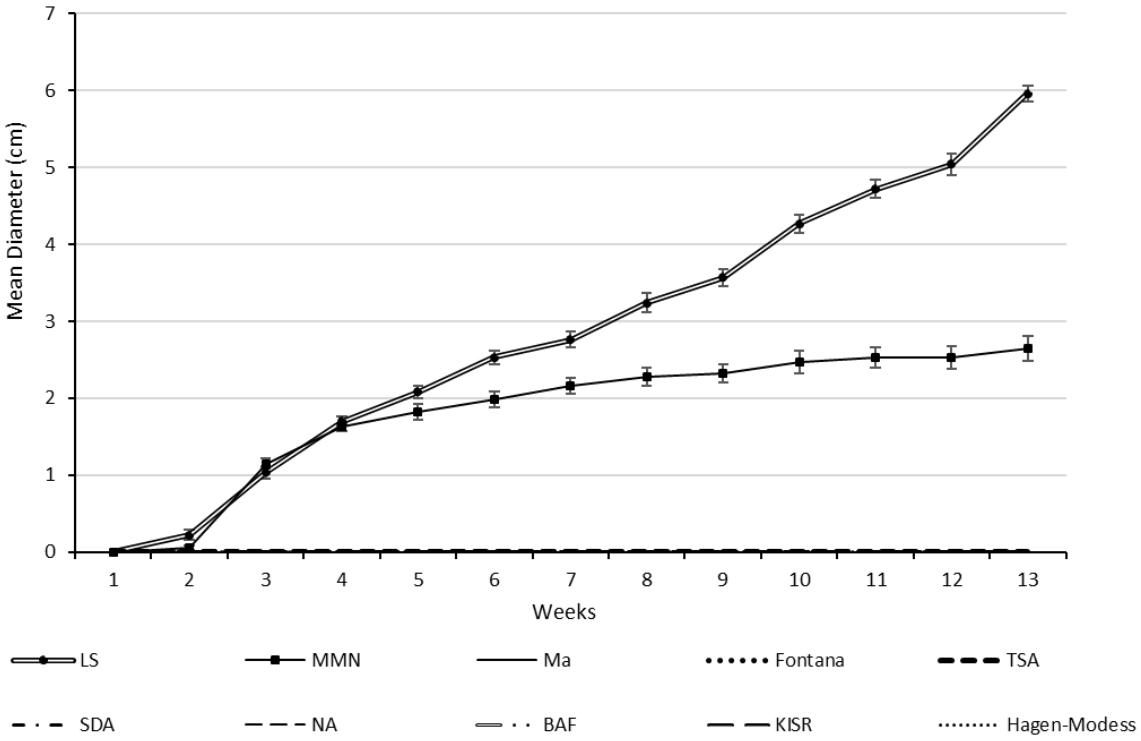
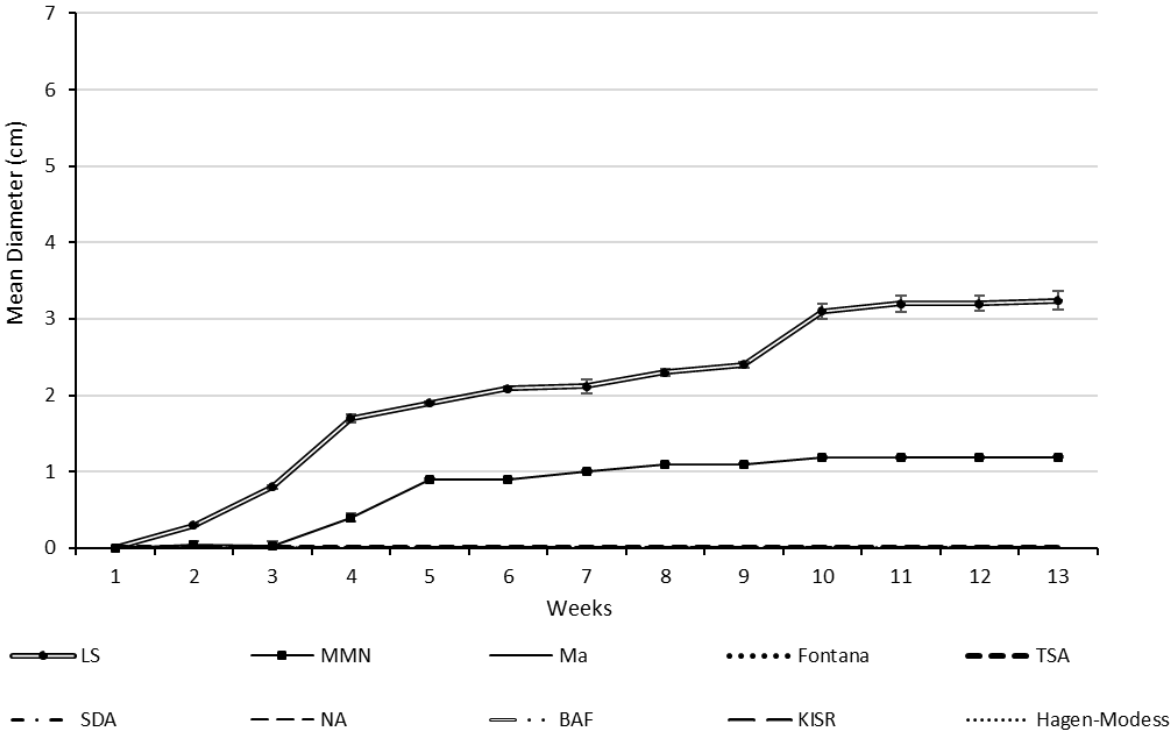

Mean Diameter (cm)
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
11
12
13
Weeks
LS
MMN
Ma
Fontana
TSA
SDA
NA
BAF
KISR
Hagen-Modess

Fig. 4


Mean Diameter (cm)
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
11
12
13
Weeks
LS
MMN
Ma
Fontana
TSA
SDA
NA
BAF
KISR
Hagen-Modess

CHAPTER VI

Morphologic characterization of the *in vitro mycorrhizae* formed between four *Terfezia* species (*Pezizaceae*) with *Cistus salviifolius* L. and *Cistus ladanifer* L.

