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ORIGINAL PAPER

Acaulospora brasiliensis comb. nov. and *Acaulospora alpina* (*Glomeromycota*) from upland Scotland: morphology, molecular phylogeny and DNA-based detection in roots

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Abstract Spores of two supposedly arbuscular mycorrhizal fungal species, new to the United Kingdom and recently described as *Acaulospora alpina* and *Ambispora brasiliensis* (*Glomeromycota*), were discovered in soil samples from moorland in upland Scotland. Soil and plant trap pot cultures were established, but attempts to establish these fungi in single-species pot cultures with *Plantago lanceolata* as host were unsuccessful. Nevertheless, based on a 1.5-kb DNA fragment spanning part of the small subunit rRNA gene, the internal transcribed spacer region and part of the large subunit rRNA gene, both these species could be detected directly in field-sampled roots, together with one uncultured species each of *Scutellospora*, *Rhizophagus* (former *Glomus* group Ab, or '*Glomus intraradices* clade') and *Acaulospora*. Whereas *A. alpina* has characteristic morphological similarities to other species in its genus, *A. brasiliensis* morphologically has little in common with any other species in *Ambispora*. The molecular phylogeny, DNA barcoding and morphological evidence clearly place *A. brasiliensis* in the genus *Acaulospora*. We therefore rename the species, reported from Brazil and Scotland, as *Acaulospora brasiliensis* comb. nov., and discuss ecological aspects of the very different environments from which *A. brasiliensis* and *A. alpina* have been reported.

Keywords Arbuscular mycorrhiza · *Glomeromycota* · *Acaulospora brasiliensis* · Molecular systematics · Molecular phylogeny · DNA barcoding · Molecular ecology

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Introduction

This study was initiated during an investigation of the mycorrhizal colonisation potential of Scottish upland soils for *Salix lapponum* cuttings (Milne et al. 2006). Natural *S. lapponum* and *S. herbacea* were sampled and examined for the occurrence of arbuscular mycorrhiza (AM). The presence of vesicles confirmed that AM fungi (*Glomeromycota*; Schüßler et al. 2001) were present, and samples were examined for the presence of glomeromycotan spores for morphological identification. Abundant spores that resembled *Acaulospora alpina* (from high altitude in Switzerland; Oehl et al. 2006) and *Ambispora brasiliensis* (from Minas Gerais State, Brazil; Goto et al. 2008) were recovered from trap cultures. The specimens of '*A. brasiliensis*' appeared to be more like an *Acaulospora* species (*Diversisporales*), than a member of *Ambispora* (*Archaeosporales*), thus conflicting with the published description. Therefore, we re-examined and expanded our data and studied the taxonomic, phylogenetic and systematic position of the Scottish organism and *A. brasiliensis* with a view to

reconciling this apparent conflict. There is no DNA sequence data for the Brazilian organism, but a morphological study was undertaken to compare it with the Scottish collections. The holotype of *A. brasiliensis*, consisting of spores preserved on microscope slides, was examined and compared with similar preparations of the Scottish specimens. The Scottish *A. brasiliensis*-like fungus was also characterised by DNA sequences providing species-level resolution, including a region that probably will cover the official DNA barcode for fungi (see also Stockinger et al. 2010). This allowed a direct detection of the fungus in the roots of plants from the Scottish upland moorland, together with *A. alpina* and additional uncultured species, one each of *Scutellospora*, *Rhizophagus* and *Acaulospora*. The discovery of the same species of arbuscular mycorrhizal fungi (AMF) in very different ecological conditions is discussed.

Materials and methods

Origin of plant and fungal material

On the 23rd of September 2003 an excursion was made to Meall nan Tarmachan (approximately 900 m altitude, UK national grid coordinates NN 58789 38612: 56° 31′ 5.82″ N 4° 17′ 48.29″ W), an upland site in Scotland, to collect fruiting bodies of ectomycorrhizal fungi associated with *Salix herbacea* along with samples of the acidic soil (pH 4.0–5.0, measurements west of Lochan na Lairige; Stevens and Wilson 1970) and vegetation. Samples were collected by removing a small patch of turf and attached soil with a hand trowel to a depth of about 10 cm. These samples came from a mainly grassy area supporting a mixed plant population of *Festuca vivipara*, *Nardus stricta*, *Salix herbacea*, *Alchemilla alpina*, *Vaccinium myrtillus*, *Vaccinium vitis-idea*, *Galium rotundifolium*, *Carex* spp. and *Rhacomitrium lanuginosum*. On 16 April 2010, six new samples were collected from Meall nan Tarmachan by National Trust for Scotland staff. Spore extractions from these yielded the same species with acaulosporoid spores as had been found in the earlier samples. Mixed plant species root samples were taken for DNA extraction. More new samples were taken from a nearby location (close to Lochan na Lairige) at a slightly lower altitude (56°31′14.20″N 4°16′ 47.60″W at approximately 500 m amsl) on 6 September 2010. The soil was thin and peaty, with a pH (in water) of 4.9, and these also contained both species.

Culture attempts

Subsamples of the soil (approximately 15 ml) were subjected to centrifugation and sucrose floatation to extract spores (Walker et al. 1982). Attempts were made to establish multi-spore pot cultures with *Plantago lanceolata* in Sunbags (Sigma-Aldrich, UK) by pipetting spores onto seedling roots in the planting hole in 10-cm diameter pots containing a heat-disinfested mixture (3:1, *v*/*v*) of horticultural sand and Terragreen™ (expanded attapulgite clay, Oil Dry Corp., USA) (Walker 1999). Further culture attempts, as ‘soil plus plant traps’ were established by mixing the soil with equal parts of Terragreen™ and replanting the sward sample to establish closed pot cultures in Sunbags (Walker and Vestberg 1994).

Morphological analyses

The holotype of *A. brasiliensis* consists of a single microscope slide, labelled ‘*Ambispora brasiliensis* 15 08 06 Serra do Cipó’. The slide was contained in a cardboard slide holder upon which was written ‘URM78879 *Ambispora brasiliensis* (typus)’. No other information was provided with the specimen except a note from URM saying ‘URM78880, also requested by Dr. Chris Walker, is not available.’ The spores on the slide were studied in detail through a Zeiss Axioskop research microscope. Digital images were captured with a Canon EOS5D camera and size measurements were made with a calibrated eyepiece reticle.

