DNA-based characterization of arbuscular mycorrhizal fungi associated with potato roots from the Andean region

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München, den 14 Oktober 2014

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Carolina Senés Guerrero

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List of abbreviations (except SI units)

~	approximately
ANOVA	analysis of variance
AM	arbuscular mycorrhiza(l)
AMF	arbuscular mycorrhizal fungi
approx.	approximate(ly)
Att	Attempt
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
bp	base pair(s)
BSA	bovine serum albumin
comb. nov.	Latin: combinatio nova (English: new combination)
DAOM	Agriculture and Agri-Food Canada National Mycological Herbarium
DAS	days after sowing
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
e.g.	Latin: exempli gratia (English: for example)
H´	Shannon diversity index
HAM-P	half-closed arbuscular mycorrhizal plant culture
HC	hyphal colonization
INSDC	International Nucleotide Sequence Databases Collaboration
ITS	internal transcribed spacer
Kb	kilo base pair(s)
LSU	large subunit
mamsl	meters above mean sea level
meq	milliequivalent of hydrogen
MID	multiplex identifiers
Mt	million tons
mtDNA	mitochondrial DNA
MSR	modified Strullu-Romand
MUCL	Mycothèque de l'Universite Catholique de Louvain
Mya	million years ago
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NPK	nitrogen, phosphorus, potassium
OTU	operational taxonomic unit
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
perMANOVA	permutational MANOVA
PVLG	polyvinyl alcohol lacto-glycerol
PVLG-M	PVLG with Melzer´s reagent
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROC	root organ culture
rRNA	ribosomal RNA
RTL	RefSeq targeted loci
SC	spore colonization

SD	standard deviation
SE	standard error
SPRI	solid phase reversible immobilization
SSU	small subunit
Taq	*Thermus aquaticus*
TE	Tris-EDTA
U	unit (of enzyme activity)
VALORAM	valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems

List of publications

Senés–Guerrero C, Schüßler A. A conserved AMF core-species community structure in potato roots from the Andes. Submitted to Fungal Diversity (28.09.2014).

Potten V, **Senés–Guerrero C**, Torres–Cortés G, Schüßler A, Walker C, Declerck S, Cranenbrouck S. Epitypification and DNA barcoding of *Rhizophagus invermaius* (=*Glomus invermaium*) with a re–description from *in vivo* and *in vitro* cultures. Submitted to Mycologia (02.07.2014).

Senés–Guerrero C, Torres–Cortés G, Pfeiffer S, Rojas M, Schüßler A. 2014. Potato–associated arbuscular mycorrhizal fungal communities in the Peruvian Andes. *Mycorrhiza* **24**: 405–417.

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson–Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grenbec T, Griffith WG, Hartmann M, Kirk MP, Kohout P, Larsson E, Lindahl DB, Lücking R, Martín PM, Matheny PB, Nguyen HN, Niskanen T, Oja J, Peay GK, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott AJ, **Senés C**, Smith EM, Suija A, Taylor LD, Telleria TM, Weiss M, Larsson HK. 2013. Towards a unified paradigm for sequence–based identification of fungi. *Molecular Ecology* 22: 5271–5277.

Contribution of the author

In this thesis I present the results of my doctoral research, carried out in Munich from September 2010 to August 2014 under the guidance of PD. Dr. Arthur Schüßler.
My thesis resulted in four manuscripts, two of them are published (Chapter 3 and Chapter 5) and two of them are submitted (Chapter 4 and Chapter 6).

3) Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson–Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grenbec T, Griffith WG, Hartmann M, Kirk MP, Kohout P, Larsson E, Lindahl DB, Lücking R, Martín PM, Matheny PB, Nguyen HN, Niskanen T, Oja J, Peay GK, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott AJ, **Senés C**, Smith EM, Suija A, Taylor LD, Telleria TM, Weiss M, Larsson HK. 2013. Towards a unified paradigm for sequence–based identification of fungi. *Molecular Ecology* 22: 5271–5277.

Carolina Senés Guerrero served as a curator for the Pluto F web-based workbench of fungal ITS sequences. The tasks included annotating AMF sequences that could be reliably used as reference sequences, improving the taxonomic annotation of the deposited sequences and filtering/removing low quality sequences.

4) Potten V, **Senés–Guerrero C**, Torres–Cortés G, Schüßler A, Walker C, Declerck S, Cranenbrouck S. Epitypification and DNA barcoding of *Rhizophagus invermaius* (=*Glomus invermaium*) with a re–description from *in vivo* and *in vitro* cultures. Submitted to Mycologia.

Carolina Senés Guerrero generated some of the published new sequences, conducted the phylogenetic analyses and wrote parts of the manuscript together with all the other authors.

5) **Senés–Guerrero C**, Torres–Cortés G, Pfeiffer S, Rojas M, Schüßler A. 2014. Potato–associated arbuscular mycorrhizal fungal communities in the Peruvian Andes. *Mycorrhiza* **24**: 405–417.

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6) **Senés–Guerrero C**, Schüßler A. A conserved AM fungal core-species community colonizes potato roots in the Andes. Submitted to Fungal Diversity.

Carolina Senés Guerrero generated all of the published new sequences, conducted the phylogenetic and statistical analyses and wrote the manuscript together with A. Schüßler.

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Carolina Senés Guerrero PD. Dr. Arthur Schüßler

Summary

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts of 70-90% of land plants, including the ten most important human food crops. In this plant-fungus interaction, AMF explore the soil with their extensive mycelium and transport water and nutrients (mainly phosphorus – P) to the plant host, which in exchange provides the AMF with up to 20% of its photosynthetically fixed carbon. Because AMF actively transport P, they can increase the utilization efficiency of fertilizers and are regarded as a potential solution to increase crop yields without aggravating the environment with high fertilizer inputs. Consequently, the use of AMF as inoculum for agricultural purposes is promising and may be most important for crops such as potato (*Solanum tuberosum*), which has a worldwide increasing value as a food crop but requires relatively high P amounts for its cultivation.

AMF are asexual clonal organisms, therefore it is not possible to define them by a biological species concept. The difficulty to define species both at the morphological and genetic levels impedes assessing the diversity of AMF communities under natural and agricultural environments and obstructs our understanding of the processes that influence the AMF-plant interactions. Thus, the principal goal of my thesis was the molecular characterization of AMF environmental samples to the species level.

My thesis was conducted in the frame of the European project VALORAM (VALORizing Andean microbial diversity through sustainable intensification of potato-based farming systems) which aimed to promote the sustainable development of potato-based agricultural systems in the Andean region by the use of native microbes that could potentially improve potato crops. Within this project, I was involved in AMF taxonomic annotation of ITS rDNA sequences in the curated sequence reference database “Pluto F”, I carried out research regarding the molecular characterization of AMF from both single-spore isolates and environmental AMF communities in potato plants from the Andes and analyzed plant related or environmental factors driving the Andean AMF community composition.

One of the main challenges of my thesis was to characterize the environmental AMF communities by using 454 pyrosequencing and annotating high throughput sequences to the species level, in order to determine preferential plant-

fungus associations and the driving forces affecting this interaction. Here, I present the molecular characterization of native AMF at the species level for single-spore isolates where morphological and phylogenetic evidence led to the identification of *Glomus invermaium*, and its transfer to the genus *Rhizophagus* as *Rhizophagus invermaius*, as well as the molecular characterization of AMF environmental communities in Peru where 50% of the detected species were unknown. Both of these results, single spores and AMF communities, were obtained by using a clone library and Sanger sequencing approach. The sequences obtained from these clone libraries, as well as other AMF reference sequences served as a base to develop a robust 454-sequencing based process to trace AMF environmental species. The method places representative 454 sequencing derived reads into a phylogenetic reference tree, which is based on a 1.5-1.8 kb DNA SSU-ITS-LSU rRNA gene region that was defined as an extended barcode suitable to delimit closely related AMF species. This approach allowed sequence annotation to both unknown and described species with high phylogenetic resolution. Members of a conserved core-species AMF community were identified. This conserved group of AMF species colonized most of the samples. Species from the genera *Acaulospora*, *Cetraspora*, *Claroideoglomus* and *Rhizophagus* colonized most of the root samples simultaneously and appear to be main players in potato AM in the Andean region. Interestingly, more than 25 AMF species can co-exist in a single root system which may be related to different complementary functions in distinct phylogenetic lineages. Moreover, the core-species AMF community of potato was conserved over a wide range of environmental conditions, even though there were many diverse factors inherent to the samples.

The methods described here for the identification of AMF species may allow the specific selection of AMF main players to be used as tailored made inocula, specifically designed to be successful colonizers of the determined plants in microbial inoculation schemes for sustainable agricultural practices.

Zusammenfassung

Arbuskuläre Mykorrhiza-Pilze (AMF) sind als obligate Symbionten mit 70-90% aller Landpflanzen, inclusive der zehn für die menschliche Ernährung wichtigsten Nutzpflanzen, assoziiert. In dieser Interaktion erschließen die AMF mit ihrem feinverzweigten Myzel den Boden und transportieren Wasser und anorganische Nährstoffe (insbesondere Phosphor, P) zur Wirtspflanze, welche im Austausch bis zu 20% des photosynthetisch fixierten Kohlenstoffes als Kohlenhydrate liefert. Da AMF Nährstoffe aktiv zur Pflanze transportieren, können sie die Nutzungseffizienz von Dünger verbessern und Ernteerträge erhöhen, was Umweltgefährdungen durch zu hohe Düngereinträge reduziert. Daher ist ihre Anwendung als Inokulum in landwirtschaftlichen Praktiken vielversprechend und kann für den Anbau von Pflanzen mit relativ hohem P-Bedarf, wie zum Beispiel Kartoffel (*Solanum tuberosum*) mit ihrer weltweit steigende Bedeutung als Nahrungsmittelpflanze, von großer Bedeutung sein.

Diese Doktorarbeit wurde im Rahmen des Europäischen Projektverbundes VALORAM (*VALORizing Andean Microbial diversity through sustainable intensification of potato-based farming systems*) durchgeführt, dessen Ziel die nachhaltige Verbesserung des Kartoffelanbaus in der Andenregion durch die Nutzung indigener Mikroorganismen war. Unser Projekt behandelte dabei die Charakterisierung von AMF Artengemeinschaften, die mit Kartoffelwurzeln in verschiedenen Wachstumsstadien und unter verschiedenen Umweltbedingungen assoziiert sind. Bisher existiert für die asexuellen AMF allerdings kein biologisches Artkonzept und es ist teilweise schwierig, sie durch morphologische und phylogenetische Artkonzepte anzusprechen. Dies erschwert, Artengemeinschaften im Feld zu bestimmen und limitiert unser Verständnis der Prozesse, welche die AMF-Pflanzen Interaktionen beeinflussen.

Im Rahmen des Projektes führte ich zunächst Annotationen von AMF ITS rDNA Sequenzen für die kurierte Sequenzdatenbank „Pluto F“ und erweitertes DNA-*barcoding* von AMF Arten sowohl aus Einzelspor-Isolaten als auch aus Umweltproben durch, um dann den Einfluss von geographischen und abiotischen Faktoren, und von Kartoffelvarietäten, auf die Kartoffel-assoziierte AMF Arten-Gemeinschaften in drei Ländern der Andenregion mittels Hochdurchsatzsequenzierung zu untersuchen.

Eine Hauptherausforderung dieser Arbeit war, AMF Artengemeinschaften im Feld mittels 454GS-FLX+ Pyrosequenzierung zu erfassen und Annotationen der Sequenzen auf Artebene durchzuführen, um präferentielle Pflanze-Pilz Assoziationen und deren treibenden Faktoren zu bestimmen. Eine initiale Charakterisierung der Kartoffel-assoziierten AMF in Peru durch Klonbibliothek-basierte Sanger-Sequenzierung zeigte, dass ca. 50% der AMF Arten zuvor nicht auf DNA Ebene charakterisiert waren. Für einen aus Kartoffel isolierten und für die Anwendung interessanten AMF wurde eine morphologische Charakterisierung und DNA-*barcoding* durchgeführt, was zur detaillierten Wiederbeschreibung von *Glomus invermaium*, als *Rhizophagus invermaius*, führte. Basierend auf den Sequenzen aus Klonbibliotheken und charakterisierten Isolaten und weiteren Referenzsequenzen wurde dann eine 760 bp Amplikon 454-Sequenzierungs Methode zum Nachweis von AMF Arten im Feld entwickelt. Dabei werden 454-Referenzsequenzen in einem phylogenetischen „Rückgrat“ (*maximum likelihood* Referenzbaum) platziert, der auf Basis von 1.5-1.8 kb SSU-ITS-LSU rRNA Gensequenzen (erweiterter AMF *DNA barcode*) berechnet wurde. Dieser Ansatz erlaubte erstmals, Sequenzen im Hochdurchsatz mit hoher Auflösung sowohl bekannten als auch unbekannten AMF Arten verlässlich zuzuordnen. Mehr als 25 AMF Arten wurden in einzelnen Wurzelsystemen nachgewiesen. Es stellte sich heraus, dass Kartoffelwurzeln mit einer konservierten AMF Kern-Artgemeinschaft assoziiert sind. In den allermeisten Wurzelsystemen kamen bestimmte Arten aus den Gattungen *Acaulospora*, *Cetraspora*, *Claroideoglomus* und *Rhizophagus* simultan vor, welche offensichtlich Hauptakteure in der Kartoffel AM in der Andenregion sind und wahrscheinlich komplementäre Funktionen haben. Diese Kern-Artengemeinschaft war über einen überraschend weiten Bereich von edapho-klimatischen Faktoren und in unterschiedlichen Kartoffelvarietäten konserviert.

Die hier beschriebene Methode zur Charakterisierung von AMF Artgemeinschaften erlaubt die spezifische Selektion von dominierenden AMF Arten mit wahrscheinlich komplementären Funktionen, um maßgeschneiderte Inokula für Kartoffel und in Zukunft auch für andere Nutzpflanzen zu entwickeln.

1. Introduction

1.1 Arbuscular mycorrhizal fungi (AMF)

Fungi forming arbuscular mycorrhiza (AM) are all members of an ancient group of fungi whose origin is estimated at about 650 million years ago (Mya) (Berbee & Taylor, 2000; Redecker et al., 2000). They form symbiotic associations with land plants which date back to the earliest existing bryophyte-like plants able to grow in terrestrial habitats. Unambiguous fossil evidence from the Devonian period (400 Mya) shows that ancient vascular plants belonging to the genus *Rhynia*, which did not yet have evolved roots, formed AM and were heavily colonized by AMF in their soil-borne shoots (Remy et al., 1994). Arbuscular mycorrhizal fungi (AMF) are still found as colonizers of “primitive” land plants (Read et al., 2000; Schüßler 2000). Thus, AMF had a profound impact on the evolution of what we nowadays know as land plants, helping them to establish in dry lands and grow in terrestrial ecosystems (Simon et al., 1993; Schüßler & Walker, 2010). During the course of evolution, AMF became obligate plant symbionts, currently forming arbuscular mycorrhiza (AM) symbioses with 70-90% of land plants, including the ten most important human food crops (Smith & Read, 2008; Brundrett 2009; FAO 2012).