to be submitted to the journal *Mycorrhiza*

ABSTRACT

Terfezia species (desert truffles) are obligate symbiotic partners of several xerophytic host plants, mainly belonging to the *Cistaceae*. Yet, their mycorrhizal associations with members of the genus *Cistus* remains poorly characterized and their potential application in desert truffle cultivation remains unexplored. This work provides the first anatomic descriptions of the *mycorrhizae* formed *in vitro* by four *Terfezia* species (i.e *T. arenaria*; *T. extremadurensis*; *T. fanfan*i, *T. cistophila*) with *C. ladanifer* and *C. salviifolius*, two of the most widespread and common *Cistus* species in acid soils. All the tested associations resulted in the formation of ectomycorrhizas with well-developed Hartig net, but with varying degrees of mantle development. Additionally, our results demonstrate that all eight *Terfezi*a-*Cistus* combinations expressed high rates of mycorrhization, however, *T. arenaria* showed significantly lower colonization rates than the other 3 mycosymbionts and *C. salviifolius* displayed a slightly better response to infection, by *Terfezia* species, that *C. ladanifer*.
In summary, the present work reports on the extraordinary compatibility of *Cistus salviifolius* and *Cistus ladanifer* with various *Terfezia* species and brings forth new data about these associations which might aid in broadening the number of situations whereby *Terfezia* spp. can be cultivated.

Keywords: Desert truffles, *Terfezia*, Mycorrhiza morphology, *In vitro* synthesis

INTRODUCTION

The term “desert truffle” commonly designate the fruitbodies produced by edible hypogeous *Ascomycota* (*Pezizaceae*), which include several species of the genera *Terfezia*, *Tirmania* and *Picoa,* found in arid and semi-arid areas throughout the world (Moreno et al., 2014; Morte et al., 2008; Navarro-Ródenas et al., 2011). These fungi establish key mutualistic associations in arid and semiarid ecosystems with the roots of several xerophytic host plants (Pérez-Gilabert et al., 2014), mainly belonging to the *Cistaceae* (e.g. *Helianthemum* spp. and *Cistus* spp.) but also of the *Fagaceae* and *Pinaceae* (i.e. oaks and pines) (Alsheikh, 1994; Díez et al., 2002; Fortas & Chevalier, 1992; Kagan-Zur & Roth-Bejerano, 2008; Morte et al., 2008).
Many *Terfezia* species are among the most prized desert truffle species; Thus, in the last decades, numerous research efforts were made in order to enable their large-scale cultivation

(Morte et al., 2008). Still, desert truffle cultivation is only now leaving its infancy, and our knowledge on the ecology, physiology and biochemistry of many *Terfezia* mycorrhizal associations still remains fragmentary (Kagan-Zur & Roth-Bejerano, 2008). One key issue that remains neglected is the choice of suitable putative hosts. In fact, so far, the only host plants tested in experimental desert truffle cultivation are perennial and annual species of *Helianthemum* and little or no attention has been given to the assessment of new potential hosts for desert truffle cultivation (Morte & Andrino, 2014). For instance, *Cistus* species have also been proposed as excellent candidates to increase the types of soil and number of places where desert truffles can be cultivated due to their wide environmental and ecological requirements (Giovannetti & Fontana 1982). In fact, the genus *Cistus* L. (*Cistaceae*) includes about 20 perennial shrub species, distributed throughout the Mediterranean region and Canary Islands. In the Iberian Peninsula the genus is represented by 12 species, all belonging to primary successional stages of many forest stands, growing readily in degraded areas or after disturbances such as fire (Águeda et al., 2006; Nuytinck et al., 2004), rendering their ectomycorrhizal ecology particularly interesting in the context of global warming and increasing desertification, in arid and semiarid areas worldwide. Also, more than 200 fungal species, belonging to 40 genera, have been reported to be associated with *Cistus* plants, among which several edible hypogeous *Ascomycota* mainly included in *Tuber* and *Terfezia* genera (Comandini et al., 2006).

Yet, despite the wide distribution of *Cistus* species in the Mediterranean basin and the awareness of their relevance as putative host for many *Terfezia* species, very few studies have been undertaken to characterize and describe these mutualistic associations (**Table 1**). Remarkably, various *Terfezia*-*Cistus* associations were not experimentally verified so far. Furthermore, with the increase of new described *Terfezia* species in recent years (reviewed in Louro et al. 2019), some of them *Cistus* specific (i.e. *Terfezia cistophila*), it is nowadays more crucial than ever to expand the current knowledge on these associations.

As a whole*, Terfezia* mycorrhizae are known to display great structural versatility depending on certain factors (i.e. host species, concentration of auxins secreted by the fungi, root sensitivity to those auxins, phosphate concentrations and drought conditions; Roth-Bejerano et al., 2014; Zitouni-Haouar et al., 2014). Research on the association of *Terfezia* and various *Cistaceae* (mostly perennial and annual species of *Helianthemum*) demonstrate the remarkable adaptability of these associations, which can result in the formation of a)

endomycorrhizas characterized by undifferentiated coil-shaped or globular intracellular hyphae penetrating the plant cells (Awameh, 1981; Gutiérrez et al., 2003; Kagan-Zur et al., 1999; Slama et al., 2010); b) ectomycorrhizas, characterized by a Hartig net, but without a true sheath (Dexheimer et al., 1985; Gutiérrez et al., 2003; Roth-Bejerano et al., 1990); and c) ectendomycorrhizas, characterized by the presence of both intercellular Hartig net and intracellular hyphae penetrating the cortex cells (Navarro-Ródenas et al., 2012, 2013).

Table 1. List of *in vivo* and *in vitro* mycorrhizal synthesis obtained so far between *Terfezia* spp. and *Cistus* spp..

Species	Putative host	References
T. leptoderma s.l.	*Cistus albidus*	Chevalier et al. (1984)
		Leduc et al. (1986)
		Zitouni-Haouar et al. (2014)
	Cistus incanus	Zitouni-Haouar et al. (2014)
	Cistus monspeliensis	Chevalier et al. (1984)
	Cistus salviifolius	Chevalier et al. (1984)
		Leduc et al. (1986)
		Zitouni-Haouar et al. (2014)
T. boudieri	*Cistus albidus*	Alsheikh (1994)
		Zitouni-Haouar et al. (2014)
	Cistus incanus	Zaretsky et al. (2006)
	Cistus salviifolius	Zitouni-Haouar et al. (2014)
T. claveryi	*Cistus albidus*	Alsheikh (1994)
		Zitouni-Haouar et al. (2014)
	Cistus incanus	Zitouni-Haouar et al. (2014)
	Cistus salviifolius	Zitouni-Haouar et al. (2014)

Lately, it has been observed that in some instances more than one of the above mycorrhizal types may be observed along the same root system of a single *Helianthemum* plant, a phenomenon that has been named “ectendomycorrhiza continuum” (Navarro-Ródenas et al., 2012). On the other hand, *Terfezia*-Cistus mycorrhizae seem to be consistently morphologically characterized as ectomycorrhizas with well-developed Hartig net, however, the presence or absence of a true sheath is still subject of debate (Alsheikh, 1994; Chevalier et al., 1984; Leduc et al., 1986; Zaretsky et al., 2005; Zitouni-Haouar et al., 2014). While most *in vitro* and *ex vitro* synthesis, enumerated in (**Table 1**), resulted in ectomycorrhizas with well-developed Hartig net but without a true mantle (Alsheikh 1994) a more recent work provided evidences on the formation of ectomycorrhizas with a thin less-developed sheath using *C. salvifolius*, *C. albidus*, *C. incanus* and three different *Terfezia* species (Zitouni-Haouar et al. 2014).

