Phylogenetic Studies of *Terfezia pfeilii* and *Choirmyces echinulatus* (Pezizales) support new genera for southern African truffles: *Kalaharituber* and *Eremiomyces*

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The ITS region including the 5.8S rRNA gene as well as the 5' end of the 28S rRNA gene of hypogeous Pezizales and Tuberaceae were studied to clarify the generic placement of two southern African desert truffles, *Terfezia pfeilii* and *Choirmyces echinulatus*. The results show that neither species belongs in the genus to which it has been assigned on the basis of morphological characters. As expected, two *Choirmyces* spp. grouped close to the representative of the Tuberaceae (*Tuber melanosporum*). However, *C. echinulatus* diverged from the other *Choirmyces* species and emerged near members of the genus *Terfezia*, being even closer to that genus than *T. pfeilii*. Two new genera and new species combinations, *Kalaharituber* gen. nov. with *K. pfeilii* (syn. *T. pfeilii*) comb. nov. and *Eremiomyces* gen. nov. with *E. echinulatus* (syn. *C. echinulatus*) comb. nov. are therefore introduced to accommodate these taxa. Both genera are closely related to *Terfezia*, and thus are placed in the Pezizales.

INTRODUCTION

Truffles are the hypogeous ascomata of fungi belonging to the Pezizales (Trappe 1979, 1990). The assignment of truffle genera to a particular family and species to a particular genus was traditionally founded on the morphology of ascomata, asc and spores (e.g. Trappe 1979). Thus, *Terfezia pfeilii* was described from Damaraland, Namibia, by Hennings (1987) and *Choirmyces echinulatus* from Cape Province, South Africa, by Marasas & Trappe (1973).

In recent years, however, molecular phylogenetic research on sequestrate fungi has repeatedly demonstrated that morphology of hypogeous fungi can be misleading. Evolution of hypogeous species typically involves a convergent reduction in macromorphological characters, often entailing loss of features otherwise useful to distinguish related epigeous taxa. More specifically, molecular analyses of the Pezizales and the phylogenetic relations among epigeous and hypogeous species have been conducted by O’Donnell et al. (1997), Norman & Egger (1999), Percudani et al. (1999), Roux et al. (1999), Hansen, Lessee & Pfister (2001), and Diez, Manjón & Martin, (2002). Some of these investigations confirm earlier morphological findings. O’Donnell et al. (1997) and Hansen et al. (2001) demonstrated that certain hypogeous members of the Pezizales show greater affinity to epigeous members than to other hypogeous ones. Similarly, the monophyletic origin of some members of *Terfezia* and *Tirmania* was confirmed by Diez et al. (2002). However, other results challenge some of the earlier placements, for example along with *Tirmania*, *Terfezia* belongs to the Pezizales rather than the Terfeziaceae (Norman & Egger 1999; Percudani et al. 1999). Further, *Choirmyces* was moved from the Terfeziaceae to the Tuberaceae (O’Donnell et al. 1997, Percudani et al. 1999), and *Terfezia terfezioides* was removed from *Terfezia* and reinstated as Mattirolomyces terfezioides (Percudani et al. 1999, Diez et al. 2002).

A preliminary molecular study of *Terfezia pfeilii* and *Choirmyces echinulatus* suggested that these two Kalahari desert truffles are molecularly close, though currently placed in different families. This apparent affinity inspired us to study the phylogenetic relationships between these two species in relation to other *Terfezia* and *Choirmyces* species. To this end, we analyzed DNA sequences from the internal transcribed...
Table 1. Species analyzed for this work.