AMF are of great economic and ecological importance, as they are able to explore the soil with their extensive mycelium and transport water and nutrients, mainly the poorly mobile phosphorus (P), to the plants. In addition, AMF can provide protection against pathogens (Wehner et al., 2011), improve soil aggregate structure (Rillig et al., 2002) and increase the utilization efficiency of fertilizers, especially the nonrenewable P, and as a consequence, preserve or improve plant yield while decreasing fertilizer inputs (Tawaraya et al., 2012). The plant host provides, in exchange, up to 20% of photosynthetically fixed carbon (Wright et al., 1998). Because of the benefits that AMF offer to plants, in the last years their popularity as biofertilizers has increased and large-scale inoculum production has expanded.

Despite the importance of AMF, many aspects of their biology are still unknown mostly because of their hidden, obligate symbiotic and asexual lifestyle. Until now, approx. 250 AMF species have been described (*Glomeromycota* species list at www.amfphylogeny.com) but a large amount of unknown species must be expected from unsurveyed ecosystems (Kivlin et al., 2011; Liu et al., 2011).

Therefore assessing the diversity of AMF from natural and agricultural environments and understanding the processes that shape AMF communities is still a major challenge. Advancements in the DNA-based characterization of AMF will have direct consequences in the application of inoculum with the purpose of improving sustainable agricultural practices (Sýkorová et al., 2012). Once the native AMF community is known and the dominant colonizers are identified, the preferential AMF-plant associations can be defined and effective inoculation schemes can be designed.

1.2 Defining AMF species

A key aspect limiting our knowledge on AMF is the difficulty to define species, both at the morphological and genetic levels (Schüßler et al., 2011). Since 2001, AMF are placed in their own fungal phylum, the *Glomeromycota*, based on the analysis of the small subunit (SSU) nuclear ribosomal RNA (rRNA) gene (Schüßler et al., 2001). The *Glomeromycota* comprise four major lineages (*Glomerales*, *Diversisporales*, *Archaeosporales* and *Paraglomerales*) and the taxonomic placement of some of their members is still a topic of controversy (Oehl et al., 2008; Morton & Msiska, 2010a; Schüßler & Walker, 2010; Redecker et al., 2013).

Because AMF are asexual clonal organisms (Walker 1992; Sanders 2002), it is not possible to define AMF species by a biological species concept. Consequently, until recently, classification was based almost entirely on spore morphology, which is a topic restricted to few experts in this field. Even though the use of molecular markers is expanding, some diversity field studies are still based only on spore surveys (Gai et al., 2009), although spores are resting stages that do not necessarily represent an active community (Merryweather & Fitter, 1998; Renker et al., 2005; Hempel et al., 2007). It is known that differences exist among AMF communities detected in rhizosphere soil or plant roots (Renker et al., 2005; Hempel et al., 2007; Senés-Guerrero et al., 2014 – Chapter 5), which could simply be caused by variation in AMF sporulation dynamics (Pringle & Bever, 2002). Spore identification can therefore be misleading when it is not combined with phylogenetic analyses (Walker et al., 2007; Gamper et al., 2009). For example, spore phenotypic characteristics like color may change by putatively simple mutations that are heritable and stable through generations (Morton & Msiska, 2010b) possibly causing misinterpretations as new species by such a single mutation. On the other hand, using only short DNA

sequences to delimit AMF species will cause low phylogenetic signal, also leading to erroneous and/or unclear taxonomic positions (as discussed in Redecker et al., 2013). Ideally, to define AMF species, a combination of careful spore observation and accurate phylogenetic analyses should be employed (Gamper et al., 2009; Krüger et al., 2011; Potten et al., 2014 – Chapter 4).

To characterize AMF taxa, whether from single-spore-isolate cultures or from complex AMF communities in the field, sequence based characterization of genetic markers is nowadays standard. AMF in symbiosis with roots cannot be identified by morphological characteristics, therefore the use of molecular methods is crucial to assess the within-roots AMF population. For molecular ecological studies with a wide taxonomic coverage, the nuclear rRNA genes are most frequently used, after PCR-amplifying the SSU (Öpik et al., 2013) and/or internal transcribed spacer (ITS) (Redecker 2000) and/or the large subunit (LSU) rDNA regions (Mummey & Rillig, 2007). Yet, due to the low variability in the SSU, an extremely high intraspecific variability in the ITS or the use of relatively short LSU fragments, most analyses of these markers led to a phylogenetic resolution above species-level, at an undefined taxonomic level between genus and species (Stockinger et al., 2009). Furthermore, some primer combinations discriminate against certain AMF lineages (Lee et al., 2008; Gamper et al., 2009), while others result in high non-specific amplification (Alguacil et al., 2009).

Besides the nuclear rDNA region, other molecular markers have been used such as the mitochondrial LSU (mtLSU) rRNA gene (Börstler et al., 2008, 2010; Sýkorová et al., 2012), the *COX1* region (Lee & Young, 2009), β-tubulin (Msiska & Morton, 2009; Morton & Msiska, 2010a, b) and elongation factor 1-α (Sokolski et al., 2010), which proved to be inadequate as they cannot be applied to all AMF taxa.

The need for reliable genetic markers combined with a solid sequence database has proved to be crucial to define AMF species, especially when using high throughput methods to describe AMF communities in natural conditions (Stockinger et al., 2009; Stockinger et al., 2010; Krüger et al., 2012; Senés-Guerrero & Schüßler, 2014 – Chapter 6). For fungi, the ITS region was defined as the official DNA barcode (Schoch et al., 2012). However because of its high variability, this region alone does not provide sufficient phylogenetic resolution to delimit AMF species. Hence, an extended DNA barcoding region with species resolution power for AMF was suggested (Stockinger et al., 2010), using primers that are specific for AMF and thus

can also be used with DNA extracts from field samples (Krüger et al., 2009). These primers were recently confirmed to have the widest taxon coverage when compared to other commonly used primers targeting a single nuclear rDNA marker (Kohout et al., 2014). The extended DNA-barcoding region comprehends a part of the SSU rRNA gene, the complete ITS region (including the 5.8S rRNA gene) and approx. 800 bp of the LSU rRNA gene. These regions can be PCR amplified as a single ~1.5 kb fragment, suitable to delimit species, also in molecular ecological field community studies (Horn et al., 2014; Senés-Guerrero et al., 2014 – Chapter 5).

1.3 Molecular characterization and deep sequencing of AMF communities

In molecular ecological studies on fungi, the use of 454 pyrosequencing is nowadays common and as a result, vast amounts of sequence data have been produced. However, satisfactory taxonomic classification to species remains problematic and several issues have been highlighted (Kõljalg et al., 2013 – Chapter 3; Lindahl et al., 2013), the main ones being the huge unknown diversity and the lack of good quality and reliable reference sequences.

For AMF the definition of a species is particularly complicated, especially in the case of molecular studies using high throughput DNA sequencing. Extended DNA barcoding of AMF species served as a platform to constitute a reliable sequence database (Krüger et al., 2012), which in turn provided a strong phylogenetic "backbone" for high throughput phylogenetic placement of AMF species (Senés-Guerrero & Schüßler, 2014 – Chapter 6). At the moment, 454 GS-FLX+ amplicon-sequencing reaches sequence lengths of 1 kb and is the high throughput sequencing method providing the best phylogenetic resolution power to monitor AMF. 454 GS-FLX+ is a relatively new improvement of the previous GS-FLX chemistry which provided sequence lengths of approx. 400 bp. Therefore, since longer reads have only been available for a short time, most ecological studies on AMF have been done by analyzing reads of an average size of ~350-400 bp.

For AMF, currently there is no consensus on how to analyze high throughput sequences in order to obtain taxonomic units that are biologically and ecologically most meaningful. The most common approach is to use similarity thresholds (usually 97%) to cluster reads into operational taxonomic units (OTUs) and BLAST them against public or curated databases (Öpik et al., 2009; Dumbrell et al., 2011; Davison

et al., 2012). Other researchers use instead evolutionary relationships by constructing phylogenetic trees in which OTUs are identified as groups forming monophyletic clades (Sýkorová et al., 2007; Horn et al., 2014, Lekberg et al., 2014). Both of these approaches require the use of reference sequences to make taxonomic annotations. Yet, global AMF molecular surveys indicate that many unknown species are living in unstudied areas (Kivlin et al., 2011; Öpik et al., 2013). This is the case of the Tibetan Plateau (Li et al., 2014) and the Andean region where approx. 50% of the found AMF species were unknown from the existing public sequence databases (Senés-Guerrero et al., 2014 – Chapter 5). This would result in many data from deep sequencing that could not be analyzed based on reference sequences, preventing therefore their accurate phylogenetic affiliation.

When dealing with pyrosequencing data, the analysis pipeline used can have a strong impact on the biological conclusions. Therefore data handling is an important concern for molecular ecologists (Bakker et al., 2012) and in the case of fungi some specific considerations have been highlighted in order to avoid artificial results (Lindahl et al., 2013). For AMF, the difficulty starts at selecting the genetic marker region, continues with the similarity threshold used to cluster the reads into OTUs and increases when trying to assign sequences into taxonomic groups.

It has been shown that the distribution of AMF communities in ordination space and their responses to environmental and spatial variables were very similar when they were analyzed by either using a 97% sequence similarity threshold or by grouping OTUs into monophyletic clades (Lekberg et al., 2014). However, with high throughput DNA sequences, delimiting species by a simple similarity threshold is not possible; furthermore, defining a similarity threshold that does not either inflate or underestimate the diversity of the sample is complicated. Moreover, defining OTU monophyletic clades of short high throughput sequences offers low phylogenetic signal. Therefore, identification of the members of the AMF community requires a deeper analysis step in which OTUs can be taxonomically classified with a better phylogenetic resolution. To achieve this, we developed a high throughput species annotation pipeline for 454 sequencing data. The method is based on reference sequences and their phylogenetic tree, allowing the individual placement of 454 reads into a phylogenetic context (Senés-Guerrero & Schüßler, 2014 – Chapter 6).

The combination of tools such as *Quantitative Insights Into Microbial Ecology* (QIIME- Caporaso et al., 2010) and the *Evolutionary Placement algorithm* (EPA –

Berger et al., 2011) with curated reference sequence databases such as the Pluto F workbench for fungi (Abarenkov et al., 2010) and published reference datasets for AMF (Krüger et al., 2012), allow more accurate taxonomic affiliation of 454 sequence reads, leaving BLAST approaches as a tool that should only be used to get a rough estimate of the members of the community. The accurate affiliation of sequences into species would be of great importance to mycorrhizal ecologists because diversity comparisons both in nature and in laboratory experiments could be made and consequently, our understanding of the composition of an AMF community and its driving forces would improve (Powell & Sikes, 2014).

1.4 The importance of potato and its sustainable production

In a world confronted with increasing human population, one of the main challenges is sustainable food production without a negative impact on the environment and natural resources. Since the green revolution, intensive agriculture secured food demands. However, this was accompanied by high environmental costs (soil destruction, biodiversity reduction, and aquatic, terrestrial and groundwater pollution) that are nowadays recognized as treats for public health and future agriculture production (Tilman et al., 2002). Moving towards sustainable agriculture is a current necessity and efforts have been made by e.g., using precision farming, low-impact pest management and improvement of soil and water management practices (Bongiovanni & Lowenberg-Deboer, 2004). In this context, sustainable potato production has become of particular interest, due to its growing importance as a staple crop.

Potato (*Solanum tuberosum*) has its origins dating back 8,000 years to the South American Andes, where landrace potato cultivars probably originated at altitudes of more than 3,000 meters above sea level (mamsl) (Spooner et al., 2005). Potato is the world's fourth-largest food crop, following maize, wheat and rice with a production of 365 Mtonnes worldwide (FAO 2012), being produced in all continents except Antarctica. The Food and Agriculture Organization (FAO) declared 2008 the International Year of the potato mainly because of its key role in the world global food system (FAO 2008) and currently, potato is considered critical for food security in a world confronted by increasing population growth and hunger rates (Birch et al., 2012).

Potato tubers are rich in several micronutrients and vitamin C; they are also an important source of starch and in many developed countries they represent a secondary staple crop. In the South American Andes potato constitutes the main staple crop, with an average family farm growing 10-12 cultivars, from the around 4,300 native Andean potato varieties (Brush et al., 1995).

Potato cultivation often is not environmentally friendly as it involves intensive soil tillage that can lead to erosion, degradation and leaching of inorganic nutrients (FAO 2008). Potato is very productive, but also a P-demanding plant (Dechassa et al., 2003), requiring relatively high amounts of fertilizer. Therefore more sustainable practices for potato cultivation are demanded and the potential use of soil microorganisms to increase plant productivity in a low input manner has not been overlooked (van Loon 2007; van der Heijden et al., 2008). For potato, the presence of a dense and diverse microbial community has been reported inside the root system and in the rhizosphere, representing a potential source of plant growth promoting bacteria and biocontrol agents (Diallo et al., 2011; Ghyselinck et al., 2013).

1.5 AMF associated with potato plants

Because potato is P-demanding and grows in symbiosis with AMF (Bhattarai & Mishra, 1984) its AM association is of great agricultural interest and economic value. The fungi can supply the plant with P and as a consequence, P-fertilization can be reduced if a well-functioning AM symbiosis is supported in the management practices.

Positive responses of *S. tuberosum* have been described when inoculated with AMF (McArthur & Knowles, 1993; Duffy & Cassells, 2000; Davies et al., 2005). For example, inoculation with *Gigaspora* species improved the yield of potato in a field experiment in Cameroon (Ngakou et al., 2006). *Funneliformis mosseae* (syn. *Glomus mosseae*) demonstrated to be a good colonizer of potato plants in trap cultures in a greenhouse experiment (Bharadwaj et al., 2007) and *Rhizophagus irregularis* (syn. *Glomus irregulare*, often wrongly named *Glomus intraradices*; see Stockinger et al., 2009) was reported to be a preferential colonizer of potato in arable soils (Cesaro et al., 2008).

Although potato is one of the most important crops in the Andean region, there is not a lot of information on AMF in potato fields. The presence of *Glomus* (sensu lato), *Gigaspora*, and *Scutellospora* spores in soil at 3,900 mamsl in Peru was

reported (Davies et al., 2005). However, identification at species level was not an aim of that study. In Peru, by using a clone library and Sanger sequencing approach, an unknown *Claroideoglomus* sp., *Funneliformis mosseae* and *R. irregularis* were found as abundant potato root colonizers (Senés-Guerrero et al., 2014 – Chapter 5), but only a maximum of two AMF species was found inhabiting single root systems. In a more detailed study including samples from Peru, Bolivia and Ecuador and using 454 GS-FLX+ sequencing, the potato roots colonizers detected in highest 454-read relative abundance and frequency were two unknown *Acaulospora* spp. In the case of this study, more than 25 AMF species were found colonizing a single root system and a conserved AMF community structure was described to be present in a wide range of altitudes, plant stages and potato varieties (Senés-Guerrero & Schüßler, 2014 – Chapter 6).

1.6 Tracing of AMF applied as inocula in the field

AMF can enhance the yield of a wide range of agricultural crops (Lekberg & Koide, 2005), therefore their use as inoculants at the field scale has increased. However, there are many factors that determine inoculation success (e.g., species compatibility and adverse environmental factors) which have not yet been elucidated (Verbruggen et al., 2013). An important starting point that would allow the understanding of these factors is the accurate identification of the AMF that are correlated to positive plant responses, can withstand specific field conditions and could integrate to a well-established local AMF community.