For the Scottish material, extracted spores were examined initially in water under a dissecting microscope, followed by study of spores in polyvinyl alcohol lacto-glycerol (PVLG) without or with Melzer’s reagent (1:4, *v*/*v*; PVLG-M) under the compound microscope as described above. Some specimens were also examined in glycerol. Spain (1990) suggested including unmodified wall structure observations from water immersed specimens, but without special objective lenses water has poor optical properties for compound microscopy, and dries rapidly in unsealed mounts. Glycerol does not affect the wall structure and gives a satisfactory refractive index. Comparisons with other glomeromycotan fungi were made from original species descriptions (e.g. Walker and Trappe 1981; Walker et al. 1993; Walker et al. 2004) and from herbarium specimens collected by Walker since 1974. Spore colour descriptions were from spores in water, either by comparison with a chart (Anon 1969; Anon 1990) or, when unmatched, by use of vernacular colour names.

The purely morphological terms ‘acaulosporoid’ or ‘acaulospore’ refer to a spore produced in the stalk or neck of a sporiferous saccule and do not imply homology with similar spores of *Ambispora* or *Archaeospora* spp. We do not use the term ‘glomerospore’ (Goto and Maia 2006) used in the protologue of *A. brasiliensis* because there are several different kinds of spores produced by glomeromycotan fungi, and they are likely not to be homologues

(Morton and Msiska 2010). Glomoid spores are found amongst widely separated systematic groups, and are unlikely to be homologous either amongst glomeromycotan higher taxa or with either acaulosporoid or gigasporoid spore morphs.

Molecular characterisation

DNA extractions from single spores, polymerase chain reaction (PCR), cloning, sequencing and sequence editing were as described in Schwarzott et al. (2001) and Krüger et al. (2009). The near full-length small subunit (SSU) rRNA gene was analysed together with the complete internal transcribed spacer (ITS) region, including the 5.8S rRNA gene and ~800-bp of the large subunit (LSU) rRNA gene.

For the SSU rDNA three clones revealing slightly different sequence variants were sequenced from sample W4699/Att1211-0, taken 19th September 2004 to obtain robust evidence on the genus level. For the ITS and LSU rDNA regions a ~1.5-kb fragment was cloned and analysed, to achieve species-level resolution (Stockinger et al. 2010) and to cover the potential official fungal primary DNA barcode (the ITS region or a combination of the ITS and the 5′ LSU regions). Part of the sequence data (clones pMK062-3; pMK064-4, 6; pMK065-4, 5, 6, 7; pMK109-1, 2) was derived from the same, stored material as the SSU rDNA (W4699/Att1211-0). The remaining clones sequenced (pCK032-1, 2, 4) came from a subculture (W5473/Att1210-5) sampled on the 5th of July 2008. DNA was extracted from 10 cm (20 randomly taken root fragments of 0.5-cm length; approximately 150 mg fresh weight) of field-sampled mixed plant roots. To cover a fraction of the intraspecific sequence variability, ten distinct sequences from two separate attempts (W4699/Att1211-0 and W5473/Att1210-5) were characterised and used for phylogenetic analyses of the ~1.5-kb SSU-ITS-LSU rDNA fragment.

The SSU rDNA sequences were submitted to the EMBL database with the accession numbers FN825898–900, those of the SSU-ITS-LSU rDNA regions with the accession numbers FN825901–912 and those for the DNA directly amplified from the roots with FR681926–936 and FR772326-334.

Phylogenetic analyses were performed with RAxML 7.2. (Stamatakis et al. 2008) hosted at the CIPRES Portal 2.2 (http://www.phylo.org/portal2/) using the GTRGAMMA model for the bootstrapping phase and for the final tree inference model, with 1,000 bootstraps. Analyses of the SSU rDNA, using sequences covering all main phylogenetic lineages in the *Glomeromycota*, clearly showed the new sequences obtained to be *Acaulospora*-related. Further phylogenetic analyses of the 1.5-kb fragment were then restricted to sequences from the *Acaulosporaceae* only incorporating all well-characterised sequences from the public databases and *Diversispora* sequences as outgroup.

The taxonomy and the sequence annotations used are adopted from the most recent systematic treatment of the *Glomeromycota* published by Schüßler and Walker (2010).