In view of these new evidences, the main goal of the present work is to provide new insights on the association between *Terfezia* species and *Cistus* spp. by reporting on the mycorrhizae formed by four *Terfezia* species -namely *T. arenaria*; *T. extremadurensis*; *T. fanfani*, *T. cistophila*- along with *C. ladanifer* and *C. salviifolius*, two of the most widespread and common *Cistus* species in acid soils. Furthermore, we aim to assess which of the above *Terfezia-Cistus* combinations are the most compatible, and open the possibility of mass production of *Terfezia* mycorrhized seedlings towards desert truffle cultivation in acid soils.

MATERIAL AND METHODS

Fungal material

Mature *Terfezia* ascocarps were harvested from different locations in the Centre and Southern Portugal, between February 2017 and April 2019. *T. arenaria* (Moris) Trappe strain Ta195 (UEVH-FUNGI 2003875), *T. extremadurensis* Muñoz-Mohedano, Ant. Rodr. & Bordallo strain Te271 (UEVH-FUNGI 2004569), *T. fanfani Matt.* strain Tf235 (UEVH-FUNGI 2004080) and *Terfezia pini Bordallo, Ant. Rodr. & Muñoz-Mohedano* strain Tp278 (UEVH-FUNGI 2004577) mycelia were isolated on LS medium (Louro & Santos-Silva, 2020) [in $mg.l^{-1}$ 475 KNO_3; 110 $CaCl_2.2H_2O$; 92,5 $MgSO_4.7H_2O$; 42.5 KH_2PO_4; 412 NH_4NO_3; 9.31 Na2EDTA; 6.96 $FeSO_4.7H_2O$; 4.22 $MnSO_4.4H_2O$; 2.15 $ZnSO_4.7H_2O$; 0.006 $CuSO_4.5H_2O$; 0.006 $CoCl_2.6H_2O$; 0.06 $Na_2MoO_4.2H_2O$; 1.55 H_3BO_3; 0.21 KI; 0.025 Thiamine hydrochloride; 0.125 Nicotinic acid; 0.125 Pyridoxine Hydrochloride; 25 Myo-Inositol; 0.5 Glycine; 0.5 6-Benzylaminopurine (BAP); 0.5 Indole-3-acetic acid (IAA)] with Sucrose 10 $g.l^{-1}$ and solidified with 10 $g.l^{-1}$ of agar and pH 5.5. All isolates were incubated in the dark at 25 ± 2 °C for 90 days. As for the remaining fragments of each specimen, half were frozen at -20 °C for further DNA characterization and the remaining half were dried at 40 °C and stored in sealed plastic bags, labelled with collection details and deposited at the Évora University Herbarium (UEVH-FUNGI), Portugal.
Molecular characterization was carried out by sequencing fragments of the nuclear ribosomal DNA region of both *Terfezia* ascocarps and mycelial cultures. DNA extractions were performed by a modified CTAB method (Nobre et al. 2018). The Internal Transcribed Spacer (ITS) region of the rDNA, including the 5.8S ribosomal gene, was amplified using the ITS5 and

ITS4 primers (White et al. 1990). Amplifications of ITS rDNA sequences were performed using a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: an initial denaturalization step for 3 min at 95 °C, followed by 35 cycles consisting of: 30 s at 95 °C, 30 s at 95 °C (annealing temp.), 1 min at 72 °C, and a final extension at 72 °C for 10 min. All reagents were acquired from NZYTech, Lda, sequencing was done commercially (STAB VIDA, Lda.) and all sequence alignments were performed with online MAFFT version 7, using the E-INS-i strategy (Katoh et al., 2017). Molecular identification was carried out by comparing our sequences with the existing ones in the GenBank database.

Plant material

Seeds from *Cistus salviifolius* L. and *Cistus ladanifer* L. plants growing in Herdade da Mitra, near Évora (Alentejo, Portugal) (38°32'N; 8°01'W; 220 m a.s.l.), were collected on November 2013 in a Montado area with natural shrub undercover dominated by *Cistus* spp. The area belongs to the Mediterranean pluviseasonal-oceanic bioclimate and is located in the low mesomediterranean bioclimatic belt. It has a dry to subhumid ombrotype with a mean annual temperature ranging from 9.2 °C to 21.5 °C and a mean annual rainfall of 664.6 mm (INMG, 1991; Rivas-Martínez, 2005). The collected *Cistus* seeds were dried at 23 °C in a Memmert forced ventilation oven (Model 600) and kept at room temperature in the dark until use. The seeds were surface sterilized by immersion in 70 % Ethanol for 2 min., followed by another immersion in a 50 % commercial bleach solution for 10-15 minutes. Afterwards, seeds were washed three times in sterilized tap water. To break seed dormancy seeds were heated at 150 °C in a bi-distilled water bath for 5 min. and left to cool down until room temperature was reach. Seeds were then placed in Petri dishes, on top of filter paper moistened with bi-distilled water, and kept in a growth chamber in the dark with 24 °C /21°C (±1 °C) day/night temperature. After germination, *C. salviifolius* and *C. ladanifer* seedlings were routinely micropropagated using the *Cistus* rapid multiplication protocol described in Louro et al. (2017).

Mycorrhizal synthesis

Mycorrhizal synthesis was performed in polypropylene transparent microboxes (90 mm Ø and 120 mm in height) with filtered polypropylene covers. Each box containing 200 ml of dried vermiculite and

100 ml of LS liquid medium was autoclaved at 121 °C and 18 psi for 20 min. After a week (to check for possible contaminants) all boxes were inoculated with 2 plugs dissected from the pre-cultivated *Terfezia* strains and incubated in the dark (25 ± 2 °C). Two weeks later, one rooted *Cistus* micropropagated plantlet was introduced in each box, near the active growing mycelia (**Fig. 1**).