One of the problems to evaluate the success of the inoculation campaigns in the field is that AMF are ubiquitous and crops naturally become colonized by native AMF, making it difficult to distinguish the inoculated fungal strains. Only molecular methods can allow the identification of AMF species or isolates within roots. Strain-specific identification has been done for *R. irregularis* (as *Glomus intraradices*) with microsatellite simple sequence repeats (SSR) analyzing colonized roots under *in vitro* conditions but not in natural habitats (Mathimaran et al., 2008). By using the mtLSU rRNA gene identification was also possible under field conditions (Börstler et al., 2008, 2010; Sýkorová et al., 2012). However only closely related *R. irregularis* isolates can be discriminated by mtLSU markers (Formey et al., 2012) and there is a limited amount of published sequence data for comparison (Thiéry et al., 2010). Sequencing the mitochondrial genome of four *R. irregularis* strains allowed the

identification of variability generating elements (homing endonucleases, DNA polymerase domain containing open reading frames and small inverted repeats) which confer genome plasticity and allow the design of specific markers for strain differentiation (Formey et al., 2012), but so far, this has only been done for *R. irregularis* and it may be that the same markers are not suited to identify other AMF species at the strain level.

At present, high throughput methods have not been used to identify AMF at the strain level, even though such knowledge would allow tracing of specific inoculants. For applied studies, tracing AMF introduced as inocula in field experiments is crucial. Therefore, another approach used by Lojan et al. (unpublished) is to use 454 GS-FLX+ to monitor the AMF species community composition of a field site and, by using control plots, analyze the success of inoculation by comparing read abundance of inoculated against non-inoculated samples. Even though this method only assesses root colonization at the species level, it may trace strains in an indirect manner if the introduced fungus can be qualitatively (species not present in native community) or quantitatively (species present in native community but abundance increased by inoculation) distinguished from the native population. The approach has the advantage of including the analysis of the native AMF community composition at the species level, which provides information on whether the inoculated AMF species was/were already part of the native AMF.

2. Aims of this study

My thesis was conducted in the frame of the European project VALORAM (valorizing Andean microbial diversity through sustainable intensification of potato-based farming systems) funded under the European Community's Seventh Framework Programme FP7/2007-2013 (Grant No: 227522, 01/02/2009-31/01/2014).

The goal of VALORAM was to promote the sustainable development of potato-based agricultural systems in the Andean region by using natural, if possible native, microbial resources as inputs to improve production of high quality potato crops.

My role in the VALORAM project was i) to determine which AMF were associated with potato plants and putatively being main players for potato growth and thus targets for application in agriculture, ii) to analyze edapho-climatic and plant related factors playing a role in the AMF-plant symbiosis and iii) to trace potato-associated AMF inocula introduced in the frame of field trials.

Therefore the general aim of my doctoral thesis regarded the molecular characterization of AMF, from both single-spore isolates and environmental AMF communities and analyzing plant related or environmental factors driving the AMF community composition.

Accurate characterization of AMF from environmental samples to the species level was essential, especially when using high throughput methods. Consequently, my work involved sequence-based characterization and annotation of *Glomeromycota*, including taxonomic annotation of ITS rDNA sequences in the curated sequence reference database Pluto F (Kõljalg et al., 2013 – Chapter 3). We achieved the molecular characterization of AMF at the species level for single-spore isolates (Potten et al., 2014 – Chapter 4) where morphological and phylogenetic evidence led to the identification of *Glomus invermaium*, and its transfer to the genus *Rhizophagus* as *Rhizophagus invermaius*. As a subsequent step, the molecular identification to the species level of AMF in environmental communities followed. Using a clone library and Sanger sequencing based approach, we detected many unknown AMF species in samples coming from Peru (Senés-Guerrero et al., 2014 – Chapter 5). The Sanger sequences obtained from this study and newly published ones from Bolivia and Ecuador (Senés-Guerrero & Schüßler 2014 – Chapter 6) along with other reference sequences, served as phylogenetic “backbone” to move towards high throughput AMF species-level identification from environmental samples. Potato root samples from Peru, Ecuador and Bolivia were afterwards analyzed for their AMF

species community composition with 454 GS-FLX+ pyrosequencing. We developed a method to annotate sequences to species by using a phylogenetic approach based on a reference sequences alignment and its phylogenetic tree (the “phylogenetic backbone data”), together with an evolutionary placement algorithm which analyses and places single representative 454-sequences individually into the “phylogenetic backbone” (Senés-Guerrero & Schüßler 2014 – Chapter 6). The previously developed high throughput species annotation pipeline would allow us to monitor the AMF introduced as inocula in potato field trials, which were carried out at the end of the VALORAM project (Lojan et al., unpublished).

3. Towards a unified paradigm for sequence-based identification of fungi

This chapter is identical to the publication:

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NEWS AND VIEWS

OPINION

Towards a unified paradigm for sequence-based identification of fungi

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Abstract

The nuclear ribosomal internal transcribed spacer (ITS) region is the formal fungal barcode and in most cases the marker of choice for the exploration of fungal diversity in environmental samples. Two problems are particularly acute in the pursuit of satisfactory taxonomic assignment of newly generated ITS sequences: (i) the lack of an inclusive, reliable public reference data set and (ii) the lack of means to refer to fungal species, for which no Latin name is available in a standardized stable way. Here, we report on progress in these regards through further development of the UNITE database (http://unite.ut.ee) for molecular identification of fungi. All fungal species represented by at least two ITS sequences in the international nucleotide sequence databases are now given a unique, stable name of the accession number type (e.g. *Hymenoscyphus pseudoalbidus*|GU586904|

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SH133781.05FU), and their taxonomic and ecological annotations were corrected as far as possible through a distributed, third-party annotation effort. We introduce the term 'species hypothesis' (SH) for the taxa discovered in clustering on different similarity thresholds (97–99%). An automatically or manually designated sequence is chosen to represent each such SH. These reference sequences are released (http://unite.ut.ee/repository.php) for use by the scientific community in, for example, local sequence similarity searches and in the QIIME pipeline. The system and the data will be updated automatically as the number of public fungal ITS sequences grows. We invite everybody in the position to improve the annotation or metadata associated with their particular fungal lineages of expertise to do so through the new Web-based sequence management system in UNITE.

Keywords: bioinformatics, DNA barcoding, ecological genomics, fungi, microbial diversity

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Introduction

The nuclear ribosomal internal transcribed spacer (ITS) region has a long history of use as a molecular marker for species-level identification in ecological and taxonomic studies of fungi (Hibbett *et al.* 2011). It offers several advantages over other species-level markers in terms of high information content and ease of amplification, and it was recently designated the official barcode for fungi (Schoch *et al.* 2012). The publicly available fungal ITS sequences vary significantly in reliability and technical quality; however, third-party annotation is not currently allowed (Bidartondo *et al.* 2008). To facilitate ITS-based molecular identification of fungi for the scientific community, the first fungal ITS annotation workshop was held on the premises of the University of Tartu, Estonia, on 29–30 January 2013. The 28 physical and online participants were chiefly fungal taxonomists whose expertise covered various lineages of Ascomycota, Basidiomycota, Glomeromycota and Neocallimastigomycota. The researchers also comprised bioinformaticians and molecular ecologists with experience in sequence quality assessment. The workshop centred on the annotation of fungal ITS sequences in the extended UNITE database (http://unite.ut.ee; Abarenkov *et al.* 2010a) through the Web-based sequence management workbench PlutoF (Abarenkov *et al.* 2010b; see also Fig. 1). Because UNITE mirrors the fungal ITS sequences in the International Nucleotide Sequence Databases (INSD: GenBank, EMBL and DDBJ), the full set of ca. 300 000 fungal ITS entries generated by the scientific community as of December 2012 served as the target data set.

The first version of the UNITE database was released in 2003 with a focus on ITS sequences of ectomycorrhizal fungi in northern Europe (Kõljalg *et al.* 2005). The database has been under continuous development since then and has become a full-blown sequence management environment with analysis and storage modules. At present, UNITE targets all fungi and geographical regions, but the founding principle – to provide reliable reference sequences for molecular identification – remains the same. Hereafter, UNITE not only refers to the original database of annotated ectomycorrhizal sequences, but also encompasses all fungal ITS sequences in the INSD database that are not of poor quality. The demand for high-quality reference sequences has risen rapidly due to the increasing use of high-throughput sequencing technologies (such as 454 pyrosequencing, Illumina and Ion Torrent; Glenn 2011; Shokralla *et al.* 2012; Bates *et al.* 2013). These approaches generate vast amounts of sequence data – hundreds of thousands to billions of reads within a few hours or days – such that various automated approaches to analysis represent the only viable option of handling the data. Several software pipelines are available for overseeing more or less the entire analysis procedure, from data cleaning to sequence clustering and taxonomic assignment (e.g. QIIME: Caporaso *et al.* 2010; MOTHUR: Schloss *et al.* 2009; Lindahl *et al.* 2013). However, satisfactory taxonomic identification remains problematic in the kingdom Fungi due to the vast, largely unexplored diversity and the lack of reliable and richly annotated reference sequences.

The ~300 000 public fungal ITS sequences constitute a poor candidate for the basis of taxonomic annotation of newly generated sequences, especially when used in conjunction with fully automated pipelines. Only about half of these sequences are annotated to the level of species (Schoch *et al.* 2012). This half represents approximately 20 000 different species (Latin binomials), which corresponds to 0.2–4.5% of the estimated 0.5–10 million extant fungal species (Bass & Richards 2011; Blackwell 2011). More than 10% of the public, fully identified fungal ITS sequences have been shown to be incorrectly annotated at the species level, making uncritical use of this data set problematic (Nilsson *et al.* 2006). Among the 50% of entries not annotated to species level, many correspond to species that are not yet formally described. There is no unified way to refer to such species, and different researchers adopt different ad hoc naming systems to such taxa compromising comparability over studies and time (Ryberg *et al.* 2008). Many of the entries furthermore suffer from quality issues such as low read quality or chimeric unions. Thus, both data structuring and filtering are needed to make the data set a useful tool for annotation of new sequences.

To generate a concise set of reference sequences, UNITE applies a two-tier clustering process, first clustering all sequences to approximately the subgenus/genus level and then to approximately the species level (Fig. S1, Supporting information). Both levels represent operational taxonomic units (OTUs) as defined in Sokal & Sneath (1963) and Blaxter *et al.* (2005), but here, we introduce the term 'species hypotheses' (SHs) for the taxa arising from the second round of clustering. An SH is normally composed of two or more sequences to avoid excessive inflation of SHs due to singleton sequences of substandard technical quality, but users can sanction individual singleton sequences to serve as SHs. A representative sequence for each SHs is chosen automatically by computing the consensus sequence of the SH and then finding the best matching sequence of the SH (Fig. S1,

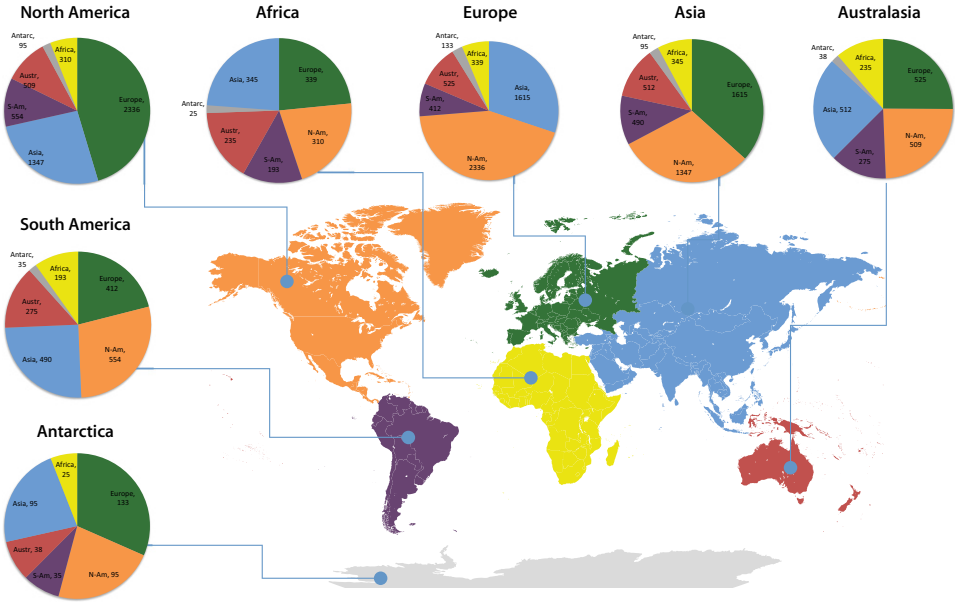
Fig. 1 Screenshot of the UNITE global key workbench depicting one of the 7470 genus/subgenus-level clusters. This cluster contains five SHs covering the well-known *Hymenoscyphus pseudoalbidus*, the causal agent of ash-dieback disease, its nonpathogenic sister species *H. albidus* and other closely related taxa. The workbench enables the users to annotate individual sequences with taxonomic and ecological metadata and to determine a reference sequence for each SH at different sequence similarity cut-off levels that represent hierarchical structures among these sequences and taxa. A reference sequence provides a proxy for the species hypothesis at user-defined cut-off levels. The coloured squares in the column SH are for the visualization of inclusiveness of SHs at five different cut-off levels (from left to right 99%, 98.5%, 98%, 97.5% and 97% similarity). Reference sequences of SHs chosen by an expert are indicated by circles. In this example, *H. pseudoalbidus* (green squares) and *H. albidus* (grey squares) fall into a single SH at 97.5% and lower sequence similarity. The reference sequence of *H. albidus* is used for the naming of SHs in these levels, because it has nomenclatural priority over *H. pseudoalbidus* that was described later (Queloz *et al.* 2011). Therefore, all sequences of these two SHs are indicated in grey at 97.5% and 97% cut-off values. It is up to the researcher to decide which cut-off values are used for identification in ecological studies. Names of SHs in publications can be hyperlinked to the cluster of sequences supplemented with metadata. The system enables saving identification results of ecological studies in a standardized and reproducible manner. The name of the SH is based on the reference or representative sequence and is compiled automatically from three data fields, viz. the taxonomic name of the sequence, the INSD or UNITE accession number of the sequence and the SH accession code. For the full description of the workbench and annotation guidelines, see Supplementary Materials. In this figure, 115 sequences of this cluster were removed for better visualization. The full cluster is illustrated in Fig. S4 (Supporting information).

Supporting information). Taxonomic experts may override the choice of representative sequence by designating a reference sequence based on type status, source of isolation and sequence quality (Fig. S2, Supporting information). Thus, all SHs have either an automatically chosen representative sequence or a manually designated reference sequence. These representative and reference sequences are released (http://unite.ut.ee/repository.php) as a reference data set for local sequence similarity searches as well as high-throughput sequencing bioinformatics platforms including the QIIME pipeline (Fig. S3, Supporting information). An annotation-aware FASTA file with all UNITE/INSD fungal ITS sequences not known to be of poor quality is also maintained at the same URL.