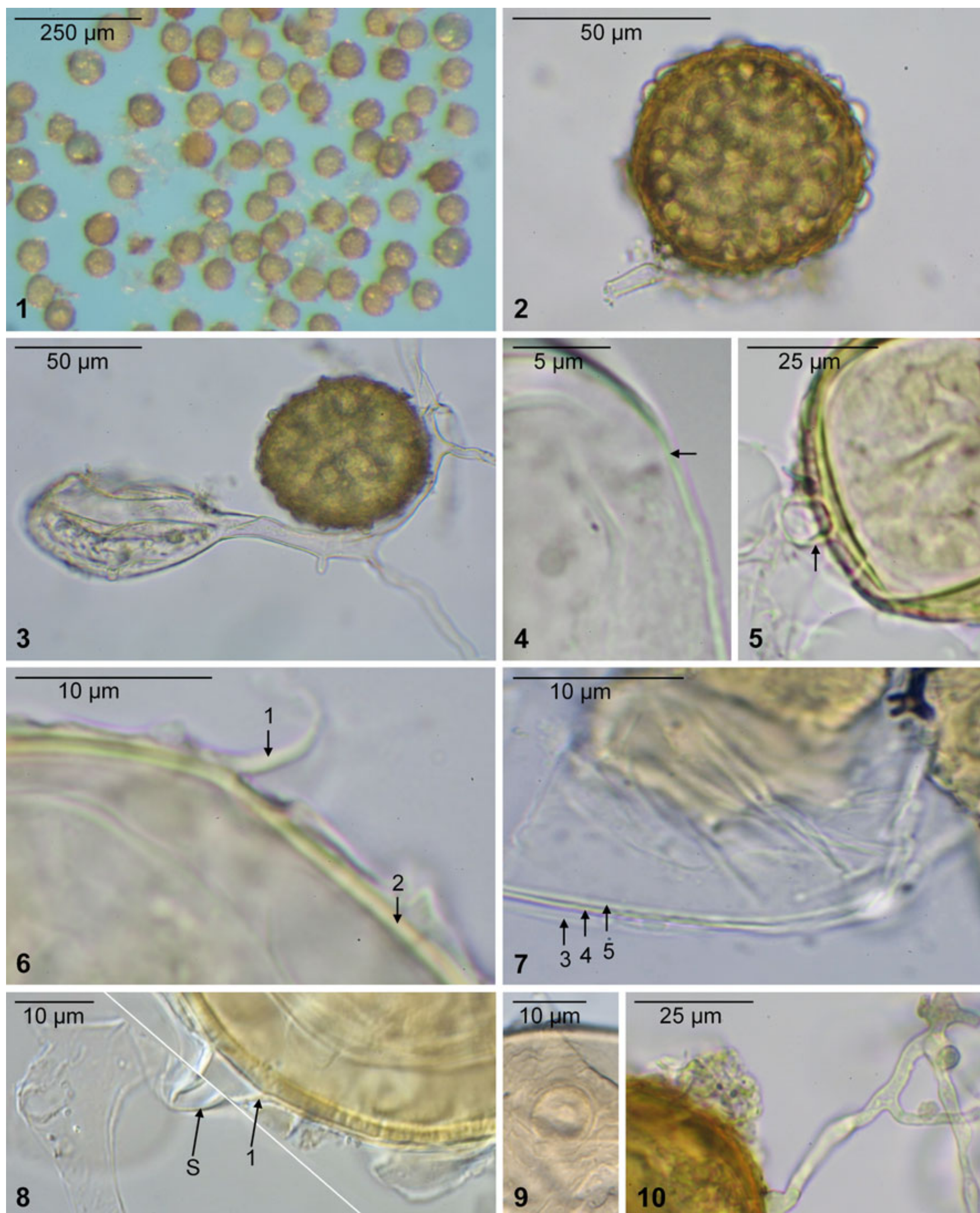
Results

The two dominantly sporulating species found in all three samplings from the upland moorland in Scotland possessed small, ornamented acaulosporoid spores. They were accompanied by a few spores of other glomeromycotan fungi. The trap cultures, in contrast, initially yielded only the two putative *Acaulospora* spp., later described as *A. alpina* by Oehl et al. (2006) and *A. brasiliensis* by Goto et al. (2008). Several unsuccessful attempts were made to isolate both these organisms in pot culture. Sporulation continued in these pots until March 2006, but when sampled again in October 2006 and in January 2008, no spore of either species was found. In November 2009, further sampling of the pot cultures revealed an *Ambispora* sp. (probably undescribed) and *Glomus ambisporum*, but all attempts at establishing subcultures of these species failed. The morphology of the spores of *A. alpina* was substantially as in the description of Oehl et al. (2006) and thus will not be discussed further herein.

Morphology of the *Ambispora brasiliensis* holotype

The holotype consists of a number of specimens mounted under two 22-mm square cover slips in what appears to be PVLG. There were 15 spores of the species concerned, as well as one spore of an undetermined species of *Scutellospora*, and two small, globose spores of an undetermined *Rhizophagus* sp. There were also a few other inclusions, but these were not glomeromycotan. All but four of the specimens were crushed, and only one had a short 'pedicel' at the point of origin. It was not possible to observe a scar or pedicel on any of the remaining spores. Because the spore base could not be identified, shortest by longest dimension of the four uncrushed specimens were measured. The resultant measurements were 72×88, 78×80, 75×83 and 69×75 μm. The crushed spores were also measured and their approximate original, uncrushed size was estimated to have been 64–88×64–88 μm. There was no saccule on the type slide, and thus no observations could be made for comparison with the original species description.

The wall structure of the type specimens was difficult to assess because, although they were crushed, in most specimens such detail was obscured and satisfactory observations were impossible. We interpret the most likely



structure to be A(UoL)B(F)C(FF), where U refers to a 'unit component', L to a laminated component, and F to a flexible component.

Morphology of the Scottish fungus

The appearance of the specimens (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) did not differ in glycerol, PVLG or PVLG-M. Because of the particularly small size of the spores, there is inevitably some doubt when interpreting the wall structure. Some components are flexible in nature, and because they wrinkle on crushing, it is often difficult to distinguish real components from artefactual ones resulting from folding. The outer component of the acaulospore wall of this species is also very difficult to see because of the ornamentation which usually obscures its origin.

The acaulospores have a sparkling brownish yellow appearance in water under reflected light (Fig. 1). The

◀**Fig. 1** *Acaulospora brasiliensis* comb. nov. Several acaulospores, some with attached saccules, extracted from substrate by swirling and decanting

Fig. 2 *Acaulospora brasiliensis* comb. nov. Individual spore, detached from the saccule, showing the collicular ornamentation on the outermost surface

Fig. 3 *Acaulospora brasiliensis* comb. nov. Spore still attached to the colourless, transparent, collapsed sporiferous saccule

Fig. 4 *Acaulospora brasiliensis* comb. nov. Detail of saccule wall, showing a single component (indicated by an *arrow*)

Fig. 5 *Acaulospora brasiliensis* comb. nov. Pedicel-like spore base (indicated by an *arrow*) formed by the thickened saccule neck at the point of spore development

Fig. 6 *Acaulospora brasiliensis* comb. nov. Point at which the spore has detached from the saccule showing a short 'pedicel' and the components of the main structural wall group (indicated by the numbers *1* and *2*, respectively)

Fig. 7 *Acaulospora brasiliensis* comb. nov. Structure of the apparent middle (*3*) and paired innermost wall components (*4* and *5*)

Fig. 8 *Acaulospora brasiliensis* comb. nov. Composite image at two depths of focus (joined at the *white diagonal line*), showing the continuous nature of the saccule wall (*S*) and the outermost component of the acaulospore (*1*)

Fig. 9 *Acaulospora brasiliensis* comb. nov. Caldera-shaped scar at the point of detachment of the spore from the saccule

Fig. 10 *Acaulospora brasiliensis* comb. nov. Germinating acaulospore; the thick, coloured outer wall components obscure the contents, and it is not possible to see if a germination shield is formed

colour of the spores varied depending on the collection. A few were more or less colourless (hyaline), but most were various shades of yellow to brown (Figs. 2, 3; Table S1). Some specimens were found with the sporiferous saccule still attached, though in all of these, it was collapsed and devoid of contents (Figs. 1, 3). The saccule wall appears to consist of just one component, about 1 μm thick (Fig. 4, arrow). The majority of spores had become detached in the manner typical of most species in the genus *Acaulospora.* We did not find a saccule with content or with young or developing spores attached.

The wall structure followed the expected pattern for members of the genus *Acaulospora* in that it consisted of a continuation of the saccule wall (Fig. 6), overlaying a laminated, pigmented, and relatively rigid, main structural component up to 4 μm thick, but mostly between 1 and 2 μm. These constitute a single wall group, A. This outer wall group is brittle and it fragments readily upon heavy crushing. Although the saccule wall itself and the mycelium from which it is formed, are smooth, component 1 is ornamented to varying degrees with large, colourless collicles (more or less rounded elevations, Fig. 2) up to 10 μm high, and in length and width up to 20×30 μm, seemingly developed from the saccule wall component (Fig. 8). In outline, the collicles may be smooth or irregular. They vary considerably in size, and their outlines in plain view also is variable, from circular to oval to irregular with smooth to jagged boundaries. Their height, even on the same specimen, can vary from about 1 to 10 μm. On some spores, they are low and quite difficult to see, whereas on others, they are immediately evident, even under the dissecting microscope. Occasional specimens are almost smooth with only a few collicles remaining attached to the structural component, indicating that perhaps this outer component may break down over time.