Figure 1. *In vitro* mycorrhizal system. **a)** detail of micropropagated *C. ladanifer* inoculated with *T. arenaria* mycelium; **b)** detail of micropropagated *C. salviifolius* inoculated with *T. arenaria* mycelium.

The boxes were then placed in a grow chamber at 24 °C/ 21 °C (±1 °C) day/night temperature and 15 h light period, under cool white fluorescent light (36 µmol-m-2s^{-1}). Mycelium pure cultures of *T. arenaria* (strain Ta195), *T. extremadurensis* (strain Te271), *T. fanfani* (strain Tf235) and *Terfezia pini* (strain Tp278) were used in the experiment. Sixteen microboxes (eight containing *C. salviifolius* plantlets and eight containing *C. ladanifer* plantlets) were inoculated by each of the four *Terfezia* species, totaling sixty four boxes. After two months, the *Cistus* plantlets were carefully retrieved from the growing medium and their roots gently washed to free them from adhering particles. The whole root system of each *Cistus* plantlets was separated from the aerial part, kept in 50 ml centrifuge tubes filled with a glutaraldehyde solution (4%) and stored at 5 °C until further examination.

Mycorrhizal morphotyping and colonization assessment

Each *Cistus* plantlets root system was washed over a 2 mm sieve and cut into segments of approximately 1 cm in length. Afterwards the root segments were spread in 2 Petri dishes containing bi-distilled water and all root tips were observed under a Stereo Microscope (WILD

M3) to determine the existent morphological types. Characterization of the mycorrhizal root tips follows Agerer (1987–2002, 1991). Prior to microscopic observation all roots fragments were cleared with a 10 % KOH solution and stained with 0.1 % trypan blue in lactophenol following the method developed by Phillips and Hayman (1970). Microscopic examination of the root fragments and characterization of the mycorrhizal system under light microscope was done, using a Leica DM750 microscope equipped with a digital camera (Leica ICC50W), according to the methodology described in Brundrett et al. (1996). The percentage of fungal root colonization was estimated based on the frequency of infection expressed by: FI (%) =100 (N−N0)/N., Where N is the total number of observed root fragments and N0 is the number of root fragments uninfected (Trouvelot et al. 1986).

Statistical analysis

Data normality was assessed using Kolmogorov-Smirnov tests. Square root and logarithmic transformations were used to achieve normality when necessary. Levene's tests were employed to assess the variance homocedasticity assumption. Mean differences in frequency of infection between plant hosts and different *Terfezia* isolates were tested through a two-way ANOVA followed by Tukey post hoc tests. All calculations were performed with IBM SPSS Statistics V 24.

RESULTS

All *Cistus* plantlets, irrespective of the host plant–fungal species combination, formed mycorrhizal associations with all the *Terfezia* isolates after 2 months using the mycorrhizal system described in material and methods section. Concerning the macroscopic morphological characterization of the mycorrhizal root tips, a single morphotype was produced by every host plant–fungal species combination (**Fig. 2**). Under the stereomicroscope, mycorrhizae are unbranched, unramified ends are straight to bent, more or less inflated, sometimes with a more enlarged apex (club shaped). Surface of unramified ends is smooth, color varies from brownish-yellow to rusty-brown ochre, with slightly darker tones on aged mycorrhizae. Emanating hyphae are infrequent, white and shiny. No rhizomorphs were observed.

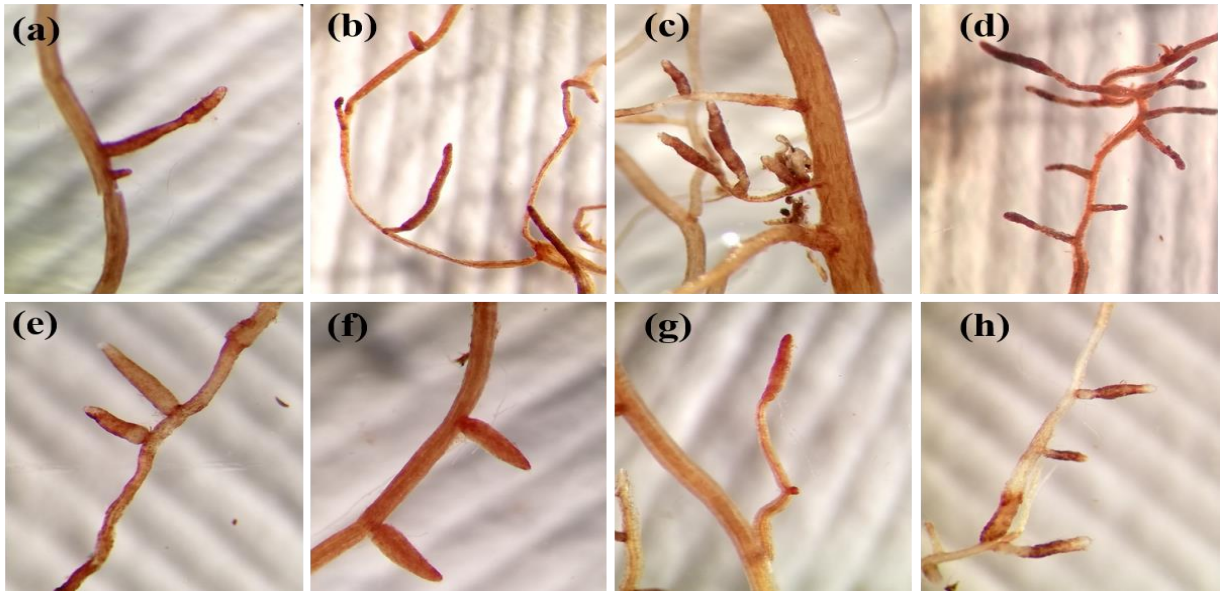


Figure 2. External characteristics of *Terfezia-Cistus* mycorrhizae: **(a)** *T. arenaria* x *C. salviifolius* mycorrhizae; **(b)** *T. extremadurensis* x *C. salviifolius* mycorrhizae; **(c)** *T. fanfani* x *C. salviifolius* mycorrhizae; **(d)** *T. pini* x *C. salviifolius* mycorrhizae; **(e)** *T. arenaria* x *C. ladanifer* mycorrhizae; **(f)** *T. extremadurensis* x *C. ladanifer* mycorrhizae; **(g)** *T. fanfani* x *C. ladanifer* mycorrhizae; **(h)** *T. pini* x *C. ladanifer* mycorrhizae.