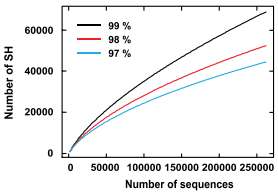
(a)

Statistics based on 98% threshold value	Total	Europe	Asia	N-America	S-America	Australasia	Antarctica	Africa	Unspecified
No of SHs*	52481	13779	8819	12941	2929	3417	313	2425	20294
No of ITS sequences	261521	50963	31560	53931	9635	8211	930	6183	100108
SH shared between continents		2832	1956	2633	686	745	130	507	N/A
Percent of unique SHs		79.5%	78%	79.6%	76.6%	78.2%	58.5%	79.1%	
No of singletons (out. clusters)	4130	694	569	742	208	266	13	182	1456
No of singletons (inside clusters)	28395	5854	3730	5825	1183	1587	97	1006	9113
* Including all singletons									

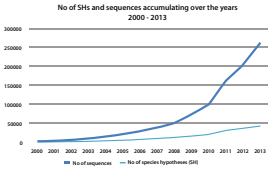
(b)



(c)



(d)



(e)

Phylum	No of SHs*	No of RefSeq
Basidiomycota	20804	1476
Ascomycota	20754	287
Chytridiomycota	285	0
Glomeromycota	1658	77
Zygomycota	602	0
Neocallimastigomycota	85	2
Blastocladiomycota	31	0
Incertae sedis	9	0
Unspecified	11387	0
*Based on 98% threshold value		

(f)

Threshold value	Number of SHs
97%	44537
97.5%	48007
98%	52481
98.5%	58594
99%	68938

Fig. 2 The statistics of the UNITE global key. Table (a) shows the number of SHs based on a 98% threshold value, the number of ITS sequences in the current version of UNITE, which passed through the quality filters, and other associated statistics. The high number of unspecified sequences and SHs that lack information on locality (more than 40%) illustrate the need for richer annotations. Circle graphs (b) illustrate the geographical distribution of those SHs that occur on more than one continent. North America, Europe and Asia are more similar to each other compared with other continents. The comparatively high number of shared SHs between Southern and Northern Hemisphere continents mark potential invasions that call for fine scale ecological studies (Antarctica has too few ITS sequences to make any sensible comparison). Table (f) provides the number of SHs for five different sequence similarity threshold values. It demonstrates how the selection of a threshold value may influence the results of studies. The new version of UNITE makes studies that employ different threshold values comparable and reproducible. Table (e) shows the number of SHs and reference sequences per fungal phylum. Basidiomycota and Glomeromycota are the most annotated phyla, reflecting the current composition of experts. Four phyla that have the smallest number of SHs are probably underrepresented in INSD databases because of difficulties to culture those fungi or find tangible reproductive/somatic structures. The graph of the subfigure (d) shows that the numbers of fungal ITS sequences in INSDs and UNITE are growing much faster than the number of SHs. This is probably biased because most sequences are still coming from North America, Europe and Asia. Potentially species-rich regions in the Southern Hemisphere are much less well represented [see also (a)]. To investigate the fungal sequencing effort at the global scale, we generated rarefied curves demonstrating the number of SHs detected vs. the number of sequences at three similarity threshold levels, viz. 97%, 98% and 99% (c). SH – species hypothesis; RefSeq – reference sequence.

The SHs can be viewed and edited in a Web browser through the PlutoF workbench (Fig. 1, Figs S4 and S5, Supporting information). Viewing sequence data by eye in the form of a multiple sequence alignment is a powerful means both to spot meaningful patterns in the data and to detect sequences of substandard quality or insufficient/incorrect annotation. Implementing changes in response to such observations in PlutoF involves only a few mouse clicks (Fig. S2, Supporting information). The user also has the opportunity to redesignate a representative sequence for any SH.

During the workshop, we targeted four aspects of sequence reliability and annotation: (i) selection of reference sequences; (ii) improving/adding taxonomic annotations; (iii) improving/adding taxonomic and ecological metadata; and (iv) tagging (and thus excluding) sequences of compromised technical quality.

Selection of representative and reference sequences

The automated choice of representative sequences in UNITE is based on nucleotide frequency, and hence, the sequence most similar to the consensus becomes representative. Although this approach is intuitively appealing and logical in most situations, there are some potential draw-

backs. For example, a single specimen may have been sequenced several times (including cloned samples), or some particular study may have exhausted a limited geographical region for records of a single species. The special authoritative standing of type specimens in systematics similarly gives rise to the desire to redesignate representative sequences on a regular basis (cf. Hyde & Zhang 2008). Not all sequences from type specimens (hereinafter 'type sequences') form ideal reference sequences though. From a bioinformatics point of view, an ideal representative sequence should cover the full ITS region and should preferably not feature many IUPAC DNA ambiguity symbols (Cornish-Bowden 1985) or manifest signs of a potentially compromised technical/read quality-related nature (cf. Nilsson *et al.* 2012). Type specimens, in contrast, might be tens to hundreds years old, making it difficult to obtain long, high-quality DNA sequences (Larsson & Jacobsson 2004).

For these reasons we re-examined the representative sequences for SHs for which we have taxonomic expertise and manually redesignated a reference sequence whenever relevant (see Fig. 1). In the absence of (high-quality) type sequences, we sought to designate a sequence that originated from the same country or geographical region as the type specimen. Sequences from vouchered fruiting bodies and living cultures were preferred over uncloned sequences from other sources (e.g. root tips and sclerotia) that in turn were given priority over cloned sequences from various complex environmental substrates where vouchering typically proves impossible. We sought to make sure that the automatically chosen representative had the most accurate taxonomic annotation possible. For example, when the automatic procedure had selected a sequence annotated as 'uncultured fungus' for a species for which the name of lower taxonomic levels (genus to phylum) was available, we made the appropriate re-annotation. We also re-annotated sequences by providing a more conservative name if the species name given by the original sequence authors did not accurately reflect recent results and findings (e.g. a misidentified *Hymenoscyphus albidus* would be annotated as *Hymenoscyphus* sp., Helotiales or Ascomycota depending on the severity of the misannotation). In recognition of the fact that no single sequence similarity threshold value – such as 97% – will demarcate intraspecific from interspecific variability in all fungi, reference sequences were set at the level they made taxonomic sense based on the results of previous studies. Many *Cortinarius* SHs were, accordingly, specified at the 99% similarity level; many lichenized fungi, in contrast, were set at the 97% similarity level.

Improving/adding taxonomic annotation

UNITE follows the Index Fungorum (http://www.indexfungorum.org) nomenclature of fungi. Approximately 84% of the sequences in UNITE are assigned at least to ordinal level, but sequences annotated as, for example, 'uncultured fungus' are assigned only at the kingdom level. If the user assigns such a sequence at a lower taxonomic level such as genus, the sequence will adopt the full hierarchical classification leading up to that genus, typically phylum, order and family. When examining the SHs, we adjusted the taxonomic annotation of the reference and representative sequences. A genus or order name was added to most sequences originally named, for example, 'cf. *Athelia*' or 'uncultured fungus'; this was only done for taxa with which we were sufficiently familiar.

Improving/adding metadata

Concurrent with the process of taxonomic annotation of sequences, we added relevant metadata such as type status, voucher specimen/culture, country of origin and host/substrate of collection. In most cases, this involved manual extraction of data from publications and sometimes contacting the original authors of the sequences.

Excluding sequences of compromised technical quality

Based on the PlutoF multiple sequence alignments, we checked the sequences for substandard quality in terms of chimeric nature and read reliability following Tedersoo *et al.* (2011) and Nilsson *et al.* (2012). During the workshop, we also made an effort to find additional chimeras using UCHIME, v. 6.0.307 (Edgar *et al.* 2011). As a reference data set, we used all representative/reference sequences from the UNITE SHs. We ran the full UNITE sequence set through UCHIME using its reference mode and then subjected sequences that exceeded the default threshold at which UCHIME considers a sequence chimeric to further scrutiny through BLAST and occasionally also through multiple sequence alignment. Sequences that were clearly unreliable or overly short were marked as such in UNITE. While all sequences marked as substandard remain searchable in the database, they are removed from BLAST searches in UNITE, the UNITE global key and the releases of representative/reference sequences.

Results and discussion

Our efforts resulted in approximately 5300 manual changes to the corpus of public fungal ITS sequences in UNITE (Fig. 2). A full 1860 of these represented redesignations of representative sequences into reference sequences (317 of which into type sequences). This means that 3.5% of the 52 481 SHs at the 98% similarity level now have a manually designated reference sequence. We implemented more than 2578 taxonomic annotations and re-annotations at the species and higher taxonomic levels. 248 sequences were excluded for being chimeric or of low quality in other regards. Finally, we added 654 items of metadata to the sequence data. It is clear that this is only the tip of the iceberg, though, and much remains to be done in all fungal phyla and the lineages covered by the present set of authors. In addition, new sequences are generated and being deposited in INSDs and UNITE at an exponential rate, such that annotation efforts will always lag behind.

The UNITE/PlutoF system offers third-party annotation capacities to all its registered users (Abarenkov *et al.* 2010b). Thus, we invite all fungal biologists to participate. In particular, we hope that all fungal taxonomists and ecologists will examine their lineages of expertise in UNITE and make sure that relevant sequences are chosen to represent SHs and that the sequences are annotated to a satisfactory level in terms of taxonomy and ecology.

The issue of naming DNA-based taxa in ecological and taxonomical studies has been debated for a long time (Hibbett & Taylor 2013). Studies that identify unknown DNA from biological samples typically apply their own ad hoc naming system (e.g. '*Tulasnella* sp. 14'; see Ryberg *et al.* 2008), which is certain to be different from that adopted by other researchers. This makes comparison among studies complicated if not impossible. Therefore, we implemented an automated, all-inclusive naming system for SHs found at various sequence similarity threshold values. The name of the SH is based on the reference or representative sequence and compiled automatically from three data fields. First is the taxonomic name of the sequence, viz. species, genus, family or higher level name. The next field is the INSD or UNITE accession code of the sequence, and the third field is the SH accession code. Thus, the name of the SH causing ash-dieback shown in Fig. 1 is '*Hymenoscyphus pseudoalbidus*|GU586904|SH133781.05FU' and its sister SH '*Hymenoscyphus albidus*|GU586876|SH114093.05FU'. In contrast to names of the '*Tulasnella* sp. 14' type, this allows for exact communication across scientific studies and time. Names in this format allow anybody to visit the same SH years later and if feasible to reproduce identification analyses based on new versions of the key. It is also easy to hyperlink those names in publication to the SH and associated information (see Fig. S3, Supporting information). Unique SH accession codes are generated automatically for all SHs at all similarity cut-off levels. The accession code begins with SH (acronym for the species hypothesis), and a unique six-digit number followed by period, a two-digit version number (version number of the key) and FU (acronym for fungi). The version number allows to place the SHs in time, and the two-letter acronym of the taxon enables quick placement of the SH in the full eukaryote classification. This would be highly useful feature if the same platform will be used for other kingdoms too.

We hope that the present effort will lead to improved taxonomic accuracy and resolution of SHs for biologists using the UNITE database, the standalone FASTA files of UNITE and the QIIME pipeline. Taxonomic precision and availability of rich metadata are clearly among the most important goals from an ecological perspective. After all, a growing number of nonmycologists now study fungi as a part of their scientific pursuit (Pautasso 2013), and it is imperative that we provide them with state-of-the-art data because they may not always be in a position to discriminate good data from bad data. For example, fully annotated ITS sequences facilitate global-scale metastudies on phylogeny, evolutionary ecology and biogeography (Bonito *et al.* 2010; Veldre *et al.* 2013). Taxonomic precision facilitates distinguishing of emerging pathogens such as *Hymenoscyphus pseudoalbidus* from their nonpathogenic close relatives (Fig. 1). Rapid and precise identification of pathogenic organisms forms a basis for efficient countermeasure, which is particularly relevant for forest, agricultural and human diseases. Arriving at the best and richest possible set of reference sequences is, however, not a question of bioinformatics or computational power but rather one of taxonomic and ecological expertise.

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Data accessibility

FASTA files of the annotated UNITE + INSD data sets are available:

1 For download at http://unite.ut.ee/repository.php;
2 Integrated into QIIME software package for comparison and analysis of fungal communities (qiime.org).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Generation of global key: technical description.

Fig. S2 Guidelines for annotating and choosing reference sequences.

Fig. S3 Format of the UNITE reference sequences FASTA file available for download at unite.ut.ee and used by QIIME.

Fig. S4 Screenshot of the UNITE global key workbench depicting the cluster UCL5_005639.

Fig. S5 Screenshot of the UNITE global key workbench depicting the species hypothesis SH155686.05FU. This workbench enables the selection of reference sequences.

4. Epitypification and DNA barcoding of *Rhizophagus invermaius* (=*Glomus invermaium*) with a re-description from *in vivo* and *in vitro* cultures

This chapter is identical to the publication:

Potten V, Senés–Guerrero C, Torres–Cortés G, Schüßler A, Walker C, Declerck S, Cranenbrouck S. Epitypification and DNA barcoding of *Rhizophagus invermaius* (=*Glomus invermaium*) with a re–description from *in vivo* and *in vitro* cultures. Submitted to Mycologia (02.07.2014).

Abstract

A new culture of arbuscular mycorrhizal (AM) fungus, originally labeled Ecu 10.2, established from a field cropped with potato (*Solanum tuberosum*), situated 2837 m above mean sea level in Carchi, Ecuador, is described and illustrated. Cultures were established in pot cultures (PC), and *in vitro* on root organ cultures (ROC) and whole plants on half-closed arbuscular mycorrhizal plant culture (HAM-P). Spores and roots colonized by this AM fungus (AMF) were sampled from all culture types for morphological observations, and from PC for DNA extraction. Sequencing and phylogenetic analyses of a 1.5 kb fragment covering the SSU-ITS-LSU rRNA gene region showed this fungus to be a member of the genus *Rhizophagus*. One line of cultures from the original pot culture (Att 1646-0) was designated MUCL 54522, and established in ROC and HAM-P. Two lines (MUCL 55341 and MUCL 55342) were subsequently established in ROC as single spore isolates, from which later pot subcultures were established. Five different spore morphotypes were observed. In PC but not *in vitro*, very small colorless 'microspores' were produced. In PC, and in one ROC, larger, almost colorless to pale yellow to brown extraradical spores produced in clusters or mats were found. In ROC and HAM-P (*in vitro* cultures), but not in PC, medium-sized, yellow to pale brown spores developed, attached to colorless mycelium surrounding the roots. In both pot- and *in vitro* cultures, colorless to very pale yellow intraradical spores were produced, but on one occasion, from a pot culture, brown intraradical spores with different morphological characteristics were produced. Morphological comparison with isotype material of *Glomus invermaium* led us to determine Ecu 10.2 as that species. From phylogenetic evidence, *G. invermaium* is transferred to *Rhizophagus* as *Rhizophagus invermaius* for which we use specimens of the strain MUCL 55342 from Att 1646-59 to designate an epitype.

***Key words*:** arbuscular mycorrhizal fungi, continuous culture, morphology, phylogeny, rDNA

Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with a huge variety of land plants (within the angiosperms, gymnosperms, pteridophytes and some members of the *Marchantiophyta*). They are a monophyletic group placed in the phylum *Glomeromycota* (Schüßler *et al.* 2001), distributed all over the world in most terrestrial ecosystems, and their ancestors have been hypothesized as instrumental in the emergence of land plants (Redecker *et al.* 2000; Schüßler & Walker 2011). These fungi depend obligatorily on carbohydrates provided by the host plant. The symbiosis is normally considered to be mutualistic because it facilitates mineral nutrition (particularly P) and water uptake for the plant. The association also may provide other benefits to the plant, such as improved resistance to biotic and abiotic stresses (Smith & Read 2008). Consequently, the AMF generate great interest in the research community as well as in the plant growth industry.