Inside the main structural wall group there sometimes appears to be a second group, B that is very difficult to observe. It is a single very thin flexible component up to, but normally considerably less than, 1 μm thick (Fig. 7). On most spores, it cannot be seen at all and might be an artefact of microscopy. It is more likely to be an ontogenetic character, as a similar group occurs in spores of some *Acaulospora* spp. that have been studied developmentally (e.g., Stürmer and Morton 1999). If it is part of a developmental sequence, it either is delicate, disintegrating when the spore is crushed, or it is ephemeral, disappearing at spore maturity. We could not resolve which is correct. Surrounded by this is a third wall group, C, consisting of a pair of apparently adherent thin components (Fig. 7). The outermost of these is very thin (<1 μm) and flexible, detaching on crushing from an innermost component (up to 1 μm thick) which encloses the spore contents.

There is either a short pedunculate stalk (Fig. 5) formed from the proximal part of the sporiferous saccule wall or a distinct caldera-shaped scar resulting from a slightly raised collar at the point of formation of the laminated wall component (Fig. 9). There was no reaction to Melzer's reagent. Glomoid spores were not found in either field samples or pot cultures. Germination was observed in one specimen (Fig. 10), but it was not possible to distinguish any pre-germination structure such as a germination shield on this spore.

Spore size comparison of holotype and Scottish material

Fungal spore size measurements should be quoted as 'length by width' (Hawksworth et al. 1983). Ours are made by taking the length as normal to the spore base (origin of spore) and the width at right angles to this. By following this convention (see e.g. Thaxter 1922; Gerdemann and Trappe 1974) it is possible to determine if spores are broader than they are long, and to compare shapes by using terms such as ovoid versus obovoid and pyriform versus obpyriform. The dimensions given by Goto et al. (2008) in the protologue of *A. brasiliensis* seem to be simply shortest dimension (presumably width) by longest dimensions (presumably length) without reference to the spore base. We have combined the dimensions given in the protologue with our own measurements for the description of the new combination.

The size range of the Scottish spores is somewhat smaller than that given in the protologue of *A. brasiliensis*. We consider the difference between 48–91×51–96 μm,

mean 66×67 μm (n=215) for the Scottish material, and 59–88×69–100(−118) μm (mean and number of specimens measured unstated in the protologue) given for the Brazilian specimens to be within the intraspecific range of glomeromycotan spores. Measurements of the images in the protologue give one complete spore at 74×84 μm, and two for which only a single dimension could be measured at 88 and 93 μm, respectively. All these values are within the range of the Scottish material as well as our measurements of the spores in the holotype (64–88×64–88, mean 75×78 μm, n=15).

Phylogenetic analyses

The phylogenetic analysis of the SSU rRNA gene sequences (Fig. 1) clearly showed that the species described as *A. brasiliensis* (Goto et al. 2008) clusters with *Acaulospora* (*Acaulosporaceae*, *Diversisporales*) and not with *Ambispora* (*Ambisporaceae*, *Archaeosporales*). Thus, the species not only belongs in a different genus from that proposed in the protologue, but consequentially it must also be placed in a different order. For achievement of species-level resolution, we analysed an approximately 1.5 kb rDNA fragment and we also characterised part of the intraspecific variability for this fragment (Krüger et al. 2009; Stockinger et al. 2010). When compared with the species for which sequence information is available, the Scottish fungus appeared most closely related to the recently published species *Acaulospora colliculosa* (Kaonongbua et al. 2010), followed by *A. alpina* (Fig. 12). We also detected the *A. brasiliensis*-like fungus in plant roots from the Scottish sampling site (sample no. 1518, Meall nan Tarmachan, 16 April 2010). Sequences representing *A. alpina* (Fig. 12), a *Scutellospora* sp. closely related to, but not conspecific with *S. gilmorei* (not shown), an unknown *Rhizophagus* sp. (not shown), and a further, unknown *Acaulospora* species also were obtained from the same plant root sample. Both the phylogenetic trees computed from the SSU rDNA and the ITS-LSU rDNA fragments, unquestionable show that the Scottish fungus, morphologically appearing conspecific with *A. brasiliensis*, clusters within *Acaulospora* (*Acaulosporaceae*) and does not belong in the *Ambisporaceae*.

Formal transfer of *Ambispora brasiliensis* to *Acaulospora*

Acaulospora brasiliensis (B.T. Goto, L.C. Maia & Oehl) C. Walker, M. Krüger & A. Schüßler comb. nov. (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12).

MycoBank no. MB 518748

Basionym: *Ambispora brasiliensis* B.T. Goto, L.C. Maia & Oehl, Mycotaxon 105: 13 (2008) (MycoBank no. 511612).

Acaulosporoid spores (acaulospores) borne singly in the soil, laterally in the neck of a hyaline sporiferous saccule, almost colourless to yellow to olive yellow to very pale brown to brownish yellow to yellowish brown to reddish yellow to yellowish red, globose to subglobose to broadly ellipsoid (rarely irregular), 48–91×51–100 μm (rarely up to 118 μm in the longest dimension). Spore wall structure of five components 1–5 in three groups, A–C. Group A of two components; outer component hyaline, originating from the neck of the sporiferous saccule, forming a collicular ornamentation of variable size, apparently arising from a continuous basal layer approximately 1 μm thick, tightly adherent to a laminated, pigmented structural component, its point of origin appearing as a slightly raised collar or occasionally as a pedicel of variable length. Wall group B of one thin, flexible, hyaline, component, <1 μm thick. Wall group C, of two components, the outermost very thin and elastic, up to 1 μm thick, juxtaposed with a more robust component, approximately 1 μm thick enclosing the spore contents. No reaction to Melzer's reagent.

Distribution and habitat Known from the Cerrado biome of Serra do Cipó, Minas Gerais State, Brazil (Goto et al. 2008) from a site described as 'mainly consisting of *Velozzia caruncularis*', and from an upland heathland in Scotland in which the dominant vegetation consists of *Festuca vivipara* and *Nardus stricta*, with *Salix herbacea*, *Alchemilla alpina*, *Vaccinium myrtillus*, *V. vitis-idea*, *Galium rotundifolium*, *G. saxatile*, *Carex* spp., and *Rhacomitrium lanuginosum*. From sequence analyses, it is known to be a member of a glomeromycotan community among the roots of these plants, including *A. alpina*, another *Acaulospora* sp., a *Scutellospora* sp. closely related to *S. gilmorei* and an undetermined *Rhizophagus* sp.

Mycorrhizal associations are unknown, but root colonisation shown by DNA-based detection in plant roots that were sampled from the field site.