Additional microscopic examination of the root fragments revealed that all four *Terfezia* species (i.e. *T. arenaria*, *T. fanfani*, *T. extremadurensis* and *T. pini*) formed ectomycorrhizas with a well-developed Hartig net but with varying degrees of mantle development (**Fig. 3-5**), both with *C. salviifolius* and *C. ladanifer*.

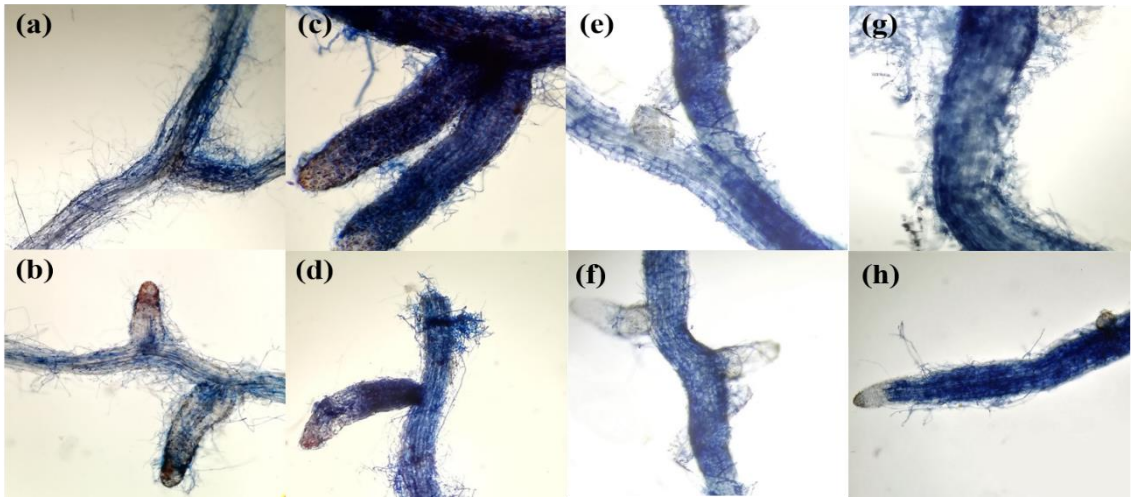


Figure 3. Light microphotographs of *Terfezia-Cistus* mycorrhizal roots, (400x): **(a)** *T. arenaria* x *C. salviifolius* root showing rudimentary sheath; **(b)** *T. arenaria* x *C. ladanifer* root showing rudimentary sheath; **(c)** *T. extremadurensis* x *C. salviifolius* root surrounded by a well-developed sheath ; **(d)** *T. extremadurensis* x *C. ladanifer* root showing well-developed sheath; **(e)** *T. fanfani* x *C. salviifolius* root showing a less-developed sheath; **(f)** *T. fanfani* x *C. ladanifer* root showing a less-developed sheath; **(g)** *T. pini* x *C. salviifolius* root surrounded by a diffuse sheath; **(h)** *T. pini* x *C. ladanifer* root showing a well-developed sheath.

Overall, the mycorrhizae formed between the different *Terfezia-Cistus* associations displayed similar microscopic characteristics irrespective of host plant-fungal species association. A general description of the anatomic features of these associations is provided below.

Outer mantle structure with a densely plectenchymatous to nearly pseudoparenchymatous structure is composed of colorless angular cells, which are more marked in *Terfezia extremadurensis* and *Terfezia pini*, but also noticeable in *Terfezia fanfani*. Inner mantle plectenchymatous is characterized by colorless hyphae forming a coarse net of irregularly shaped hyphae, tightly glued together, which sometimes begin as small star-like arrangements, as in the case of *Terfezia arenaria* (**Fig. 4a, 4b**).

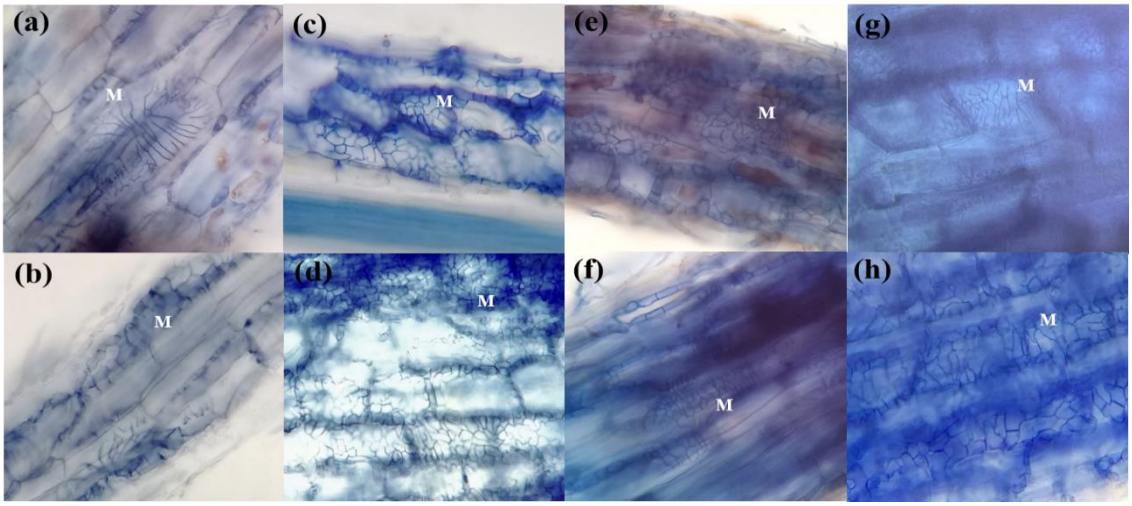


Figure 4. Light microphotographs of *Terfezia-Cistus* mycorrhizal roots, (400x): **(a)** detail of *T. arenaria* x *C. salviifolius* ectomycorrhizae mantle structure (M); **(b)** detail of *T. arenaria* x *C. ladanifer* ectomycorrhizae mantle structure (M) **(c)** detail of *T. extremadurensis* x *C. salviifolius* ectomycorrhizae mantle structure (M); **(d)** detail of *T. extremadurensis* x *C. ladanifer* ectomycorrhizae mantle structure (M); **(e)** detail of *T. fanfani* x *C. salviifolius* ectomycorrhizae mantle structure (M); **(f)** detail of *T. fanfani* x *C. ladanifer* ectomycorrhizae mantle structure (M); **(g)** detail of *T. pini* x *C. salviifolius* ectomycorrhizae mantle structure (M); **(h)** detail of *T. pini* x *C. ladanifer* ectomycorrhizae mantle structure (M).