It is not possible to define AMF species by the biological species concept because of the presumed absence of sexual stages (Morton 1990 a & b; Riley & Corradi 2013; Walker 1992). Until about a decade ago, classification was based almost entirely on spore morphology. Depending on the genus, spore color, size, shape, presence and characteristics of the subtending hypha and of the germination shield, thickness and number of layers or components of the wall, along with the presence of vesicles or spores inside the host roots and presence and nature of soil-borne auxiliary cells have all been used as taxonomic characters (Thaxter 1922; Nicolson & Schenck 1979; Nicolson & Gerdemann 1968; Trappe 1982; Morton 1990 a & b; Walker 1992).

The limits of this purely morphological approach might already have been reached because there is some evidence that spore characteristics may be modified by external factors (Morton 1985; Schenck & Smith 1982) and, for example, pigmentation may perhaps be lost through a single mutation (Morton & Msiska 2010). Cryptic speciation in which spores of very similar morphology may belong to widely divergent taxa is also known (Walker et al. 2007). Stockinger *et al.* (2009) compared spores of *Rhizophagus intraradices* (as *Glomus intraradices*) and *R. irregularis* (as *G. irregulare*) and underlined the necessity of assessing the degree of variation in spore morphology before making a new species description in this genus, as some species show considerable morphological plasticity. Declerck *et al.* (2000) used ROC to produce spores used for a species description of *Glomus proliferum* in which they

combined sequencing of the SSU rDNA, spore sterols and fatty acid profiles, optical and electron microscopy and morphological features of the extraradical mycelium, but did not establish pot cultures of the species, which would have allowed morphology of the fungus in whole plants grown in a soil-like substrate to be examined.

The development of various molecular approaches brought additional tools useful for species classification. For many fungi, the internal transcribed spacer (ITS) ribosomal DNA (rDNA) region (ITS region) is the most widely used locus for species determination and became the official DNA barcode for fungi (Nilsson *et al.* 2008; Schoch *et al.* 2012) but for AMF it is exceptionally variable and has proved inadequate for resolving closely related species (Stockinger *et al.* 2010). The sequencing of a 1500-1700 bp fragment covering a part of the small subunit (SSU), the complete ITS region and approx. 800 bp of the large subunit (LSU) rDNA region (SSUpart-ITS-LSUpart) was proposed by Krüger *et al.* (2009) and Stockinger *et al.* (2010) as a barcode region to separate and identify AMF species. Nevertheless, these authors also stressed the need for taxonomic expertise when translating sequence information into species names. The combination of appropriate molecular and morphological methods can result in pertinent and congruent conclusions regarding species identity and classification (Schüßler & Walker 2010).

To date, more than 230 species of glomeromycotan fungi have been described, mostly based on morphology alone (Smith & Schenck 1985, Kramadibrata & Hedger 1990, Sieverding & Oehl 2006), though some consider a combination of morphological characters and molecular analysis (Declerck *et al.*, 2000, Blaszkowski *et al.* 2006, Oehl *et al.* 2006, Walker *et al*. 2007), and a few, such as some members of *Paraglomus*, are based entirely on DNA sequence (Renker *et al*. 2007).

In this study, we described an AMF strain (catalogued as MUCL 54522, initially designated Ecu 10.2), which was cultivated both *in vitro* on root organs or on whole plants, and in pot culture. We compared spore morphology amongst specimens from the different cultures types and studied the phylogenetic position of the fungus by molecular analysis, also providing sequence data suitable to determine the species identity in the field. The nomenclature used follows that of Redecker *et al*. (2013) and Schüßler & Walker (2010).

Material & Methods

Sample collection

A soil sample was collected in early 2003 from a *Solanum tuberosum* (potato) field at San Luis, Carchi Province, Ecuador, approx. 0° 50' 21.54" N; 77° 41' 6.25" W; 2837 m above mean sea level. The soil was analyzed by standard methods (Carter & Gregorish 2006) at the Plant Protection Department of the National Institute of Agricultural Research (INIAP, Ecuador).

Establishment of cultures

Soil was received at the Université catholique de Louvain (Louvain-la-Neuve, Belgium) in February 2003 and immediately used to initiate an open trap pot culture with *Allium porrum* L. (leek) as the host plant (Gilmore 1968). A 9-cm plastic pot (approx. 250 mL volume) was approximately half-filled with expanded clay (Agsorb 8/16 LVM-GA, Chicago, Illinois, USA) that had been autoclaved twice (15 min at 121 C) at 12 hour intervals. A layer of unsterilized sample soil (approx. 100 g) was added and the pot was then topped up with more sterilized expanded clay. The trap cultures were maintained in a greenhouse, heated to 20 C minimum from October to March, but otherwise unregulated for temperature and with natural lighting. The plants were fertilized intermittently with a nutrient solution (10% P (0.13 mM) with full-strength concentrations of the other nutrients [Valentine *et al.* 2001]) and watered weekly with tap water (Walker & Vestberg 1994). This culture was designated Attempt (Att) 1646-0, and subsequent subculture attempts were numbered Att 1646-1, Att 1646-2, and so on (Fig. 1). Later subcultures in pots were established with substrate (including roots and spores) from this pot, or with spores and roots from *in vitro* cultures established as described below.

Spores and root fragments were extracted from pot cultures by wet sieving through a series of sieves (38, 106 and 250 µm aperture). The supernatant on each sieve was examined in water under a dissecting microscope, and spores, clusters of spores and pieces of roots were separated into a dish of water with fine forceps. From the *in vitro* cultures a gel plug containing spores and colonized root was extracted and dissolved using citrate buffer as described in Cranenbrouck *et al.* (2005).

To establish root organ culture (ROC), root pieces containing spores that could be seen under a dissecting microscope were disinfested following the method of

Cranenbrouck *et al.* (2005). Root fragments (5-10 mm) were incubated in 9-cm diameter Petri plates containing modified Strullu-Romand medium (MSR) (Declerck *et al.* 1998) (594 mg l^{-1} MSR powder (Duchefa Biochemie, Haarlem, Netherlands), 256.14 mg L^{-1} $Ca(NO_3)_2$ (Sigma-Aldrich, St. Louis, MO, USA) and 10 g L^{-1} sucrose (Sigma-Aldrich, St. Louis, MO, USA), pH 5.5, solidified with 4 g L^{-1} Phytagel [Sigma-Aldrich, St. Louis, MO, USA]). The plates were incubated in the dark at 27 C and checked daily for extraradical AMF mycelium growth or contamination during four weeks. Contaminated plates were discarded. The root fragments producing hyphal re-growth were associated on MSR medium with a 3-5 cm piece of transformed carrot (*Daucus carota*, DC2) root (Declerck *et al.* 1998) on Petri plates and incubated at 27 C in the dark for six months.

Half-closed arbuscular mycorrhizal plant (HAM-P) *in vitro* cultures (Voets *et al.* 2005) were established with *Solanum tuberosum* (potato) 'Bintje' on MSR medium without vitamins and sucrose (Declerck *et al.* 1998). The ROC were incubated in the dark at 27 C; the HAM-P were incubated in an illuminated growth chamber (22/18 C (day/night), 70% relative humidity, 16-h day and an average photosynthetic photon flux density of 225 µmol m^{-2} s^{-1}) (IJdo *et al.* 2010).

In May 2009, the plant from Att 1646-0 was removed, assessed for AMF colonization, washed free of substrate under running tap water and transferred to autoclaved substrate (2:2:1 v/v/v expanded clay (Agsorb 8/16 LVM-GA, Chicago, Illinois, USA): fine quartz (0.5-1.0 mm): coarse quartz [1.5-3.0 mm]) as Att 1646-1. The remaining substrate was stored briefly at 12 C, until a sample (approx. 150 g of substrate, including roots) was used to extract spores and roots for use in establishment of other cultures, one of which, Att 1646-4 (MUCL 54522) was a ROC initiated with a single root fragment. Subsequent subcultures (ROC, HAM-P and pot culture (PC), with different hosts) were initiated with spores, root fragments, or both (Fig. 1).

In June 2010, a sample of root fragments and spores from Att 1646-7 (multi-spore ROC) was used to initiate a new pot culture (Att 1649-14) with *Plantago lanceolata* in autoclaved substrate. In November and December 2011, material from the pot culture Att 1646-14 was used to produce two single-spore ROCs with carrot (Att 1646-33, MUCL 55341) and *Cichorium intybus* (chicory) (Att 1646-58, MUCL 55342) and pot cultures of these isolates were subsequently established.

Molecular characterisation

For phylogenetic analyses, DNA was extracted from large brown spores and root fragments from PC Att 1646-14 and used to amplify a 1.5 kb fragment covering the SSU-ITS-LSU rDNA.

DNA from extraradical or intraradical spores (including root tissue) was extracted with the FastDNA Spin Kit for soil following manufacturer instructions (MP Biomedicals, Heidelberg, Germany). Single spores were crushed manually in 1 µL sterile water under a dissecting microscope using a sterile needle, 9 µL of sterile water were added to reach a final volume of 10 µL (Kramadibrata *et al.* 2000) for direct PCR amplification.

Whole genome amplifications (Illustra GenomiPhi DNA Amplification Kit, GE Healthcare, Buckinghamshire, UK) were also successfully achieved on crushed single spore, following manufacturer instruction preceding PCR amplification.

Polymerase chain reactions (PCR), cloning and clone selection with restriction fragment length polymorphism (RFLP) analyses were performed as described in Krüger *et al.* (2009) using 35 cycles for the first PCR and 30 cycles for the nested PCR, 37.5 µg/mL of BSA and 0.375 µM of each primer mixture in the first PCR reaction. Nested PCR products were visualized on 1% agarose gels (120 V, 1× TAE buffer) after staining in ethidium bromide solution (1 µg mL^{-1}). For each RFLP pattern, 2 clones were sequenced using M13 primers, at the Genomics Service Unit (Ludwig-Maximilians University (LMU), Germany) on an ABI capillary sequencer with the BigDye v3.1 sequencing chemistry. The sequences were assembled and proof read with the program SEQASSEM (SequentiX, Klein Raden, Germany) and deposited at the EMBL database under accession numbers HG969374 - HG969392.

The sequences from this study and sequences from Krüger *et al.* (2012) were used for phylogenetic analyses. Data were aligned with MAFFT version 6 (Katoh *et al.* 2002) and manually checked using the program ALIGN (SequentiX, Klein Raden, Germany). In total, 224 (1500 bp) sequences were analysed phylogenetically (including 19 sequences of Att 1646-14, MUCL 54522) using *Pacispora scintillans* as outgroup. The analyses included the ITS region with the highly variable ITS1 and ITS2. The maximum likelihood phylogenetic tree was calculated through the CIPRES science gateway (http://www.phylo.org/portal2) with RAxML version 7.3.2 (Stamatakis 2008), with 1000 bootstraps and the GTRGAMMA model for both

bootstrapping and tree inference. Phylogenetic trees were processed with FigTree version 1.3.1 (http://tree.bio.ed.ac.uk) and edited in Microsoft Powerpoint (Microsoft Office System, Washington, USA).

Spore morphology

Initial observations of *in vitro* cultures, and of extracted spores and root fragments were made through Leica MZ8 and MZ95 dissecting microscopes (Leica Microsystem AG, Heerbrugg, Switzerland), under incident illumination from a quartz-halogen fibre optic source with a color temperature of 3200 K (Kramadibrata *et al.* 2000). Spore color was assessed on fresh specimens in water and compared with Munsell color standards (Anon 1990). Specimens were mounted in glycerol or polyvinyl alcohol lacto-glycerol (PVLG) (Omar *et al.* 1979) with or without addition of Melzer's reagent (4:1 v/v: PVLG-M) on microscope slides for observation through a compound microscope. Roots were stained as described in Walker (2005).

Measurements and morphological characteristics of the spores and their wall structure were determined by examination through Optiphot (Nikon, Melville, NY, USA) and Axioskop (Carl Zeiss, Oberkochen, Germany) compound microscopes with bright field illumination. Intact spores were measured (with particular care being taken to identify the true length and width) (Schüβler *et al.* 2011) with a calibrated eyepiece graticule through the compound microscope, and spore wall structures and other microscopic characteristics were assessed from spores crushed by application of pressure to the cover glass (Kramadibrata *et al.* 2000). Photomicrographs were taken with digital cameras (Leica DFC320R, Leica Microsystem AG, Heerbrugg, Switzerland) and Canon EOS 60D, 6D and 5D [Canon, Tokyo, Japan]). A comparison of size range of spores from clusters of one particular culture was made using a 2-tailed t-test, assuming unequal variance.

Results

Soil Characteristics

The soil had an approximate bulk density of 0.93, 11.30% organic matter, pH 4.9 (measure: 1:2.5 in water), 75.67 µg mg^{-1} available P, 182.33 µg mg^{-1} N, 0.71 meq 100 g^{-1} K, 6.33 meq 100 g^{-1} Ca and 0.90 meq 100 g^{-1} Mg.

Mycorrhiza establishment

The initial trap culture, Att 1646-0 produced intraradical glomeromycotan spores (W5917) similar to those produced by species of *Rhizophagus* (Fig. 2 a) along with extraradical spores of a *Funneliformis* sp. but attempts to establish subcultures in pots, including the transferred leek (Att 1646-1) all failed. However, the ROC from a root fragment (Att 1646-4) was successful, though after use for subculture attempts, it became contaminated. The subcultures Att 1646-6a and b were both successful, producing small yellow spores. Subsequent subculturing resulted in successful cultures in ROC, HAM-P and pot. In pot culture, mycorrhizas were arbuscular (Fig. 2 b) and no thin-walled vesicles were observed. Thick-walled spores were produced in the roots (Fig. 2 c) in pot culture, ROC and HAM-P.

Spore morphology

Five spore morphs were observed, the most obvious being fascicles or mats of pale yellow to dark orange to dark brown extraradical chlamydospores in or on the surface of the substrate of PCs. In both ROC and HAM-P culture, almost colorless to pale yellow to pale brown extraradical spores were developed in very loose clusters. In all three types of culture, almost colorless to pale yellow intraradical spores developed, and in PC only, dark brown intraradical spores and very small colorless extraradical spore-like structures (termed microspores) were produced. The lengths and widths (Table I) and the shape of the spores (Table II) tended to be very variable, depending somewhat on the type of culture.

Extraradical spores

Extraradical spores from ROC

The first spores noted were in ROC (un-vouchered specimens from Att 1646-4, W5921 from Att 1646-5, W5923 from Att 1646 -7, W5927 from Att 1646-8, W5937 from Att 1646-13 and W6057 from Att 1646-33). These were very small, almost

colorless to (mainly) yellow (Munsell 2.5Y 8/4-8/6 to 10YR 8/6-8/8) and are formed singly or in loose clusters (Fig. 2 d) close to the tissue-cultured roots (Fig. 2 e).