Specimens examined Typus: Brazil. Minas Gerais. Serra do Cipó, beneath cerrado vegetation (dominated by *Velozzia caruncularis*). Microscope slide (URM78879) dated 15 Aug. 2006. In the protologue, the collection date is given as 'July 2004'.

United Kingdom, Scotland, Perthshire, Ben Lawers National Nature Reserve, Meall nan Tarmachan (Hill of the Ptarmigan), approximately 900 m amsl, from within 200 m of UK National Grid Reference: NN58789 38612 (latitude, 56.518284N; longitude, 4.296748W) from soil beneath heathland vegetation or from subsequent pot cultures. C. Walker (voucher numbers preceded by W). W4514 from sample 1136 on 23 Sep 2003; W5748 from Sample 1517; W5751 from sample 1518; W5755 from sample 1519; W5759 from sample 1520; W5762 from sample 1521; W5765 from sample 1522, all collected 16 April 2010. W5827 from sample 1527, close to Lochan na Lairige (56°31′14.20″N 4°16′47.60″W) at approximately

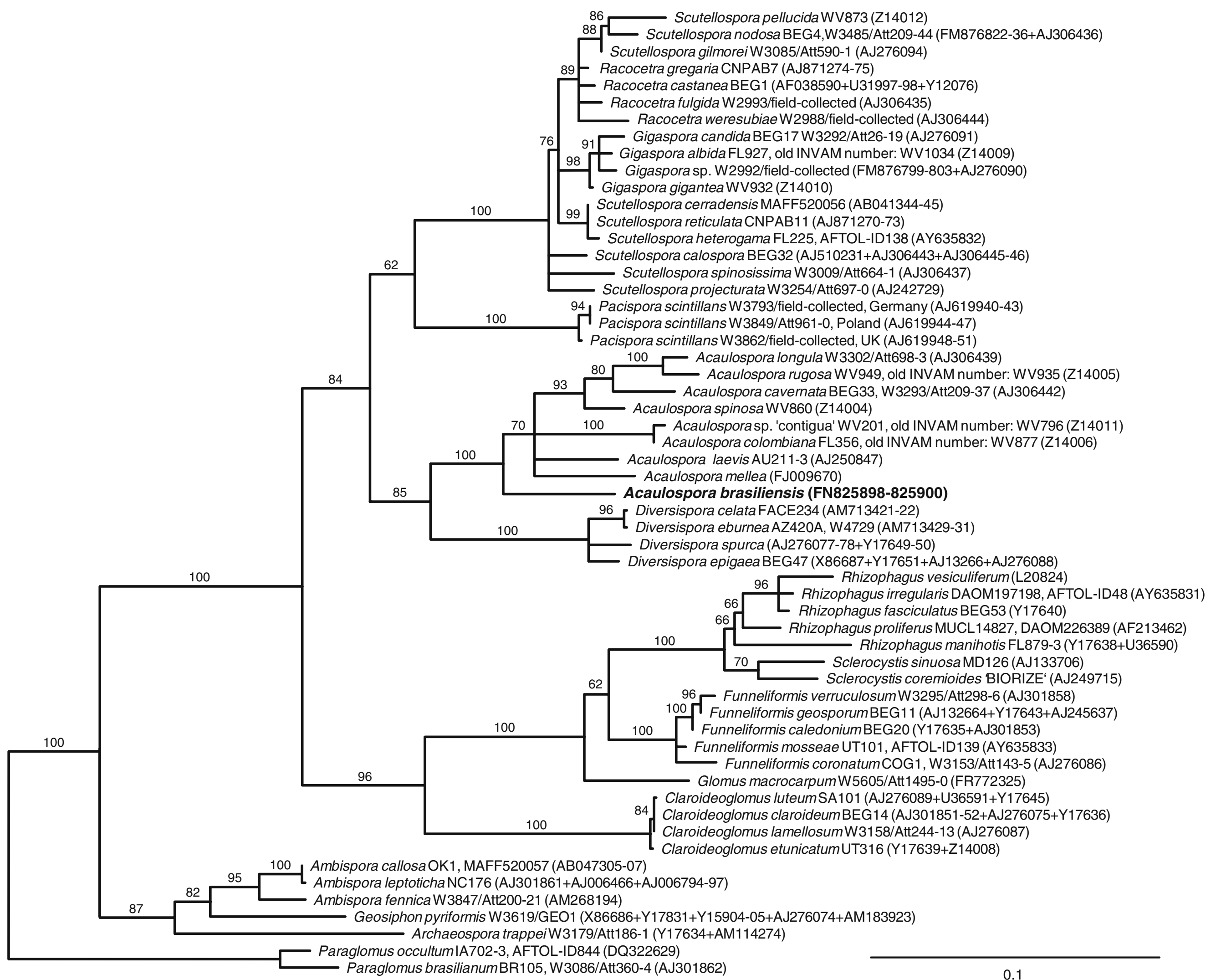


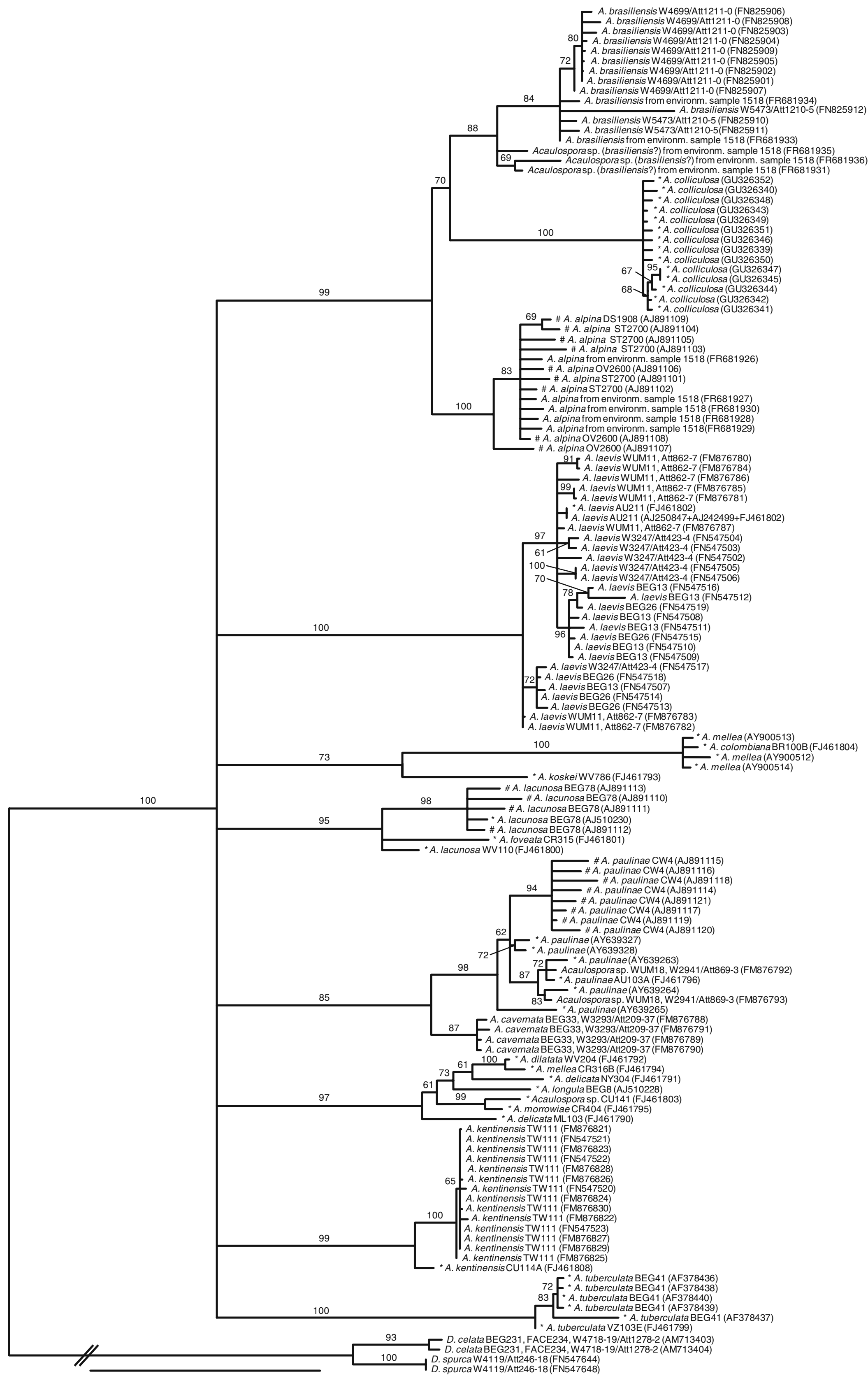
Fig. 11 Phylogenetic maximum likelihood tree computed with RAxML from individual or consensus sequences of near full-length SSU rRNA gene sequences, including all main lineages of the *Glomeromycota*. New taxa are adopted from Schüßler and Walker (2010). Support values derived from a 1,000-fold bootstrapped analysis are shown on the branches; values below 60% were considered as unresolved and the respective topologies were collapsed to polytomies. *Paraglomus* sequences were used as outgroup