<table>
<thead>
<tr>
<th>Truffle</th>
<th>Collection site</th>
<th>Date</th>
<th>Provided by</th>
<th>DNA fragment</th>
<th>GenBank accession no.</th>
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<tr>
<td>Choiromyces alveolatus</td>
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<td>1998</td>
<td>J. Trappe</td>
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<td>(Trappe 22830)</td>
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<td>C. echinulatus (voucher no. 12)</td>
<td>Ghanzi, Botswana</td>
<td>1999</td>
<td>V. Kagan-Zur</td>
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<td>1996</td>
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<td>ITS</td>
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</table>

In this study we present molecular data that supports removal of Terfezia pfeilii from Terfezia and Choiromyces echinulatus from Choiromyces. We describe two new genera to accommodate these truffles, still within the Pezizaceae.

MATERIALS AND METHODS

The fungi

The origin of fruit bodies obtained for the analysis presented in this work is summarized in Table 1. Voucher collections of the specimens studied are deposited in the Department of Botany Mycological Herbarium, Oregon State University (OSC). Previously published sequences used in this work are presented in Table 2.

DNA extraction and amplification

The method described by Grube et al. (1995) was employed to obtain small amounts of amplifiable DNA. DNA was amplified with primers ITS 1 and ITS 4 (White et al. 1990) for ITS or LR 1 and U2 (van Tuinen et al. 1998b and Sandhu et al. 1995, respectively) for the large subunit (LSU). Primers were synthesized by Rhenium, Jerusalem. Both ITS and LSU amplifications were performed as described for ITS by Kagan-Zur et al. (1999).

Cloning

PCR amplified ITS or the S' end of the LSU fragments were cloned into InSt/Aclone™ PCR Product Cloning kit No. K1214 (MBI Fermentas, Lithuania).
Phylogenetic evolutionary trees based on these data were constructed along with 1000 bootstrapping repeats by MEGA version 2.1 (Kumar et al. 2001). Several reshuffled lists of sequences were presented to both the ClustalX and Mega programs. The Mega program’s ‘Subtree Swapping’ option was used to adjust outputs. Trees were built with and without deleting gaps. Trees presented here were constructed using the gap delete options. Phylogenetic analysis of the ITS and the large subunit (LSU) sequences were carried out by the neighbour-joining (NJ) and maximal parsimony (MP) methods.

**Morphological analyses**

Dried specimens were hand-sectioned with a razor blade and mounted in 5% KOH, Melzer’s reagent and cotton blue in lactic acid, respectively. Spore dimensions are based on at least 30 randomly selected spores plus the smallest and largest seen, excluding the ornamentation. Spores were measured in cotton blue mounts, because the walls or ornamentation of many ascomycetes tend to swell in KOH. Other tissues were measured in KOH.

**RESULTS AND DISCUSSION**

**Phylogenetic analyses**

**ITS analyses**

The ITS phylogenetic tree presented as Fig. 1 was the output result of both NJ and MP methods of analysis, although there are differences in percent bootstrapping. In this tree some of the *Terfezia* group nodes, notably separation of *T. claveryi* and *T. arenaria* (40/30) as a distinct clade and grouping of the latter with the *T. boudieri* clade (47/36), were only weakly supported by either method. Including sequences leading to extensive gap formation resulted in an NJ tree very similar to Fig. 1. The only placing changes observed involved the weakly supported branches (data not shown) within the *Terfezia* clade. Reshuffling the input data did not change the grouping presented in Fig. 1 but in one or two cases changed the placing order (e.g. putting *T. boudieri* first, or separating *T. claveryi* from *T. arenaria*). These are easily achieved or corrected by ‘Subtree Swapping’ at will. However, examining the best bootstrapping tree (data not shown) revealed several other differences: *Tuber melanosporum*, chosen as an out group, which forms a *Tuber-Choriozymes* clade together with *C. venosus* (submitted to the GenBank as *C. meandriformis*) and *C. alveolatus* in Fig. 1, emerges as a true out-group, though close to the above mentioned *Choriozymes* spp.

The separation of *T. boudieri* 2 (and 42a2) from *T. boudieri* 1 and 3 is well supported in Fig. 1, but all 3 *T. boudieri* ITS forms (Holdengraeber et al. 2001) stem from the same root in the bootstrapping tree.