Their overall size was 34-141×32-108 (mean 73×71) µm, SD length 14.84, SD width 14.03 (Table I) (n=281). The shape of the spores (Table II) was predominantly globose or subglobose, but a few spores were found that were, ellipsoid, broadly ellipsoid, bladder-, pip- or lemon shaped, pyriform or peanut-shaped. No subangular or irregular spores were found. The spores have a 3 components wall structure, with an outer colorless evanescent component up to 1 µm thick, a colorless component that mostly appears to lack laminations, but in some spores is clearly laminated up to 2.5 µm thick, and a colored (yellow to brown), inner coarsely laminated components 2-5 µm thick. They are developed from a persistent more or less straight subtending hypha and are either open-pored (Fig. 2 f) or occluded by a basal or distal septum (Fig. 2 g, h). Because individual spores did not detach from the clusters, it was impossible to decide at what point the 'subtending hypha' could be distinguished from the somatic mycelium. Normally, such a measurement is made from spores that have become detached during the extraction process. Consequently, defining a subtending hyphal length was meaningless. Some spores were formed on a short branch, as little as 8 µm long, whereas on others the distance from the spore base to the nearest branching point was more than 200 µm. At the base, the hypha could be straight (more or less parallel-sided), slightly flared, or slightly constricted, and up to 8 µm wide with walls up to 2 µm thick, tapering to less than 1 µm (the width of the mycelial wall). These spores did not react to Melzer's reagent.

In one culture, Att 1646-33, in addition to the spores described above, near-colorless (10YR 8/2) to pale yellow to brownish yellow to brown (10YR 6/8 to 7.5YR 4/4-3/4) spores, W6057, were produced (Fig. 2 i) attached to clusters of near-colorless (10YR 8/2) to pale yellow spores. These spores normally had a relatively thin wall and were larger than the more typical spores found in ROC. Fifty-three such spores were measured, the dimensions being 40-77 × 38-77 (mean 58× 58) µm, SD length 8.67, SD width 8.86. Their spore wall structure is of a single wall group with two components, a colorless unit component up to 2 µm thick and a pale yellow to brown component usually about 3 µm thick, but occasionally up to 7 µm thick. Component 2 does not appear to be laminated, and the spores did not react to Melzer's reagent. The subtending hypha is prominent, and yellow (concolorous with the yellow spores, but for the brown spores, sometimes paler than the spore walls

themselves) (Fig. 2 i). The spores remain attached in clusters, on thick-walled subtending hypha that are indeterminate in length as they remain attached to the mycelial network (for example, one spore had developed from a hypha 450 µm long to the point of branching from its parent hypha). The subtending hypha is 5-10 µm wide at the base with walls 3-4 µm thick proximally, tapering to 1 µm thick distally. They are mostly straight, but some may be constricted at the base, tapered or flared distally and they may be slightly sharply curved. The spores were mostly open-pored, but occasionally occluded a thin distal septum.

Extraradical spores from HAM-P

The spores from HAM-P (Att 1646-9 (W5928) and Att 1646-10 [W5929]) were pale yellow to reddish yellow (2.5Y 8/4 to 7.5 YR 6/8) (Fig. 2 j, k). They had a somewhat different appearance to those in ROC. No evanescent component could be seen, and the colored wall components were laminated. The colorless component was 1-2 µm thick and persistent. The overall wall thickness was 1-6 µm. Spores measured (37-64 × 24-64 (mean 40 × 39) µm, SD length 5.57, SD width 7.64 (n=37). They were mostly globose, with a few that were subglobose, ellipsoid or broadly ellipsoid. Only five spores were broader than long and no spore was eccentrically attached to the subtending hypha. As in ROC, the subtending hypha was of indeterminate length because they remained attached together in clusters. The distance from the spore base to the nearest branching point varied from 5 to 220 µm. At the base, the hypha could be straight (more or less parallel-sided), slightly flared, or slightly constricted, and 3-5 µm wide with walls up to 2 µm thick, tapering to less than 1 µm (the width of the mycelial wall). Most spores were open-pored, but a few were occluded by a thin curved distal or proximal septum (Fig. 2 g, j). The extraradical spores from HAM-P cultures did not react to Melzer's reagent. No brown spores were produced in this type of culture.

Extraradical spores from pot cultures

Pot cultures produced large numbers of large, opaque, brown to reddish black spores (7.5 YR 5/8 to 2.5YR 3/6) formed singly or in loose or dense clusters or mats covering substrate particles on the surface of the pots (Fig. 2 l, m). These clusters or mats were of indeterminate size, had no obvious locus of origin, and lacked a peridium. Such large, dense clusters of dark colored spores were not produced in any of our *in vitro* cultures, whether ROC or HAM-P. Pale-colored spores were not noted except that in Att 1646-42, one of the daughter pot cultures of the single spore

ROC (Att 1646-33), brown specimens were produced within (and attached to) clusters of predominantly colorless to pale yellow spores (Fig. 2 n). The majority of spores did not react to Melzer's reagent, though in two collections, (W6177 from Att 1646-60 and W6248 from Att 1646-59), both the inner surface and the laminated components (the main structural spore wall) reacted to become red (Fig. 2 o).

Extraradical spores were not found in the original trap culture (other than a few specimens that belonged in the genus *Funneliformis* and a few very badly degraded brown spores that could not be determined to species with any confidence), but they were abundant in Att 1646-14 (W5924, W5938, W6258), Att 1646-27 (W5941), Att 1646-42 (W6176), Att 1646-59 (W6248), Att 1646-60 (W6177) and Att 1646-63 (W6178). Overall the extraradical spores in pot cultures measured 43-141×32-108 (mean 73× 71) µm, SD length 14.84, SD width 14.03 (n=285). Of these specimens, 50 were broader than long, most were globose or subglobose and a few were ellipsoid, broadly ellipsoid, bladder-, pip- or lemon shaped, obovoid, ovoid, reniform, subreniform, flattened by juxtaposition with another spore or subangular. Only two intercalary spores of this type were found. It was noted that the spores (W6250) from two separate fascicles of spores produced from the same subculture of an isolate of Ecu 10.2 (Att. 1646-63) appeared to be different in size. Eighteen spores were measured from each of these and the data analyzed with a two-tailed t-test. The spores from these clusters were significantly different in both length and breadth ($p<=0.001$), (cluster 1, 56-78 × 54-67 (mean 78 × 78) µm: cluster 2, 74-117 × 77-128 (mean 88 × 87) µm).

The spore wall structure (Fig. 2 p, q) is very difficult to determine. It is in total 4-7 µm thick, and appears to have three components (Fig. 2 p, q) though the innermost, thin component is not easily detected on many spores. The outermost component (up to 2 µm thick) is colorless and generally persistent (though it may disintegrate in old spores), and is tightly adherent to the main structural component of the spore which is laminated and 1-6 µm in thickness. A possible third component (approx. 1 µm thick) is less easily observed because, in the spore, it remains very close to the inner surface of the laminated component and does not normally separate easily on crushed specimens. Consequently, in many spores it cannot be distinguished from an innermost lamina. This third component is more clearly observable in darker spores in the clusters. It becomes clearly evident on mature spores (Fig. 2 r, Fig. 3 a, b) and it is possible that it is generated *de novo* during the

germination process (Fig. 3 c). In some instances (Fig. 3 a, b) it can appear to form a septum occluding the spore contents that develops into a germination tube (Fig. 3 c). Germination is by regrowth through the subtending hypha.

The prominent subtending hypha may be colorless, concolorous with the spore wall or paler distally (Fig. 2 r, Fig. 3 a-c). The spores mostly remain attached in clusters, but on rather short straight, distally tapered or distally flared branches (subtending hypha) 5-75 µm in length and 11-15 µm wide at the base with walls 4-7 µm thick proximally, tapering to approx. 2 µm thick distally. These are mostly straight, but some may be constricted at the base, tapered or flared distally and they may be slightly to sharply recurved sometimes to the extent that they follow the curve of the spore itself. The spores were open-pored, occluded or partially occluded by thickening (ingrowth) of the inner wall component (Fig. 2 r), or by a septum formed by an inner component that seems to form *de novo* from within the spore (Fig. 3 a, b).

Very small colorless extraradical 'microspores' from pot culture

These were found only in pot cultures (W6176 from Att 1646-42, W6177 from Att 1646-60 and W6178 from Att 1646-63). These are produced either terminally (blastically) (Fig. 3 e) or sometimes (23.6%) intercalarily on fine colorless mycelium (Fig. 3 d). A few (9.6%) were eccentrically developed from their subtending hypha. They have been observed attached to mycelium surrounding roots extracted gently from pot cultures by a process of swirling and decanting through a 50 µm sieve. They are produced singly (no clusters were observed), and are 18-40 (-59) × (10-) 16-38 (-72) (mean 27 × 25) µm SD length 7.75, SD width 8.65 (n=114) and thin-walled (<1.5 µm). Very few (7%) were broader than long. Spore shape included specimens that were irregular, globose, subglobose, ellipsoid, broadly ellipsoid, oval, bladder, pip- or lemon shaped, obovoid, pyriform, reniform and subangular. We did not find these small, thin-walled, colorless spores in either ROC or HAM-P.

Intraradical spores

Intraradical spores (Fig 2 a, c, Fig. 3 f-l) were formed in all three culture types.

Intraradical spores from ROC

Intraradical spores (Fig. 3 f) were found in only one ROC, Att 1646-8 (W5927), and these measured 29-101 × 27-70 (mean 55 × 52) µm, SD length 12.87, SD width 10.19, n=50. These spores were colorless to yellow (2.5Y 8/6), and their shape was mostly globose or subglobose, with only one broadly ellipsoid and 1 oval spore

deviating from this. Just three spores were broader than long. The wall structure of these spores is of a very thin outer, colorless component (approx. 1 µm thick) and two laminated, colored inner components (2-6 µm and 1-5 µm thick respectively), all in a single wall group. These spores were of a similar appearance and wall structure to the extraradical spores found in ROC and HAM-P described above.

Intraradical spores from HAM-P

As for ROC, intraradical spores were noted in only one HAM-P culture (Fig. 3 g), Att 1646-10 (W5929). These were pale brownish yellow in color (10YR 6/8). The measurements for this collection were 24-120 × 19-109 (mean 53 × 47) µm, SD length 16.69, SD width 16.24, n=100. There was a high proportion, 24% that were broader than long, and the spore shape varied from irregular, globose, subglobose, ellipsoid, broadly ellipsoid, oval, fusiform, obovoid, ovoid, and subangular. The wall structure of the spores in roots of HAM-P cultures was identical with that of the extraradical spores, consisting of two clearly visible components, a colorless unit component <1-2 µm thick, and a pale yellow laminated component 1-3 µm thick. Some spores reacted to Melzer's reagent, the inner surface of the spore becoming pink (Fig. 3 h), perhaps suggesting a thin inner component that is not resolvable with the light microscope, but this was inconsistent, perhaps indicating differences in physiological state of individual spores.

Intraradical spores from pot culture

Colorless or very pale yellow spores formed sparsely within the roots of the original trap culture, Att 1646-0 (W5917) (Fig. 2 a). Thirty-one spores were measured, resulting in a size range of 27-96 × 21-50 (mean 52 × 32) µm, SD length 15.52, SD width 7.38. There was no spore broader than long and only one that was eccentrically developed, but the shape was very variable with very few globose or subglobose specimens and the majority being irregular, ellipsoid, broadly ellipsoid, oval, fusiform, pyriform or obovoid.

Intraradical spores were abundant in all subsequent successful pot cultures (W5925 and W5939 from Att 1646-14, W5940 from Att 1656-27, W6176 and W6247 from Att 1646-42, W6179 from Att 1646-35, W6249 from Att 1646-59, W6177 from Att 1646-60 and W6178 from Att 1646-63) (Fig. 2c, Fig. 3 i-k).

Overall spore measurements were 27-180 × 18-208 (mean 80 × 52) µm, SD length 23.94, SD width 19.11 (n=258). Only nine spores were broader than long and four were intercalary. Shape was very variable (Fig. 3 j, k), with very few globose or

subglobose spores. Other spore shapes included ellipsoid, broadly ellipsoid, oval, fusiform, spatulate, bladder-, pip- or lemon shaped, pyriform, peanut-shaped, obovoid, ampuliform, flattened by juxtaposition with another spore, subangular or subtriangular. Wall structure is the same as for HAM-P intraradical spores, being made up of two components, (each 1-3 µm thick), and outer colorless one and an inner colored one, though in some specimens mounted in PVLG it was impossible to resolve two spore wall component and the laminae could not be distinguished. No evanescent component could be detected. Occasional spores are occluded by a very thin septum close to the spore base, but most spores are open-pored.

A second kind of intraradical spore (Fig. 3 l-o) from pot culture was found in one sample (W6249 from Att 1646-59) with spores that were dark brown (5YR 5/8 to 7.5YR 5/8), 53-107 × 42-104 (mean 82 × 60) µm SD length 11.37, SD width 11.21 (N=63) with a thick colorless to pale yellow outer component (1-3 µm thick) overlaying a colored laminated component (2-6 µm thick) (Fig. 3 m, o), and an innermost unit component (approx. 1 µm thick) that may form a septum at or near the spore base (Fig. 3 n), though most are open-pored. Spore shape was very variable, many of the spores being misshapen by juxtaposition with their neighbors in the cortical cells of the root.

Extaradical spores from the type of *Glomus invermaium*

The few specimens examined from the type were brown (Fig. 3 q, r) and in a single fascicle similar to those found with Ecu 10.2 (Fig. 3 p). These were examined in the 1980s by C. Walker and photographed on film through a Zeiss photomicroscope. The English translation of the Latin diagnosis is as follows: ‘Spores hypogeous globose 50-75 µm diam, light brown to brown formed in loose sporocarps up to 1 mm across. Peridium lacking. Spore wall double, outer colorless, 1-1.5 um thick, inner light brown to brown, 3-6 µm thick. Outer wall extending down the subtending hypha for up to 100 µm. Walls inseparable. Subtending hyphae 6-13 µm diam, colorless to brown, slightly pinched-in at the point of attachment. Pore 1-4 µm wide, without septum’. It should be noted, however, that a septum is present occluding one of the type specimens (Fig. 3r)

External mycelium growth in *in vitro* systems (ROC and HAM-P)

It was observed that the mycelium growth was very slow and appearing in patches. After the mycelium had contacted the root and established colonization, a

few running hyphae were observed. Some of the hyphae grew in a corkscrew form ("spiral growth" as described by Mosse & Hepper 1975). In most of the cultures, the AMF did not explore all available substrate, and large spaces remained mycelium-free, even when they were occupied by roots. Where sporulation was high, the mycelium grew along the roots, but in 3 dimensions, without any clear growing structure or any obvious organization or any apparent preference for surface, bottom or center of the medium. On such mycelium, many single spores or small clusters of spores (containing each 3 to 5 spores) grew close to each other. Sometimes, some so-called branched absorbing structures (small groups of dichotomous hyphae formed by the extraradical mycelium [Bago *et al.* 1998 a]) were produced.

Molecular analyses

Maximum likelihood phylogenetic analyses of 19 sequence variants revealed that MUCL 54522 was phylogenetically affiliated with members of the genus *Rhizophagus* (Schüßler & Walker 2010). MUCL 54522 was clearly resolved as being separated from other *Rhizophagus* species.

In the phylogenetic tree, all *Rhizophagus* sequences clustered together in a monophyletic clade with 100% bootstrap support. A *Rhizophagus* sp. is placed basal to the other, more closely related species in the genus (*Rhizophagus proliferus*, *R. intraradices*, *R. clarus*, *R. irregularis*, a fungus annotated as *R. fasciculatus* and MUCL 54522 [Ecu 10.2]) [Fig. 4].