500 m amsl, collected 6 September 2010. *From trap pot cultures* from containing *Festuca vivipara*, *Nardus stricta* and *Galium rotundifolium*: W4699 from Att1211-0 from sample 1136 on 19 September 2004; W4786 from Att1210-0 from sample 1136 on 6 February 2006; W4796 from Att1210-0 from sample 1136 on 21 February 2006; W4833 from Att1210-0 from sample 1136 on 15 July 2006.

Discussion

We showed first records of two *Acaulospora* spp., *A. alpina* and *A. brasiliensis* from a Scottish upland. The latter species was initially described as *Ambispora brasiliensis* (Goto et al. 2008) and is transferred to *Acaulospora* (*Acaulosporaceae*) based on molecular evidence and morphological characterisation.

To study its morphology, isotypes of *A. brasiliensis* were requested as a loan from the herbaria OSC and Z+ZT (Oregon State University and Zurich), but neither of them could locate the specimens concerned. Nevertheless, it is clear from the holotype and the protologue of *A. brasiliensis* that there are no significant differences between spores of the Brazilian and Scottish organisms, and we conclude they are conspecific. Goto et al. (2008) described, but did not illustrate, one glomoid spore of 25–30 μm in diameter attached to a germinating hypha from a single acaulosporoid spore. The Scottish collections contained glomoid spores of an *Ambispora* sp., but these were very large (~300 μm in diameter) in comparison with those of *A.*