**Table 2. Sequences obtained from the National Center for Biotechnology Information (NCBI).**

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Species</th>
<th>Origin</th>
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<td>AF003910</td>
<td><em>C. venosus</em> (as <em>meandriformis</em>), ITS</td>
<td>Italy</td>
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<td>AF27668</td>
<td><em>Maitriotremyces</em> terfezioides, ITS</td>
<td>Hungary</td>
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<td>AF276680</td>
<td><em>M. terfezioides</em> 2, ITS</td>
<td>Hungary</td>
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<td>AF276679</td>
<td><em>Terfezia leptoderma</em> 1, ITS</td>
<td>Spain</td>
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<td><em>T. leptoderma</em> 2, ITS</td>
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<td><em>Tiramia pinoyi</em>, ITS</td>
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<td><em>T. nivea</em>, ITS</td>
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<td><em>T. pinoyi</em>, LSU partial sequence</td>
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<td><em>T. nivea</em>, LSU partial sequence</td>
<td>Kuwait</td>
</tr>
</tbody>
</table>

* O’Donnell et al. (1997).
* Amicacch, Stochi & Martin (Direct submission).
* Diez, Manijis & Martin (2002).
* Gnusclierz, Honrubia & Morte (Direct Submission).
* Hansen et al. (2001).

The plasmid mixture was transformed into *Escherichia coli* XLI-blue in accordance with the manufacturer’s recommendations. Transformants were plated on agar solidified Luria Broth (LB) plates (Miller 1972) containing 50 μg ml⁻¹ ampicillin.

**Inserts sequencing**

Single colonies were picked up and grown in LB overnight. Plasmids were extracted from these cultures by the alkaline method (Birnboim & Doly 1979). Plasmids containing inserts were sent to the sequencing unit of the Hebrew University of Jerusalem, where the Big Dye Terminator kit (Perkin-Elmer, Branchburg, NJ) was used in conjunction with the primer pair M13 (Forward) and pUC (Reverse) (Sambrook & Russell 2001) for bi-directionally sequencing of inserted fragments directly from each plasmid. Primers were synthesized at the Molecular Biology Laboratory, Hadassa Hospital, Jerusalem. The sequence was determined with an ABI prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were deposited in the National Center for Biotechnology Information (NCBI) databases (Table 1).

**Alignments and phylogenetic analysis**

Several sequences were obtained for phylogenetic analysis from the National Center for Biotechnology Information (Table 2). Sequence alignments were submitted to TreeBASE PIN no. 12889. Sequences were aligned with ClustalX version 1.81, downloaded for the MacIntosh from http://www.ebi.ac.uk/clustal/darwin/. The results were manually checked and adjusted.
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Fig. 1. Neighbour-joining consensus phylogenetic tree of ITS sequences. Bold numbers are % of 1000 bootstrapping replicates supporting the NJ tree presented here. Non-bold numbers are % of 1000 bootstrapping replicates supporting the same node by the MP method.

(no hierarchy). Some of the less supported nodes were also eliminated from the latter, resulting in the loss of the less supported information. Both, however, essentially support removal of C. echinulatus and Terfezia pfeili from the genera to which they were assigned solely on morphological grounds.

C. echinulatus, emerges closer to the Tirmania clade than to the other Choiromyces species, being placed within the in-group. Most of the Terfezia spp. group together to constitute a monophyletic Terfezia clade, the closest neighbour of which is the Tirmania lineage. T. leptoderma and T. obiensis form a distinct group as sister to T. boudieri, T. arenaria and T. claveryi. However, T. pfeili, the Kalahari Terfezia, is distant from other Terfezia species and forms a separate lineage. Mattirolomyces terfezioides, T. pfeili and C. echinulatus, belonging to separate clades, are placed between the Tirmania and Tuber-Choiromyces groups.

The data support reinstatement of Mattirolomyces terfezioides (Fischer 1938) as a replacement for Terfezia terfezioides and representative of a separate genus distinct from Terfezia, as suggested by Percudani et al. (1999) and supported by Diez et al. (2002).