Discussion

Although the original soil trap produced two species of AMF, only one of these produced extraradical spores, and these were identifiable as a species of *Funneliformis*. The culture also produced spores in the roots that were recognized as possibly belonging to a species of *Rhizophagus*. Attempts to establish subcultures with conventional pot culture spore traps failed, but the utility of the *in vitro* ROC method was confirmed when a root fragment attempt resulted in a successful subculture. Purification of this by use of single spores, and re-establishment in pot culture from more than one culture line confirmed that a single species, designated as Ecu 10.2, or MUCL 54522, was present and that its spore morphology was very variable. Molecular evidence confirmed the monospecificity of the cultures, and demonstrated that it should be placed in the genus *Rhizophagus*. It also showed that the fungus occupied a clade separate from all other known species in the *Glomeraceae*.

Spore morphology

In pot cultures with *P. lanceolata*, intraradical spores were produced that were similar to those in the original soil trap culture, but along with these, prominent clusters (fascicles) of colorless to yellow to brown extraradical spores were formed at or near the surface of the substrate on coarse, differentiated thick-walled mycelium that reacted in Melzer's reagent to become pink, along with tiny, hypogeous colorless 'microspores' formed on fine mycelium that did not react to Melzer's reagent. Single spore *in vitro* isolation and subsequent re-establishment in pot culture proved that this morphological variation was inherent within a single organism. However, in one pot culture dark brown spores with a different morphology were produced within some of the roots in addition to the pale intraradical spores.

The small, colorless 'microspores' are similar to those already noted in other species of glomeromycotan fungi (Taylor et al. 2014). Whilst it is not possible to be certain that these are nothing more than very early stages of 'normal' spore formation, some specimens were occluded by a septum, and therefore might be considered to be 'spores' in their own right. With the light microscope, their spore wall can only be resolved to a single component. In clusters of the larger spores colorless specimens, regardless of spore size, possessed a wall structure that was at least double. In addition, there were no specimen of intermediate size or with more

complex wall structure (that is no evidence of development into the larger, colored spores) and they do not react in PVLG-M. We have so far not been able to show if these can germinate and act as propagules. Such spores were not seen in either ROC or HAM-P cultures. Wu (1993) and Wu & Sylvia (1993) observed that small spores of *R. irregularis* (Chabot *et al.*, 1992) and *R. clarus* (de Souza and Berbara 1999) developed into mature spores. Small, "vegetative spores" were observed in ROC of *Funneliformis mosseae* (Mosse & Hepper 1975) and on whole seedlings of *Trifolium repens* and *T. parviflorum* in monoxenic conditions with *F. caledonius* (Hepper 1981), but these apparently did not develop further. Dalpé & Declerck (2002) interpreted small structures observed *in vitro* as a "pre-sporulation step" in a transitory stage of colony development, ensuring resources storage to support further sporulation.

In HAM-P (W5929 from Att 1646-9), slightly darker (brownish yellow) small (approx. 40 µm diam.) spores were observed (Fig. 2j), but none of these was dark brown. In one monosporal ROC (Att 1646-33) larger (approx. 50 µm diam) brown spores (W6057) were noted in the same cluster as colorless to pale yellow spores (Fig. 2i), with the appearance and wall structure of the fascicular spores found at or near the surface of PC, rather than the yellow spores in the HAM-P or ROC. The pale spores from this cluster did not darken to brown during continued observation of the Petri plate over more than two months. Large, dark brown spores were produced in only this one *in vitro* culture. Fascicular spore clusters from an almost 10-month-old PC (W6176 from Att 1646-42) similarly varied from almost colorless to brown (Fig. 2n).

In ROC and HAM-P, the predominant extraradical spores produced were small and pale yellow in color and formed in very loose clusters. The extraction process from gel substrates is gentle, and it is likely that such spores would detach from the mycelium with more normal extraction methods, and thus be interpreted as 'spores borne singly in the substrate'. Such spores were not seen in pot culture, despite careful examination of gently extracted samples. It has not been possible to explain why different types of spore can be produced in different conditions, but nevertheless, it provides proof that the spore morphology is plastic, which may act as a warning against species descriptions from field collections or without establishment of cultures of any kind. It also highlights the advantages of using different types of cultures to reveal any such differences.

In one ROC only, relatively large, more or less colorless to yellow to brown extraradical spores, similar to those produced in pot cultures, were produced in clusters on coarse differentiated mycelium. Such spores were not produced in the HAM-P culture system, and indeed were uncommon within ROC, though normal in PC. No dark brown intraradical spores were produced in ROC, but it is noteworthy that such spores were found only once in PC. The intraradical spores from ROC were very small, resembling the extraradical spores in the same cultures, whereas those in HAM-P and pot culture appeared identical with the intraradical spores from the root fragments from the original trap culture. This behavior also cannot be explained from the research so far carried out on this species.

There is no species in the *Glomeromycota* described as producing five different spore morphs. However, most descriptions have been made either from single collections of field-collected specimens, or from only one single type of culture. The use of different culture types (PC, ROC and HAM-P) provided additional information on the plasticity of an AMF, but because taxonomy is a comparative endeavor, only if similar broad comparisons are made, can such variability be fully exploited for understanding the relationship amongst species. Dimorphism of spores, apparently due to whether produced hypogeously or epigeously was shown for *Diversispora epigaea* (Schüßler et al. 2011), though it had not been noted in the original species description (Daniels & Trappe 1979).

Two species that might morphologically belong to the genus *Rhizophagus* have been reported to develop brown extraradical spores and colorless intraradical spores. *Glomus ambisporum* (Smith & Schenk 1985) and *G. heterosporum* (Smith & Schenk 1985) were described from field collections. Neither living pot cultures nor molecular evidence is available for comparison with Ecu 10.2. Their spores were described as being produced in ‘sporocarps’ lacking a peridium and with spores formed radially from a sterile central plexus in the manner described for species of *Sclerocystis*. Extraradical spores of *G. ambisporum* were described as dark brown to black, globose to subglobose, 85-166 × 85-157µm, with three walls [components] (inner: 1, middle: 3-14, outer: 2-4 µm). The outer component is also described as being covered by a ‘reticulum’, though the authors write that they could not illustrate this feature so it is hard to understand what was meant by this. The intraradical spores were described as hyaline [colorless], more variable in shape and 54-197 × 44-163 µm. The spores were described as having no reaction to Melzer’s reagent.

For *G. heterosporum*, the extraradical spores were described as light to dark brown, obovoid to ellipsoid, occasionally globose, measuring 99-206 × 61-201 µm, with 2 walls (inner: 3-10; outer: 2-7 µm). The intraradical spores are described as hyaline (colorless), globose to highly variable in shape, measuring 31-102 × 27-68 µm. Neither spore morph reacted to Melzer's reagent.

These two species are similar to Ecu 10.2, but are described as having spores (consistently) radiating from a central plexus have much larger spores than the 40-77×38-77 (mean 58×58) size of Ecu 10.2 in PC. It would seem that the 'reticulum' described for *G. ambisporum* spores could not be illustrated by the original authors, and there was no such structure noted in the spores studied here. In contrast to *G. ambisporum* and *G. heterosporum* spores, which did not react to Melzer's reagent, some extraradical and intraradical spores of Ecu 10.2 reacted to become pink or red in this substance, although this was not consistent, perhaps related to the age and condition of the spores. The spores of *G. ambisporum* are described as being dark brown to black - darker than our specimens. Although the spores of *G. heterosporum* may be the same color (described as 'light to dark brown', the spores are much larger. Therefore, Ecu 10.2 could not be assigned to either of these species.

The original species description of *G. invermaium* (Hall 1977) was also based on field-collected material. No culture was established and mycorrhizal associations were not confirmed. An Australian culture (WUM 10) was considered to represent the species, and was registered as BEG 44 in the International Bank for the *Glomeromycota* (www.i-beg.eu), but this culture is not available. However, vouchers in the C. Walker's herbarium (personal communication) allowed morphological comparison, although only of the colored morph. The species was described as producing spores in 'loose sporocarps'. Spores of *G. invermaium* are described, in the protologue, as light brown to brown, 50-75 µm diameter (implying that they were globose only). Size range from field-collected specimens, however, is probably not a reliable taxonomic character, and measurements among different cultures of a species and even different subcultures within a species can differ considerably (Walker & Vestberg 1998). The spores (W6250) from two separate fascicles of spores produced from the same subculture of an isolate of ECU 10.2 (Att. 1646-63), that were observably of different sizes, confirmed on statistical analysis as having significant size differences. The subtending hypha is described as colorless to brown and lacking a septum. The wall structure is described as 'double' with outer colorless

and inner colored components. However, one specimen from the isotype shows a three-component wall structure, the innermost of which forms a kind of septum. The species was described only from field-collected material, so it remains unknown if it formed colorless spores in roots. There are sufficient close similarities between the type material of *G. invermaium* for us to consider that Ecu 10.2 represents that species. The alternative, which would have been to describe a new species, was considered, but there seems no need to add to the proliferation of species names when Ecu 10.2 fits both the description in the protologue, and the characteristics of WUM10, which was acknowledged widely to represent *G. invermaium*.

Molecular analysis

Neither the type of *G. invermaium* nor WUM10 has been sequenced, and because no living material is available, such sequencing is unlikely. The molecular analyses of the SSU-ITS-LSU region were performed with MUCL 54522 material (different morphotypes: large dark spores and intraradical spores from pot cultures). All these produced sequences that clustered together in a single terminal clade. Results placed Ecu 10.2 as clearly distinct in comparison with other known and sequenced AMF species. The greatest similarity occurred with *R. intraradices*, *R. irregularis*, *R. fasciculatus* and *R. proliferus*. From the morphological point of view, all these species are easily distinguished from our strain as none of these produces dark brown spores. In consideration of its phylogenetic position, *G. invermaium* is transferred to the genus *Rhizophagus* as *R. invermaius*.

Conclusions

To date, five successive *in vitro* generations have been successful, but the sixth failed to establish and re-establishment from PC will be needed if *in vitro* (ROC or HAM-P) cultures are required. It is not unusual for ROC to fail after a time, and to overcome this, intermittent re-cycling through a plant in pot culture is advisable. This loss of potency in ROC reinforces the need to have back-up PCs for species cultivated *in vitro*. Although *in vitro* cultures offer experimental approaches providing a different way of understanding AMF biology and behavior (Bago *et al.* 1998 b), the loss of valuable germplasm is a serious potential problem for all kinds of continuous culture collections. For example, *Acaulospora rehmii* was established in ROC (Dalpé

& Declerck 2002), but could not be maintained through successive generations and is now lost to science.

In vitro cultures alone are unlikely to display the whole gamut of AMF behavior or morphological characteristics. Pawlowska *et al.* (1999) and many others recommend not to base a species description on *in vitro* cultures only, but the work presented here shows that pot cultures alone also may not provide a comprehensive view of the morphological variation within a species. It is even less likely that a few specimens from a field collection will provide an adequate basis for a comprehensive description of an AMF. In this example, the differing behavior of Ecu 10.2 *in vitro* and in pot cultures underlined how complex is the process of species description and highlighted that a multiple approach (with different hosts and culturing systems) is desirable to circumscribe the species variability and provide useful information for future taxonomic and systematic comparisons and AMF research in general.

It is impossible to know if the yellow spores from the ROC and HAM-P cultures would have developed into the brown, clustered spores, but this seems unlikely. Att 1646-33 produced clustered spores, and yet it was younger than any of the other successful *in vitro* cultures. There was no difference in nutrients in the ROC plates, so it is unlikely that the different behavior was related to nutritional status. The yellow spores were not seen in any pot culture, despite rigorous efforts to find them (careful sieving followed by detailed scrutiny). Consequently, it is concluded that these two morphs are distinctive, and not just two different developmental phases. This is reinforced by the spatial differences: the fascicles of spores are produced at or near the surface of the substrate, on differentiated mycelium, whereas the yellow spores are produced as individual spores attached to non-differentiated hyphae, round the roots. The wall structure may have been affected by the growing conditions. In particular, the outer colorless component is much thicker and more persistent on spores produced *in vitro* than on those from pot culture. Perhaps this is because of the lack of microorganisms in the former to break it down.

When we compare the three culture systems, it is not possible to decide if the differences in morphology between one type of culture and another were generated by the host or the environment, or a combination of both. Nevertheless, it is now clear that the morphology of these spores, and by inference, of those of other AMF species, is variable, and the simplistic view that any morphological variation of spore can be treated as a new species is questionable. Multiple spore morphologies within

a species might occur in other species, which were described from cultures of only one kind, or maintained for only a short time on a single host or in only one type of substrate. The results presented here highlight the desirability of comparing different living conditions (different kind of cultures, different hosts) when describing a species to embrace the range of inherent variation.

Nomenclature

Rhizophagus invermaius (Hall) Walker comb. nov. Figs. 2-3

Mycobank Number: MB809417

≡ *Glomus invermaium* Hall (1977) Trans. Brit. Mycol. Soc. 68: 345

TYPE: from a field collection under *Trifolum repens*, Hall 425 (PDD; isotype OSC)

Epitype: W6248 & W6249 (two voucher numbers relating to different spore morphs) 4 May 2014 in the C. Walker's collection in E, here designated. Derived from the single-spore origin pot culture with *Plantago lanceolata* with designator Attempt 1646-59. Subcultures of this isolate (MUCL 55342) are available by request from BCCM^{TM}/MUCL/GINCO collection (http://bccm.belspo.be/catalogues).

Overall spore description (Taxonomy)

Spores or spore-like structures of five different morphological types produced from arbuscular mycorrhizas in which thin-walled vesicles have not been observed. Singly or in loose clusters in the substrate, or in roots of host plants.

Morph 1. Known only from pot cultures. Small, colorless, thin-walled (<1.5 µm) terminal or intercalary 'microspores' produced from hypogeous mycelium around the roots of a mycorrhizal plant host. Produced singly. 18-40 × 16-38 (mean 27 × 25) µm. Variable in shape.

Morph 2. Known only from pot cultures, root organ cultures, or field collections. Near colorless to pale yellow to brownish yellow to brown (to opaque brown to reddish black in pot culture) extraradical spores. In loose to dense clusters or fascicles, sometimes in mats (known only from PC). 40-77 × 38-77 (mean 58 × 58) µm in ROC, 43-141 × 32-108 (mean 73 × 71) µm in pot culture. Mostly globose to subglobose (but other shapes observed). Spore wall of three components: a 2µm thick colorless and generally persistent unit component overlaying a main structural laminated component, 1-7 µm thick 7µm and an innermost flexible component up to 1

µm thick apparently produced at spore maturity. Subtending hyphae prominent (5-10 µm long), thick walled, concolorus or paler than the spore walls. No reaction to Melzer's reagent (some reactions observed on spores from pot cultures).

Morph 3. Known only from *in vitro* cultures. Small, near colorless to pale yellow to pale brown or reddish yellow spores. Singly to very loosely clustered extraradical spores, close to the root; 34-141×32-108 (mean 73×71) µm in ROC, 37-64 × 24-64 (mean 40 × 39) µm in HAM-P or intraradically in ROC; 29-101x27-70 (mean 55x52) µm. Predominantly globose or subglobose shaped (other shapes observed). The spore wall has 2 (HAM-P), to 3 (ROC) components, outer colorless evanescent component up to 1µm thick in ROC only, colorless mostly without lamination, 1 to 2,5 µm thick component and inner colored (yellow to brown) laminated component, 2 to 5 µm thick. Persistent, more or less straight subtending hyphae, up to 8 µm wide with walls up to 2 µm thick. No reaction to Melzer's reagent.