The Hartig net is usually composed by a single row of hyphae that protrudes deeply towards the endodermis, enveloping completely 1 to 3 rows of cortical cells, but never touching the endodermis or the central cylinder. Hyphal segments around cortical cells, initially of constant width, but later forming a beaded or pearl-like structure (**Fig. 5c**, **5e**). In respect to root colonization, significant differences were found in the mean frequency of infection between the different *Terfezia* isolates ($n = 64$, $p < 0.001$). Regarding those differences, *Terfezia arenaria* showed significantly lower mean frequencies of infection on both *Cistus salviifolius* and *Cistus ladanifer* when compared with the other 3 *Terfezia* species (**Fig. 6**).

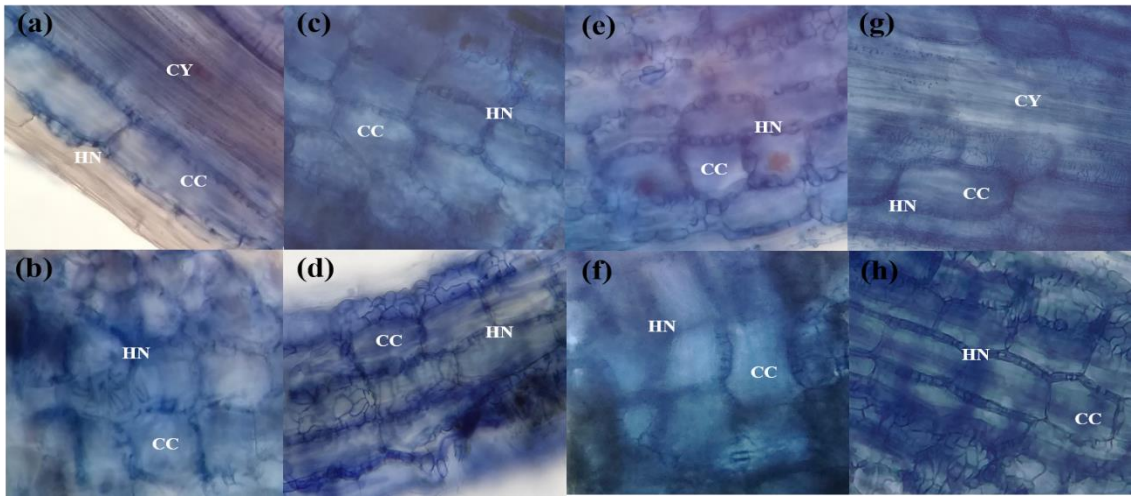


Figure 5. Light microphotographs of *Terfezia-Cistus* mycorrhizal roots, (400x): **(a)** *T. arenaria* x *C. salviifolius* ectomycorrhizae showing Hartig net (HN) restricted to cortical cells (CC), (CY) central cylinder; **(b)** *T. arenaria* x *C. ladanifer* ectomycorrhizae showing well-developed Hartig net (HN) surrounding cortical cells (CC); **(c)** *T. extremadurensis* x *C. salviifolius* ectomycorrhizae showing intercellular hyphae pearl structure (Hartig net (HN)) between cortical cells (CC); **(d)** *T. extremadurensis* x *C. ladanifer* ectomycorrhizae showing well-developed Hartig net (HN)) between cortical cells (CC); **(e)** *T. fanfani* x *C. salviifolius* ectomycorrhizae showing the characteristic pearl structure of the Hartig net (HN)) surrounding root cortical cells (CC); **(f)** *T. fanfani* x *C. ladanifer* ectomycorrhizae well-developed Hartig net (HN)) between cortical cells (CC); **(g)** *T. pini* x *C. salviifolius* ectomycorrhizae showing well-developed Hartig net (HN) surrounding cortical cells (CC)), (CY) central cylinder; **(h)** *T. pini* x *C. ladanifer* ectomycorrhizae showing a widespread Hartig net (HN) surrounding cortical cells (CC).

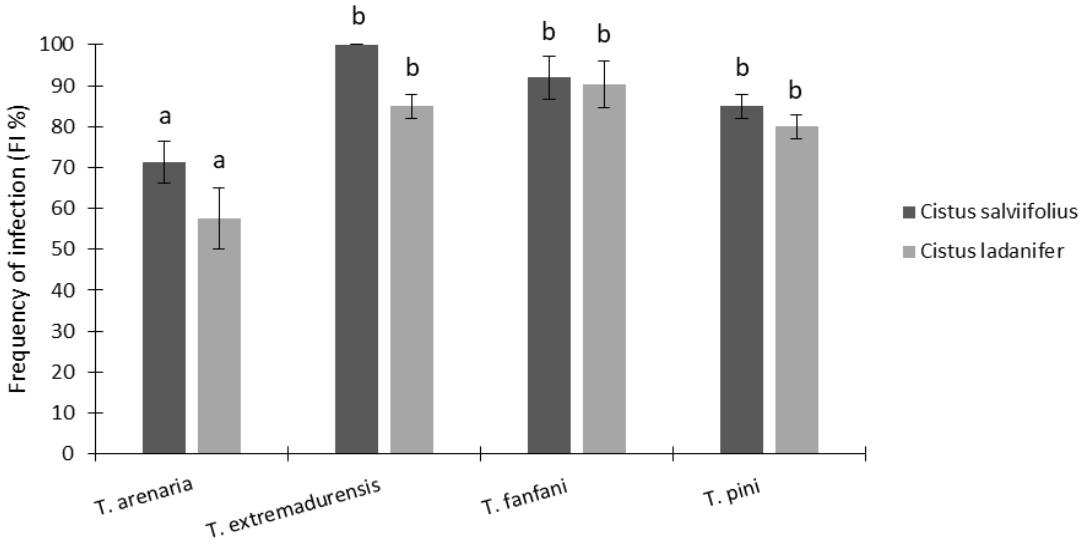


Fig. 6. Colonization frequency of the four *Terfezia isolates* on root tips of *C. salviifolius* and *C. ladanifer*. Values are the mean of 8 replicates ± standard error per treatment. Different letters within columns indicate a significant difference (P<0.01).