We also performed multiple alignment of the 5.8S. Table 3 shows that out of the 155 bp analyzed for the 5.8S gene, 23 sites exhibited variability, representing 22 independent events. Of these, ten were transitions (5 C-T), eight were transversions, two were inversions of dinucleotides, one was an insertion and one deletion of two nucleotides.

The above sequences fall into two main groups, one including T. melanosporum, C. alveolatus, C. venosus (as meandriformis) and C. venosus, and the other Terfezia spp., C. echinulatus, Tirmania spp., and
M. terfezioides. Both show relatively small differences within and relatively large differences between the groups. Detailed analysis of the 5.8S gene revealed three variable sites within the Tuber-Choiromyces group (three sequences), and eight sites variable solely within the Terfezia-Tirmania group (ten sequences). An additional seven sites were identical within each group but varied between them. T. pfeili and C. echinulatus displayed greater conformity to the 5.8S gene consensus sequence (emerging from this study) than T. arenaria and T. claveryi.

Analysis of the S' end of the LSU

To further test these results, the divergent domains D1 and D2 of the LSU rRNA gene were also analyzed. This DNA region is better conserved than the ITS but still variable enough to show differences between species (Hillis & Dixon 1991, van Tuinen et al. 1998b). The LSU NJ tree (Fig. 2), like the ITS tree, shows C. alveolatus and C. venosus branching together with T. melanosporum to form a Tuber-Choiromyces clade, an outgroup; this tree is supported by the bootstrapping. As in the ITS tree, the genus Tirmania is placed closer to the Terfezia clade, while C. echinulatus, M. terfezioides, and T. pfeili, form separate clades where C. echinulatus is closer to Terfezia spp. than to other Choiromyces spp. However, the MP trees were somewhat different. One of the better supported trees (Fig. 3) shows a non-hierarchical relationship of M. terfezioides, C. echinulatus, Tirmania spp. and the Terfezia clade, while T. pfeili emerges in a hierarchically once removed clade. The non-hierarchical branching is rather poorly supported (59% bootstrap value), but the separation of T. pfeili is well supported. All MP trees, however, placed T. melanosporum on its own, as an outgroup, while C. alveolatus and C. venosus formed a separate clade. Essentially, the two rRNA gene cluster regions examined in our phylogenetic analysis of Terfezia and Choiromyces species, namely the ITS region including ITS1, 2 and the 5.8S rRNA gene, as well as the S' end of the LSU gene, generated similar results. However, as GenBank contains rather few 28S sequences, the range of the 28S (LSU) gene tree was necessarily limited as compared with the ITS tree. The LSU result concerning Terfezia boudieri was somewhat unexpected, as this region is rather better conserved than the ITS, we had expected a resolution along species lines rather than within the species. While T. pfeili a and b had identical 28S sequences and T. boudieri ITS forms 1 and 3 were also almost identical to each other in their 28S sequences, T. boudieri ITS 2 deviated from the latter. The hypothesis that these forms, though they may seem morphologically indistinguishable, actually represent two different species, deserves further investigation.

All trees presented here provide strong support for a close relationship between T. melanosporum and the Choiromyces clade, while equally strongly supported is
Fig. 2. Neighbour-joining consensus phylogenetic tree of the D1–D2 5' end of the 28S (LSU) rRNA gene sequences. Numbers are % of 1000 bootstrapping replicates supporting the tree presented here.

Fig. 3. A representative Maximal Parsimony (MP) phylogenetic tree of the D1–D2 5' end of the 28S (LSU) rRNA gene sequences. Numbers are % of 1000 bootstrapping replicates supporting the tree presented here.

the placing of C. echinulatus outside this clade within the ingroup. T. pfeilii, although located within the ingroup is phylogenetically farthest from the Terfezia clade.