A. brasiliensis W4699/Att1211-0 (FN825906)
A. brasiliensis W4699/Att1211-0 (FN825908)
A. brasiliensis W4699/Att1211-0 (FN825903)
A. brasiliensis W4699/Att1211-0 (FN825904)
A. brasiliensis W4699/Att1211-0 (FN825909)
A. brasiliensis W4699/Att1211-0 (FN825905)
A. brasiliensis W4699/Att1211-0 (FN825902)
A. brasiliensis W4699/Att1211-0 (FN825901)
A. brasiliensis W4699/Att1211-0 (FN825907)
A. brasiliensis from environm. sample 1518 (FR681934)
A. brasiliensis W5473/Att1210-5 (FN825912)
A. brasiliensis W5473/Att1210-5 (FN825910)
A. brasiliensis W5473/Att1210-5(FN825911)
A. brasiliensis from environm. sample 1518 (FR681933)
Acaulospora sp. (brasiliensis?) from environm. sample 1518 (FR681935)
Acaulospora sp. (brasiliensis?) from environm. sample 1518 (FR681936)
Acaulospora sp. (brasiliensis?) from environm. sample 1518 (FR681931)
* A. colliculosa (GU326352)
* A. colliculosa (GU326340)
* A. colliculosa (GU326348)
* A. colliculosa (GU326343)
* A. colliculosa (GU326349)
* A. colliculosa (GU326351)
* A. colliculosa (GU326346)
* A. colliculosa (GU326339)
* A. colliculosa (GU326350)
* A. colliculosa (GU326347)
* A. colliculosa (GU326345)
* A. colliculosa (GU326344)
* A. colliculosa (GU326342)
* A. colliculosa (GU326341)
# A. alpina DS1908 (AJ891109)
# A. alpina ST2700 (AJ891104)
# A. alpina ST2700 (AJ891105)
# A. alpina ST2700 (AJ891103)
A. alpina from environm. sample 1518 (FR681926)
A. alpina OV2600 (AJ891106)
A. alpina ST2700 (AJ891101)
A. alpina ST2700 (AJ891102)
A. alpina from environm. sample 1518 (FR681927)
A. alpina from environm. sample 1518 (FR681930)
A. alpina from environm. sample 1518 (FR681928)
A. alpina from environm. sample 1518(FR681929)
A. alpina OV2600 (AJ891108)
A. alpina OV2600 (AJ891107)
A. laevis WUM11, Att862-7 (FM876780)
A. laevis WUM11, Att862-7 (FM876784)
A. laevis WUM11, Att862-7 (FM876786)
A. laevis WUM11, Att862-7 (FM876785)
A. laevis WUM11, Att862-7 (FM876781)
* A. laevis AU211 (FJ461802)
A. laevis AU211 (AJ250847+AJ242499+FJ461802)
A. laevis WUM11, Att862-7 (FM876787)
A. laevis W3247/Att423-4 (FN547504)
A. laevis W3247/Att423-4 (FN547503)
A. laevis W3247/Att423-4 (FN547502)
A. laevis W3247/Att423-4 (FN547505)
A. laevis W3247/Att423-4 (FN547506)
A. laevis BEG13 (FN547516)
A. laevis BEG13 (FN547512)
A. laevis BEG26 (FN547519)
A. laevis BEG13 (FN547508)
A. laevis BEG13 (FN547511)
A. laevis BEG26 (FN547515)
A. laevis BEG13 (FN547510)
A. laevis BEG13 (FN547509)
A. laevis W3247/Att423-4 (FN547517)
A. laevis BEG26 (FN547518)
A. laevis BEG13 (FN547507)
A. laevis BEG26 (FN547514)
A. laevis BEG26 (FN547513)
A. laevis WUM11, Att862-7 (FM876783)
A. laevis WUM11, Att862-7 (FM876782)
* A. mellea (AY900513)
* A. colombiana BR100B (FJ461804)
* A. mellea (AY900512)
* A. mellea (AY900514)
* A. koskei WV786 (FJ461793)
# A. lacunosa BEG78 (AJ891113)
# A. lacunosa BEG78 (AJ891110)
# A. lacunosa BEG78 (AJ891111)
* A. lacunosa BEG78 (AJ510230)
# A. lacunosa BEG78 (AJ891112)
* A. foveata CR315 (FJ461801)
* A. lacunosa WV110 (FJ461800)
# A. paulinae CW4 (AJ891115)
# A. paulinae CW4 (AJ891116)
# A. paulinae CW4 (AJ891118)
# A. paulinae CW4 (AJ891114)
# A. paulinae CW4 (AJ891121)
# A. paulinae CW4 (AJ891117)
# A. paulinae CW4 (AJ891119)
# A. paulinae CW4 (AJ891120)
* A. paulinae (AY639327)
* A. paulinae (AY639328)
* A. paulinae (AY639263)
Acaulospora sp. WUM18, W2941/Att869-3 (FM876792)
* A. paulinae AU103A (FJ461796)
* A. paulinae (AY639264)
Acaulospora sp. WUM18, W2941/Att869-3 (FM876793)
* A. paulinae (AY639265)
A. cavernata BEG33, W3293/Att209-37 (FM876788)
A. cavernata BEG33, W3293/Att209-37 (FM876791)
A. cavernata BEG33, W3293/Att209-37 (FM876789)
A. cavernata BEG33, W3293/Att209-37 (FM876790)
* A. dilatata WV204 (FJ461792)
* A. mellea CR316B (FJ461794)
* A. delicata NY304 (FJ461791)
* A. longula BEG8 (AJ510228)
* Acaulospora sp. CU141 (FJ461803)
* A. morrowiae CR404 (FJ461795)
* A. delicata ML103 (FJ461790)
A. kentinensis TW111 (FM876821)
A. kentinensis TW111 (FN547521)
A. kentinensis TW111 (FM876823)
A. kentinensis TW111 (FN547522)
A. kentinensis TW111 (FM876828)
A. kentinensis TW111 (FM876826)
A. kentinensis TW111 (FN547520)
A. kentinensis TW111 (FM876824)
A. kentinensis TW111 (FM876830)
A. kentinensis TW111 (FM876822)
A. kentinensis TW111 (FN547523)
A. kentinensis TW111 (FM876827)
A. kentinensis TW111 (FM876829)
A. kentinensis TW111 (FM876825)
* A. kentinensis CU114A (FJ461808)
* A. tuberculata BEG41 (AF378436)
* A. tuberculata BEG41 (AF378438)
* A. tuberculata BEG41 (AF378440)
* A. tuberculata BEG41 (AF378439)
* A. tuberculata BEG41 (AF378437)
* A. tuberculata VZ103E (FJ461799)
D. celata BEG231, FACE234, W4718-19/Att1278-2 (AM713403)
D. celata BEG231, FACE234, W4718-19/Att1278-2 (AM713404)
D. spurca W4119/Att246-18 (FN547644)
D. spurca W4119/Att246-18 (FN547648)
0.1

◀ **Fig. 12** Phylogenetic maximum likelihood tree computed with RAxML from approximately 1.5 kb sequences covering approximately 250 bp of the SSU rRNA gene, the whole ITS region and an approximately 800 bp of the LSU rRNA gene, of members of the *Acaulosporaceae*. Some shorter sequences from the public databases were also included for comparison and are marked as follows: *number sign*, covering partial SSU and whole ITS region; *asterisk*, covering partial LSU. Support values derived from a 1,000-fold bootstrapped analysis are shown on the branches; values below 60% were considered as unresolved and the respective topologies were collapsed to polytomies. The tree was rooted with *Diversispora* sequences as outgroup; the root was shortened by 50%, as indicated by *diagonal slashes*

brasiliensis, and corresponded with the descriptions given for members of *Ambispora* (Walker et al. 2007). No glomoid spores have been found linked to the Scottish acaulospores. Therefore, more evidence is needed before the asserted dimorphic nature of this organism can be verified.

The Brazilian acaulospores have a slightly larger maximum dimension than those from Scotland, but similar differences even occur among subcultures of single-spore AMF isolates (Walker and Vestberg 1998). Though the Brazilian spores are described as being 'hyaline to light yellow', images in the protologue show them to be yellow to brown. The range of colour for the Scottish collections is almost colourless to yellow to pale yellow brown or reddish brown. Such differences are likely to result from different perceptions and methods of comparison and, as the slight size differences, are not sufficient to separate species. The 'pedicel' used to place the organism in *Ambispora* is not a feature confined to that genus being present on members of *Acaulospora* and *Entrophospora infrequens* (Hall 1977). Some specimens of *A. brasiliensis* from Scotland had a short stalk although most had only a circular or oval scar as seen in most *Acaulospora* spores. The illustration of a 'collar' in the Brazilian species description (Goto et al. 2008) is similar to those typical of spores in the genus *Acaulospora*, showing that both scars and short 'pedicels' may be present.

We could not reconcile the wall structure in the species description with either the holotype specimens or those in our own collections. Even with large-spored species, it usually is impossible to follow spore development from field-collected material. In our collections and trap cultures, we have so far found spores either completely sessile or attached only to empty and collapsed saccules. Thus, it was impossible to follow the development of the saccules or spore wall structure. The thickened and uneven ornamentation on the acaulospore surface makes it difficult to determine wall structure or to see internal structures such as a germination shield.