As for the other three mycosymbionts, the best frequencies of infection were obtained on *Cistus salviifolius* plantlets inoculated with *Terfezia extremadurensis*, with hardly no

differences between the 8 replicates. Comparably, *Terfezia fanfani* and *Terfezia pini* also achieved high frequency of infections on both *Cistus* hosts, though slightly lower than those of *Terfezia extremadurensis*.

DISCUSSION

The present work has brought forward compelling evidences on the ability of *T. arenaria*, *T. extremadurensis*, *T. fanfani* and *T. pini* to engage in mycorrhizal association under *in vitro* culture conditions, with two of the most widespread *Cistus* species (i.e. *C. salviifolius* and *C. ladanifer*). Furthermore, it provides for the first time a comprehensive macro and microscopic descriptions of the mycorrhizae formed between *T. arenaria*, *T. extremadurensis* and *T. pini* on the abovementioned *Cistus* species.

One interesting question that needed answering is the presence or absence of a true sheath in these mycorrhizal associations. We can now ascertain that all four *Terfezia* species analysed (i.e. *T. arenaria*, *T. fanfani*, *T. extremadurensis* and *T. pini*) do form ectomycorrhizas with a true sheath, and therefore agree with the work of Zitouni-Haouar and collegues (2014). However, differences in mantle development were observed between the mycorrhizae formed by the four mycosymbionts, with *T. arenaria* colonized roots showing only a sparsely rudimentary sheath, whereas on the other end, *T. extremadurensis* colonized roots where surrounded by a profuse well-developed sheath, under the same experimental conditions. Although these differences might represent true differences on the morphologic characters of those particular associations, another possible explanation is that the observed differences might just be a reflection of the flexibility of each *Terfezia* species to colonize different plant hosts. In other words, although capable to enter into mycorrhizal association, the time or the conditions required to form full developed mycorrhizas may differ between *Terfezia* species. For instance, Kovács et al. (2003) noted that the colonization of *Helianthemum ovatum* roots by *T. terfezioides* increased with increasing phosphate content, whereas Kagan-Zur et al. (1994) showed that mycorrhization between *H. sessiliflorum* and *T. leonis* (= *T. arenaria*) was inhibited at low phosphate concentration without exogenous sugar. Likewise, the lower colonization rates observed in *T. arenaria* inoculated *Cistus* roots might be a reflection of its different nutritional requirements or indicative of a slower interaction under the tested conditions.

In summary, given that modern truffle cultivation is largely based on mass production of

adequately colonized plants raised under controlled conditions (Zambonelli et al. 2015), our results are encouraging since all eight *Terfezia-Cistus* combinations expressed high rates of mycorrhization (comparable to those obtained in previous works) (Morte et al., 2012; Zaretsky et al., 2005; Zitouni-Haouar et al., 2014). Nevertheless, our experimental data seems to indicate that *Cistus salviifolius* is a better option than *Cistus ladanifer* as potential host for the production of *Terfezia* inoculated plants. Regarding the mycosymbiont, our data failed to discriminate between *T. fanfani*, *T. extremadurensis* and *T. pini*, which implies that all three represent suitable fungal partners for the two *Cistus species* and may be applied for the production of desert truffle inoculated *Cistus* plantlets. In general, the choice of the fungal partner in truffle cultivation depends on various factors (e.g. sporocarp size, edibility, plantation purpose, interactions with other organisms, etc.). In that context, *Terfezia fanfani* represents probably the best choice, mostly due to its superior gastronomic value and ascocarp size (Morte et al. 2009). Yet, *T. extremadurensis* and *T. pini* inoculated *Cistus* seedlings may also be interesting alternatives for reforestation programs and/or to prevent soil erosion after intense disturbances.

ACKNOWLEDGMENTS

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FINAL CONSIDERATIONS

Terfezia hypogeous ascocarps are indisputably among the most prized desert truffles, highly appreciated in North African, Middle Eastern and Eastern European countries, for their bland and sweet taste. In addition, *Terfezia* species have also great ecological value due to their position as key symbiotic partners of several xerophytic host plants in arid and semiarid ecosystems. These attributes have driven many researchers to focus their research interest in developing desert truffles as alternative crops for arid and semiarid zones. However, *Terfezia* cultivation is still in an initial stage, as only *T. claveryi* and *T. boudieri* were successful domesticated. Indeed, many aspects of *Terfezia* life-cycle have not been completely elucidated yet and knowledge on the ecology, physiology and biochemistry of many *Terfezia* mycorrhizal associations is rudimental at best.

Within this context, the primary aim of present work was to study the mycorrhizal associations formed between *Terfezia* species and *Cistus* spp., and explore their potential application in desert truffle cultivation. However, while trying to achieve that goal, some unexpected research questions regarding the identity and taxonomic placement of some of the collected *Terfezia* specimens were raised. The awareness of the pressing necessity to solve these taxonomic issues led to the redefinition of the initial research goals to encompass an additional assessment of the diversity of *Terfezia* species in Portugal.

Given the interdisciplinary nature of the present research, this thesis was divided into two sections, so that, **section I** addresses the topic of the diversity of genus *Terfezia* and **section II** focus on the study of the mycorrhizal associations between *Terfezia* and *Cistus* and its application on desert truffle cultivation.

In what concerns the topic of the diversity of genus *Terfezia*, it became evident soon after the beginning of this work that *Terfezia* taxonomy was far from resolved and that the use of molecular techniques was mandatory for accurate specimen's discrimination. However, the increasing amount of published sequence data also produced a great number of undescribed *taxa* without the needed adjustment and updating towards minimization of data base errors. Consequently, most popular current nucleotide search databases, possess numerous errors, poor-quality sequences, and many deposited sequences with little or no associated taxonomic nor ecological information (Nilsson et al., 2006). Within this framework, the present work presents the most comprehensive *Terfezia* phylogeny to date. As reviewed in

Chapter I, our reconstructed phylogeny based on public *Terfezia* ITS data available on the custom-curated UNITE database allowed us: 1) to discriminate at least 17 distinct lineages within the genus, and 2) to successfully resolve some of the more pressing taxonomic issues, namely, the *T. leptoderma*/*olbiensis* complex and some misapplied synonymy (e.g. *T. trappei*). Additionally, an identification key to *Terfezia* genus, highlighting the importance of morphological and ecological characterization was proposed towards the establishment of a consensual *Terfezia* classification.