To conclude, our findings evidence that the assignment of C. echinulatus to the genus Choiromyces and therefore to the Tuberaceae is incorrect. It is also clear that T. pfeilii does not belong to the Terfezia clade and its classification needs to be revised. In spite of the molecular resemblance between T. pfeilii and C. echinulatus, which initiated this study, the finding that the two species occupy distinct clades in all types of analyses precludes their placement in a single genus. We therefore propose two new genera, described below, to accommodate the above findings.

**TAXONOMY**

Kalabarituber Trappe & Kagan-Zur, gen. nov.

_Etym.:_ Kalahari–+–tuber (Latin, ‘truffle’), ‘truffle of the Kalahari.’

A Terfezia sports spinis minutissimis dense congestis, ordinibus DNA abhorrentibus, distributione in Hemisphaerio Meridionali differt.

_Typus: Terfezia pfeilii_ Henn.

Kalabarituber pfeilii (Henn.) Trappe & Kagan-Zur, _comb. nov._

Etym.: in honor of Count Pfeil, the original collector of the type species.

Ascomata hypogeous, smooth, unpolished, minutely pubescent, turbinate to obpyriform or subglobose, to 6.5 x 6 cm. Peridium ca 1 mm thick, wrinkled (especially on the upper ascoma surface), dark brown, the wrinkles yellowish. Glēba solid, fleshy, marbled with sterile, white veins and yellowish white to brown, fertile pockets, Odour strongly fungoid. Spores globose, 16–22 (–26) μm diam, at first hyaline, in age light brown, the walls + 1.5 μm thick, 2-layered, light yellow to light orange in Melzer’s reagent; ornamentation appearing in KOH as a minutely papillose or granulose, mucilaginous epispore but in cotton blue clearly seen as densely crowded, acute spines 0.5–1.5 (–2) x ≤ 0.5 μm. Asci nonamyloid, with (4–) 5–8, loosely arranged spores, globose to ellipsoid or obovoid, 70–100 x 50–80 μm, sessile to subtuplicate, the walls ± 1 μm thick at maturity, randomly arranged in fertile pockets. Ectal excipulum 180–220 μm thick, of brown hyphae 4–12 μm broad at septa but the cells inflated to 15–30 μm. Ental excipulum of generally circumferentially aligned, hyaline to pale yellow hyphae 4–10 μm broad at the septa, the cells often slightly inflated. Global hyphae of sterile veins and fertile pockets undifferentiated, hyaline, thin-walled, 5–12 μm broad at septa but the cells generally inflated to 20 μm. Distribution, Habitat and Season: Kalahari Desert of southern Africa: Namibia, Botswana, and adjacent areas of South Africa, In compact to fairly compact, calcareous, white and pink sands (Leistner 1967), associated with Acacia spp. (Marloth 1913, as Terfezia claveryi, Storey 1958, Taylor et al. 1995) and various other plants, including Citrullus vulgaris (Kagan-Zur et al. 1999), April through June.

Illustrations (as Terfezia claveryi or T. pfeilii): Pole-Evans (1918: pl. 7), Mattirolo (1922: fig. 28), Leistner (1967: pl. 50), Marasas & Trappe (1973: fig. 2), Alsheikh (1994: pl. 2–18).

Comments: The spore ornamentation of minute, densely crowded spines of K. pfeilii was recognized as different from that of any other Terfezia spp. by Mattirolo (1922), Marasas & Trappe (1973), and Alsheikh (1994). Nonetheless, it has been variously misidentified as Tefezia boudieri or T. claveryi (Marloth 1913, Pole-Evans 1918, Ceruti 1960). Marasas & Trappe (1973) ascribe the early confusion of K. pfeilii with other Terfezia spp. to the paucity of good specimens for study and the nature of its spore ornamentation: spines so minute and crowded that they can be discerned only when stained, even with the oil immersion objective. Use of K. pfeilii for food by natives of the Kalahari was reviewed by Trappe (1990).