The sporiferous saccule wall is described by Goto et al. (2008) as being two-layered, but their illustrations do not convincingly illustrate more than one layer, and saccules are completely lacking from the holotype material available to us. Their 'evanescent outer layer' appears to be soil particles adherent to the collapsed and decaying saccule. We have been unable to see more than a single wall component in our specimens, and from the images in the protologue, the wall structure seems the same as that observed in the Scottish material. In our interpretation, the main structural wall group of the spore consists of two components. The first is colourless and seems to be continuous with the wall of the saccule. It is ornamented to varying degrees with pustule-like collicles which occur only around the spore and not on the saccule itself. However, the limitations of light microscopy on such small specimens must be considered. The illustration of the pedicel in Goto et al. (2008) as continuous with the main structural spore wall ('outer wall') does not adequately illustrate such a feature. Although one specimen on the holotype slide does have a short pedicel, it is presented in such a way that its structure and relationship to the wall components of the acaulospore could not be determined. We interpret it as part of the outermost component (the saccule wall). Tightly adherent to it is the coloured outer component of the spore itself. This is probably 'laminated', though in many specimens it is so thin that layers cannot be seen. Many spores of glomeromycotan species seem to have such a laminated component as the main structural component or layer. We, therefore, interpret the wall structure of wall group 1 as consisting of one component originating from the saccule wall and a second component, the structural wall of the acaulospore, that is probably produced de novo within a lateral swelling in the saccule neck. Goto et al. (2008), however, consider that the saccule has two components (layers) that later differentiate into two separate 'walls', the outermost having three layers and the innermost having two layers. From examination of many specimens, it is clear that the inner wall groups lack any attachment to either the saccule wall or the main structural wall group of the acaulospore. Spores of both *Acaulospora* spp. and *Ambispora* spp. develop their main structural wall de novo within the saccule wall (Kaonongbua et al. 2010; Stürmer and Morton 1999; Walker et al. 2007).

Moving towards the interior of the spore, Goto et al. (2008) describe a 'middle wall' that consists of two layers (formed by differentiation from the saccule wall). Such a development has not been recorded for any species in the *Glomeromycota*, and in particular is different from the structure of either *Ambispora* or *Acaulospora* (Kaonongbua et al. 2010; Walker et al. 2007). We could see only a very thin flexible component that we consider to be a second wall group because sometimes, upon crushing the spore, it remains close to wall group 1, and sometimes to the innermost group (group 3). Goto et al. (2008) illustrate a third 'wall' consisting of three layers. We interpret the third

wall group as having two distinct components of more or less equal thickness, though sometimes only a single one could be seen. We were able to see what we thought might be a germination shield from a lateral view on one specimen (not shown), but we were not certain that we were interpreting it correctly. Goto et al. (2008) described (but did not illustrate) a germination shield on one spore only as being a lobed structure similar to that present in spores of species in *Scutellospora* or *Racocetra* (Morton and Msiska 2010). We could not find a germination shield on any of the holotype specimens.

With the exception of *A. colliculosa*, no other member of the *Acaulosporaceae* has small, yellow to brownish yellow acaulosporoid spores possessing collicular ornamentation. The spores of *A. brasiliensis* lack reaction to Melzer's reagent, even after the most vigorous crushing on a microscope slide with PVLG/Melzer's (4:1, *v*/*v*) and in pure Melzer's reagent. Although most *Acaulospora* species react to this reagent, producing a pale purple to dark purple colour associated with at least one internal component, a few species, such as *A. laevis*, and *A. colliculosa* (Kaonongbua et al. 2010) lack such a reaction. However, *A. alpina*, which is a close relative of *A. brasiliensis*, possesses an inner wall component that becomes purple when spores are crushed in PVLG/Melzer's (Oehl et al. 2006; C. Walker unpublished). This provides support for the opinion that the reaction to Melzer's reagent may not be a phylogenetically informative character (Kaonongbua et al. 2010).

Neither ourselves nor Goto et al. (2008) have been able to establish the fungus in pure culture or to isolate it by single-spore culturing attempts. Spores of *A. brasiliensis* have been produced only in pot cultures established from field soil and natural plants, but these could not be maintained even by moving entire plants to a new pot of sterilised substrate. However, we could directly detect the presence of *A. brasiliensis* in field-collected roots from the Scottish location by molecular biological methods, together with *A. alpina* and one undetermined AMF species each of *Scutellospora* (closely related to *S. gilmorei*), *Rhizophagus* (different from any other species yet sequenced from this genus), and *Acaulospora* (clustering in a monophyletic clade with *A. colliculosa*, *A. brasiliensis* and *A. alpina*). It will still be necessary to establish it in pure culture before its mycorrhizal nature can be confirmed through the application of Koch's postulates.

Acaulospora alpina was previously known only from altitudes above 1,300 m amsl in the alpine region of mainland Europe. Although the Scottish locations are at much lower altitude (500–900 m amsl), the climatic conditions in Scotland are also very severe, but soil conditions and plant communities clearly are very different in these ecosystems. The Scottish samples came from a thin, peaty soil of approximately pH 5, overlaying a 'Ben Lawers schist'. In contrast, the bedrock in the alpine areas from which *A. alpina* is known seems to be very variable. Spores of *A. alpina* were found in '...acidic sandstones, siliceous gneiss and granite rocks, up to ultrabasic serpentinite and calcareous "Bündner Schiefer" schists and carbonatic and dolomitic limestones ...' (Oehl et al. 2006). The pH value given is five for the sample from which the type material came. However, it is much more unexpected to find a fungus, *A. brasiliensis*, reported from a dry, cerrado ecosystem with predominantly summer rainfall (Minas Gerais State, Brazil) on almost permanently wet, cold, peaty Scottish moorland. Nevertheless, the bedrock in the Serra do Cipó also seems to be igneous, and has a low pH of 4.7 (Goto et al. 2008), as does the Scottish site (pH 4–5). Low pH has been shown as a likely key factor in affecting populations of glomeromycotan fungi in agricultural conditions (Wang et al. 1985).

The distribution of some species in the *Glomeromycota* is known to be very wide with respect to different site conditions (Börstler et al. 2010), even to the point of speculation that humans have been responsible for spread through agricultural practices (Rosendahl et al. 2009). *A. brasiliensis* to date is known only from two sites that are not so heavily influenced by humans and its occurrence in such widely different ecosystems could lead to suggestions that it may be very widespread. On the other hand from two records, it is certainly too early to draw conclusions about its ecological preferences as a species, and it is not too far from the truth that the known distribution of organism may reflect the distribution of people interested in them rather than their true spread. As far as we can discover, the only common factor seems to be igneous bedrock with low soil pH, and this might be one of the problems in relation to establishing pot cultures. Molecular tools with species-level resolution should soon provide a better basis for interpreting such ecological and biogeographical information at the level of species on a secure foundation.

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