Also relevant in the framework of **Section I,** two new *Terfezia* species were described for the first time on the basis of their distinct morphology and unique ITS-rDNA sequences, respectively, *Terfezia lusitanica* (in Chapter II) and *Terfezia solaris-libera* (in Chapter III). Prior to this work, the available information on *Terfezia* species richness in Portugal was scarce and outdated. Only four *Terfezia* species were known to occur in the country, namely, *T. alsheikii*, *T. arenaria*, *T. fanfani* and *T. olbiensis* (Bordallo et al., 2013, Chevalier, 2014). Thus, *per se* the discovery of these two new *Terfezia* taxa on national ground represents a significant contribution to the knowledge of *Terfezia* species richness and to the knowledge of the Portuguese mycobiota. Furthermore, the intensive ascocarp sampling conducted, from February 2013 till May 2020, allowed us to update the existing knowledge and raise the number of known *Terfezia* species occurring in the country to 10 species (i.e. *T. alsheikii, T. arenaria, T. cistophila, T. extremadurensis*, *T. fanfani*, *T. grisea*, *T. lusitanica, T. pini, T. olbiensis and T. solaris-libera*) (unpublished data). Altogether, these results seem to emphasize the occurrence of a rich assembly of *Terfezia* species in the semiarid conditions of the western Iberian Peninsula, where a wide variety of soils occur (both basic and acidic) and the climatic conditions allow for an abundance of plant hosts such as therophytes, chamaephytes and phanerophytes of the family of *Cistaceae* but also of phanerophytes of other families (oaks, pines) (Chevalier, 2014).

On the topic of the mycorrhizal associations between *Terfezia* and *Cistus* and its application on desert truffle cultivation, for decades, desert truffle cultivation was hindered by difficulties in obtaining good inoculum sources and due to erratic seed germination and low plant survival rates in nursery conditions (Morte et al., 2009; Morte et al., 2008). With the perspective of such challenges, much attention was invested in finding the best solutions to overcome those particular issues throughout the experimental phase of the present work. As a result, Chapter IV and Chapter V (**Section II)** respectively feature an optimized *in vitro* micropropagation

protocol for rapid multiplication of true-to-type *Cistus salviifolius* and a new fully-synthetic culture media and process for improved isolation and maintenance of *Terfezia* spp. mycelium cultures.

In relation to *Cistus* plant propagation, seed germination and induction of axillary and adventitious shoots, by tissue culture techniques, were both found to be adequate options for the propagation of *Cistus* plants (data not shown). However, *Cistus* seeds need to be pre-treated (e.g seed scarification) and even then, seed germination rates are erratic. Therefore, the proposed methodology for *in vitro* micropropagation of *Cistus* (discussed in Chapter IV) proved to be more advantageous over seed germination, particularly when the purpose is mass production of *Cistus* plantlets for the mycorrhization assays. Moreover, it represents an innovation over previously established multiplication protocols, given that allows rapid multiplication of mature explants using smaller quantities of plant growth regulators. It is also worth mention that since its publication, the same methodology was successfully employed for multiplication of yet another *Cistus* species (i.e. *Cistus ladanifer*) as refered to in Chapter VI.

Not surprisingly, the most challenging biotechnological issue that had to be overcome in this research was establishing a suitable isolation media for *Terfezia* spp. It is widely recognized that *Terfezia* mycelium cultures are difficult to obtain and maintain (Fortas & Chevalier 1992). Plus, the growth rates are slow and extremely variable between species (Iotti et al. 2012; Navarro-Ródenas et al. 2011). Indeed, while trying to solve the abovementioned issue, several attempts were made using various semi-synthetic and synthetic formulations, at different pH levels ranging from pH = 5 and pH = 8. Yet, none of the tested culture media led to the isolation of a single *Terfezia* strain. So, a different strategy had to be adopted: the development of a new fully-synthetic culture media which has been object of a patent, the LS culture media (European Patent Application n. 19204730.6 – 1118 (10.01.2020)). In the end, this strategy worked rather well, seeing that it led to the successful isolation of 20 *Terfezia* strains belonging to: *T. arenaria* (9), *T. fanfani* (8), *T. extremadurensis* (2) and *T. pini* (1). Furthermore, it has been clearly showed that the new LS medium also obtained better results in the maintenance *Terfezia* spp. mycelia pure cultures than the standard MMN medium (as discussed in Chapter V).

The importance of the above biotechnological breakthroughs cannot be overlooked, since without them it would be impossible to implement the subsequent *in vitro* mycorrhizal trials

described in Chapter VI. In this regard, it is known that mycorrhizal synthesis under controlled conditions is the key step of any mycorrhization program, and of great importance for verification of the symbiotic compatibility between the intended fungi and their putative host plants (Repáč, 2011). From that perspective, the research introduced in Chapter VI allowed us to provide the first anatomic descriptions of the mycorrhizae formed *in vitro* by four *Terfezia* species (i.e. *T. arenaria*; *T. extremadurensis*; *T. fanfani* and *T. pini*) with *C. ladanifer* and *C. salviifolius* and to compare and discuss the suitability of the two plant hosts towards the viable production of *Terfezia* inoculated seedlings. In accordance, it was found that all studied plant-mycosymbiont combinations expressed high colonization rates, thought *T. arenaria* showed significantly lower colonization rates than the other three mycosymbionts. *C. salviifolius* displayed a slightly better response to infection, by *Terfezia* species, that *C. ladanifer*. Overall, the present research clearly demonstrates that *Cistus* spp. are indeed promising candidates for desert truffle cultivation and that different *Terfezia*-*Cistus* combinations can be used to enable desert truffle cultivation over a wide range of situations depending on the plantation purposes.

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FUTURE PERSPECTIVES

Following the lines of the present research, and due to the demonstrated importance of a correct species identification, designing specific primers for each *Terfezia* species would provide a quick way to identify the mycosymbiont on *Cistus* root tips and directly in soil. This tool would become crucial in the understanding of host-symbiont specificity dynamics in this symbiosis, leading also to a better understanding of co-evolutionary processes that might eventually have led to co-speciation events. Now that the most pressing taxonomic issues have been solved, and in the presence of an appropriated species identification framework and a suitable rearing media (both achieved through this work), the time is ripe for research towards a better understanding of *Terfezia* life cycle and its determinants. This fundamental knowledge can aid in the fine tunning of rearing methods and on understanding system sustainability in the long run. Also, and resulting from the achievements in the development of the basic biotechnological tools for the production of *Cistus* plants inoculated *Terfezia*, the next logical phase would be the establishment of various experimental plots, in which to assess plant quality, site suitability, plantation frame and other management practices, all critical factors for obtaining good desert truffle yields.