Specimens examined: Botswana: Ghanzi, G. Scholtz (PRE 41869). Namibia: Damaraland, Graf Pfeil (S – holotype of Terfezia pfeilii); Gibeon, Burger (PRE 42082, 42203); Gobabis, Verbuchtzlein (PRE 4390); Klein Karas, 20 Apr. 1921, A. E. Hill (OSC, TO); loc. cit., 8 June 1923 (BPI 38083, PRE 17799, OSC, TO); Windhoek, Gies (PRE 42076). South Africa: North Cape: Kakamas, June 1931, M. J. Oosthuizen (PRE 26335, OSC); Kalahari Gemsbok National Park, May 1956, R. Story 5616 (PRE 41602); Postmasburg, May 1911, F. L. Hunter (PRE 11293, OSC); Prieska, MacCloud (PRE 11619); Upington, O. A. Leistner 2610 (PRE 42201); Vryburg, Stephens 527 (PRE 36103); unspecified locality.
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Le Riche (PRE 41870) - Kalahari Desert: unspecified locality, Bottomley (PRE 44310); herb. G. Breda (BPI, as Tepesia boudieri); 1913, R. Marloth (K, as Terfezia claverty); Nash (PRE 44254); Weintraub (PRE 32394).

Eremiomyces Trappe & Kagan-Zur, gen. nov.

Etym.: Eremi- (Greek, 'desert') + -myces (Greek, 'fungus'), 'desert fungus.'

A Choiromyces sporis virgis conios rectis obtusis, cellulis magnopere inflatis, ordinibus DNA abhorrentibus (ad Pezizaceae, non Tuberaceae, affinis) et distributione in desertis Hemisphaerio Mendionali differt.

Typus: Choiromyces echinulatus Trappe & Marasas.

Eremiomyces echinulatus (Trappe & Marasas) Trappe & Kagan-Zur, comb. nov.


Etym: 'echinulatus' (Latin, 'having small spines').

Ascomata pale cream colored and subglobose when fresh, as dried with a black peridium and solid gleba marbled by dark brown veins embedding hymenial palisades; opposing palisades deformed from pressing against each other.

Sporae globose, 10–14 μm diam, light yellow to light brownish yellow, the walls 1 μm thick, light blue in cotton blue; ornamentation of straight, obtuse rods and cones 1–2 x 0.5–1 (~1.5) μm, pale yellow in KOH and light blue in cotton blue, ca 20–25 around the spore circumference, unconnected except for some spores on which barely perceptible lines on the spore surface join occasional ornamentals. Asc. hyaline, thin-walled, mostly 8-spored, in youth cylindric to ellipsoid or saccate, by maturity mostly cylindric, 140 x 12–17 μm, astipitate or with a short basal projection, the spore arrangement occasionally uniseriate but mostly incompletely biseriate to irregular. Paraphyses thin-walled, hyaline, ±4 μm broad. Ectal excipulum of light yellow, thin-walled hyphae 4–8 μm broad at the septa; ental excipulum similar but the hyphal cells often inflated to 15–50 μm broad. Glebal veins of subparallel-interwoven hyphae 3–6 μm broad at the septa, the cells mostly inflated.

Distribution, Habitat and Season: North Cape, South Africa, and Botswana, June, in sand dunes.

Illustrations (as Choiromyces echinulatus) Marasas & Trappe (1973: fig. 1), Trappe (1979: fig. 24a).

Comments: This rarely collected fungus mimics Choiromyces of the Tuberaceae in its macro-morphology. However, its spore ornamentation of straight, obtuse, rods and cones differs from the sinuous rods with apical depressions of Choiromyces venosus, the type species of that genus. The other Choiromyces spp. have pitted spore surfaces (Trappe 1979). Eremiomyces echinulatus is a desert dweller, and like most other desert truffles it has tissues with greatly inflated cells, a character not found in Choiromyces species of temperate forests. Of course, the DNA sequences place E. echinulatus in the Pezizaceae, whereas Choiromyces sensu stricto is in the Tuberaceae (O'Donnell et al. 1997, this study).


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