Morph 4. Known from both *in vitro* cultures and pot cultures. Spores intraradical, colorless to pale yellow to pale brownish yellow. 29-101 × 27-70 (mean 55 × 52) µm in ROC, 24-120 × 19-109 (mean 53 × 47) µm in HAM-P and 27-180 × 18-208 (mean 80 × 52) µm in PC. Shape mostly globose or subglobose (other shapes observed) in ROC, very variable in pot cultures and HAM-P. Wall structure difficult to determine with a colorless unit component <1-2 µm thick, and a pale yellow laminated component 1-3 µm thick, but sometimes appearing as if of only a single unit component, presumably due to very fine laminations. Sometimes reaction to Melzer's reagent (inner component becomes pink).

Morph 5. Known only from pot culture. Intraradical spores, dark brown, occasionally pale yellow. 53-107 × 42-104 (mean 82 × 60) µm. Irregular in shape, often due to pressure from juxtaposition with neighbouring spores within the root cortex. Thick-walled, colorless to pale yellow outer component 1-3 µm thick, overlaying a colored laminated component 2-6 µm thick, and an innermost unit component approx. 1 µm thick.

Acknowledgements

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Figures

Fig. 1 - Culturing history of the epitype and subcultures of *Rhizophagus invermaius* (Ecu 10.2), from February 2003 to September 2012. Each rectangle represents a subculture, named Att 1646-x. Vouchers are designated Wxxxx. PC=pot culture (blue), ROC=root organ culture (orange), HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture (red). MUCL numbers represent a strain. All cultures have the same MUCL number between major isolation steps. The first MUCL strain is 54522 (subculture Att 1646-4) and all cultures linked by green arrows are considered as the same MUCL strain. MUCL 55341 (linked by purple arrows) and MUCL 55342 (linked by burgundy arrows) are monosporal isolates. Material from Att 1646-14 was used for molecular analyses. Culture Att 1646-59 produced the epitype.

Fig. 2 - Arbuscules and spores from different subcultures of *Rhizophagus invermaius*. Subcultures are identified with a unique attempt and subculture numbers (Att 1646-x). Voucher specimens are identified by unique numbers (Wxxxx). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

a) A pale yellow intraradical spore, , similar to those produced by species of *Rhizophagus*, from Att 1646-0 (original trap, PC) W5917.

b) Arbuscular mycorrhiza stained in blue ink, from Att 1646-60 (PC) W6177,.

c) Thick-walled pale yellow intraradical spores, from Att 1646-14 (PC) W5925.

d) Extraradical spores in loose clusters, from Att1646-8 (ROC) W5927.

e) Extraradical spores develop close to the tissue-cultured roots, in ROC. Root and spores stained in blue ink.

f) Small pale yellow extraradical spore, from Att 1646-8 (ROC) W5927. The pore remains open.

g) Small pale yellow extraradical spore, from Att 1646-5 (ROC) W5921. The pore is occluded by a distal septum (arrow).

h) Small pale yellow extraradical spore, from Att 1646-5 (ROC) W5921. The pore is occluded by a basal septum (arrow).

i) Extraradical brown spore, in the same cluster as pale yellow spores, from Att 1646-33 (ROC) W6057. The subtending hypha of the brown spore is much paler than the spore walls themselves.

j) Pale yellow extraradical spore, from Att 1646-9 (HAM-P) W5929. The spore is occluded by spore wall thickening.

k) Wall structure of a pale yellow extraradical spore, from Att 1646-10 (HAM-P) W5929 showing a persistent colorless outer component and a laminated colored inner component.

l) Dense cluster of extraradical brown to reddish black spores, from Att 1646-14 (PC) W5924.

m) Extraradical orange to brown spore from a dense cluster (fascicle), from Att 1646-14 (PC) W5924.

n) Extraradical brown and colorless to pale yellow spores produced within the same fascicle, from Att 1646-42 (PC) W6176.

o) Extraradical spore and mycelium reacting to Melzer's reagent, from Att 1646-60 (PC) W6177. Both the inner surface and the laminated components (the main structural spore wall) of the spore reacted to become red.

p) Structure of the wall of an extraradical spore, from Att 1646-14 (PC) W5927. The structure appears to have three components. The outermost component is colorless and generally persistent and adherent to the main structural component of the spore, which is laminated. There is a possible third component.

q) Structure of the wall of an extraradical spore, from Att 1646-60 (PC) W6177. The third component of the wall is less easily observable in paler spores.

r) Prominent subtending hyphae of an extraradical spore, from Att1646-14 (PC) W5924. The third component of the wall is more clearly observable in mature spores.

Fig. 3 - Spores of *Rhizophagus invermaius*. Images a-p were taken with material from different sub cultures which are identified with unique attempt and subculture numbers (Att 1646-x). Voucher collections are identified by unique identifiers (Wxxxx). PC=pot culture, ROC= root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

a) Prominent subtending hypha, paler distally, of an extraradical spore from Att1646-60 (PC) W6177 showing an innermost wall component that develops before germination.

b) Prominent colored subtending hyphae of an extraradical spore, from Att1646-14 (PC) W5924 with a septum apparently formed *de novo* from within the spore (arrow).

c) Germination by regrowth through the subtending hypha, from Att 1646-14 (PC) W6258, showing the third wall component developed into a germination tube.

d) Intercalary colorless ‘microspore’ on fine colorless mycelium, from Att 1646-42 (PC) W6176.

e) Terminal colorless ‘microspore’ on fine colorless mycelium, from Att 1646-42 (PC) W6176.

f) Pale yellow globose intraradical spores, from Att 1646-8 (ROC) W5927.

g) Pale yellow mis-shapen intraradical spores, from Att 1646-10 (HAM-P) W5929.

h) Pink reaction to Melzer’s reagent of an intraradical spore, from Att 1646-10 (HAM-P) W59279.

i) Colorless to pale yellow intraradical spores protruding through the root cortex, from Att1646-14 (PC) W5939.

j) Very variable (irregular) shapes of pale yellow intraradical spores, from Att1646-14 (PC) W5925.

k) Very variable (irregular) shapes of colorless intraradical spores, from Att1646-42 (PC) W6176.

l) Brown intraradical spores with the shape affected by juxtaposition with other spores, from Att1646-59 (PC) W6249.

m) Brown somewhat mis-shapen intraradical spore from Att1646-59 (PC) W6249. The thick colorless to pale yellow outer component overlays a colored laminated component.

n) Brown intraradical spore, from Att1646-59 (PC) W6249. A septum occludes the base of the spore.

o) Structure of the wall of a brown intraradical spore, from Att1646-59 (PC) W6249. The thick colorless to pale yellow outer component overlays a colored laminated component.

p) Cluster of extraradical brown spores, from Att 1646-14 (PC) W5938 in a single fascicule.

q) Cluster (fascicle) of spores from the type of *G. invermaium*.

r) Spore from the type of *G. invermaium*. The spore is closed by a septum (arrow).

Fig. 4 - Phylogenetic maximum likelihood tree computed with RAxML, based on an alignment of 1500 bp sequences covering the SSU-ITS-LSU rDNA fragment, showing that all sequences of *Rhizophagus invermaius* cluster together with 100% bootstrap support. The 19 sequences of *Rhizophagus invermaius* were obtained from extra- and intraradical spores from MUCL 54522, Att 1646-14. Other sequences are from Krüger *et al.* (2012). Support values derived from a 1,000-fold bootstrapped analysis are shown on the branches. The GTRGAMMA model was used for both bootstrapping and tree inference. Branches with < 60% bootstrap support are not shown, and a long branch was shortened by 50%, which is indicated with two diagonal slashes. The scale bar indicates the number of substitutions per site. Sequences of *Pacispora scintillans* were used as outgroup.

Tables

Table I - Measurements of *Rhizophagus invermaius* spores from different subcultures identified with unique attempt number (Att 1646-x), and specimen vouchers numbers (Wxxxx). Measurements are in µm (length (L) × width [W]). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

Table II - Different shapes of *Rhizophagus invermaius* spores from different subcultures, identified by voucher (Wxxxx) numbers: ext (extraradical), int (intraradical) and mic ('microspore'). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

Fig. 1 - Culturing history of the epitype and subcultures of *Rhizophagus invermaius* (Ecu 10.2), from February 2003 to September 2012. Each rectangle represents a subculture, named Att 1646-x. Vouchers are designated Wxxxx. PC=pot culture (blue), ROC=root organ culture (orange), HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture (red). MUCL numbers represent a strain. All cultures have the same MUCL number between major isolation steps. The first MUCL strain is 54522 (subculture Att 1646-4) and all cultures linked by green arrows are considered as the same MUCL strain. MUCL 55341 (linked by purple arrows) and MUCL 55342 (linked by burgundy arrows) are monosporal isolates. Material from Att 1646-14 was used for molecular analyses. Culture Att 1646-59 produced the epitype.

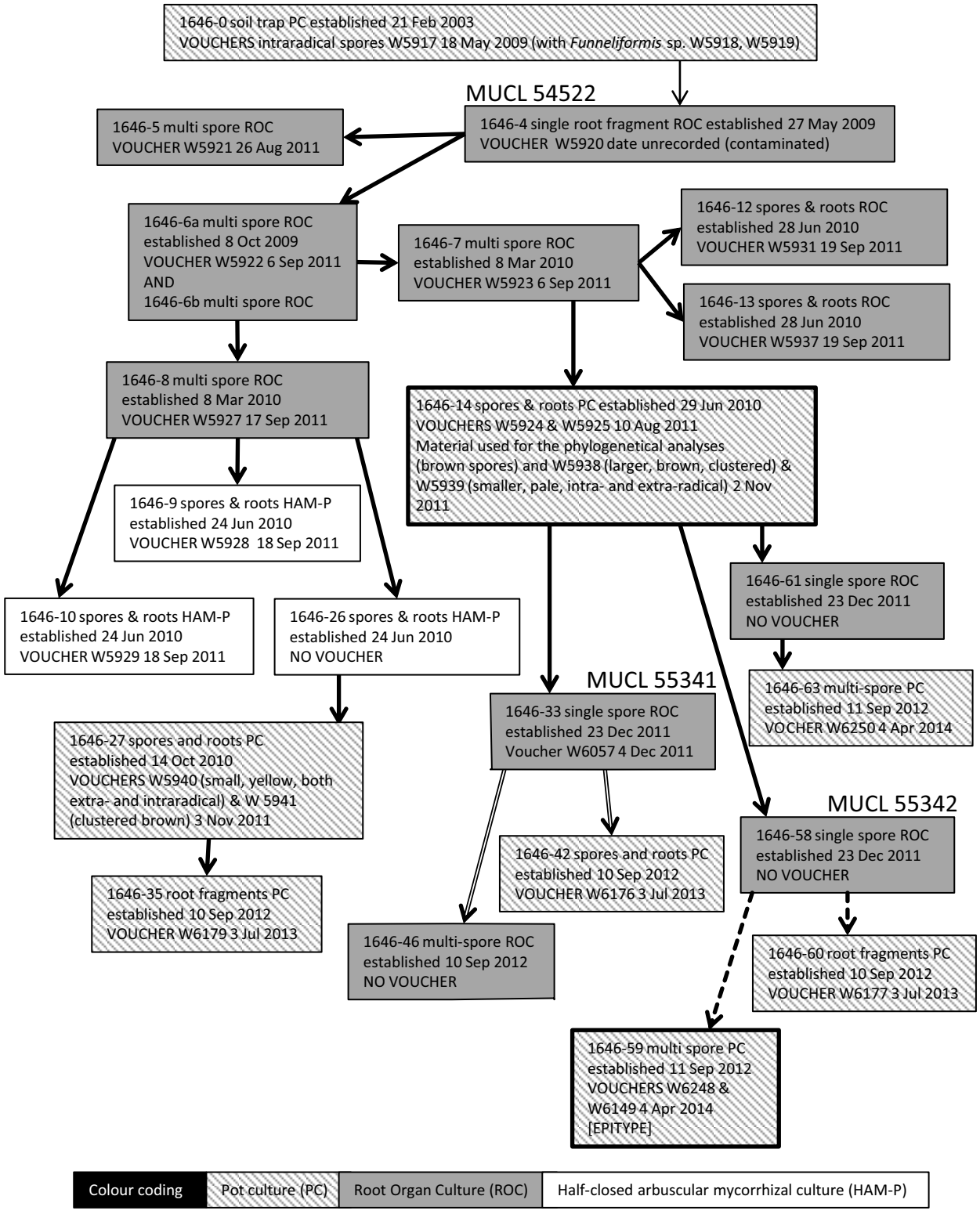
1646-0 soil trap PC established 21 Feb 2003
VOUCHERS intraradical spores W5917 18 May 2009 (with *Funneliformis* sp. W5918, W5919)
MUCL 54522
1646-5 multi spore ROC
VOUCHER W5921 26 Aug 2011
1646-4 single root fragment ROC established 27 May 2009
VOUCHER W5920 date unrecorded (contaminated)
1646-6a multi spore ROC
established 8 Oct 2009
VOUCHER W5922 6 Sep 2011
AND
1646-6b multi spore ROC
1646-7 multi spore ROC
established 8 Mar 2010
VOUCHER W5923 6 Sep 2011
1646-12 spores & roots ROC
established 28 Jun 2010
VOUCHER W5931 19 Sep 2011
1646-13 spores & roots ROC
established 28 Jun 2010
VOUCHER W5937 19 Sep 2011
1646-8 multi spore ROC
established 8 Mar 2010
VOUCHER W5927 17 Sep 2011
1646-14 spores & roots PC established 29 Jun 2010
VOUCHERS W5924 & W5925 10 Aug 2011
Material used for the phylogenetical analyses
(brown spores) and W5938 (larger, brown, clustered) &
W5939 (smaller, pale, intra- and extra-radical) 2 Nov
2011
1646-9 spores & roots HAM-P
established 24 Jun 2010
VOUCHER W5928 18 Sep 2011
1646-10 spores & roots HAM-P
established 24 Jun 2010
VOUCHER W5929 18 Sep 2011
1646-26 spores & roots HAM-P
established 24 Jun 2010
NO VOUCHER
1646-61 single spore ROC
established 23 Dec 2011
NO VOUCHER
MUCL 55341
1646-63 multi-spore PC
established 11 Sep 2012
VOUCHER W6250 4 Apr 2014
1646-33 single spore ROC
established 23 Dec 2011
Voucher W6057 4 Dec 2011
1646-27 spores and roots PC
established 14 Oct 2010
VOUCHERS W5940 (small, yellow, both
extra- and intraradical) & W 5941
(clustered brown) 3 Nov 2011
MUCL 55342
1646-58 single spore ROC
established 23 Dec 2011
NO VOUCHER
1646-42 spores and roots PC
established 10 Sep 2012
VOUCHER W6176 3 Jul 2013
1646-35 root fragments PC
established 10 Sep 2012
VOUCHER W6179 3 Jul 2013
1646-46 multi-spore ROC
established 10 Sep 2012
NO VOUCHER
1646-60 root fragments PC
established 10 Sep 2012
VOUCHER W6177 3 Jul 2013
1646-59 multi spore PC
established 11 Sep 2012
VOUCHERS W6248 &
W6149 4 Apr 2014
[EPITYPE]
Colour coding
Pot culture (PC)
Root Organ Culture (ROC)
Half-closed arbuscular mycorrhizal culture (HAM-P)

Fig. 2 - Arbuscules and spores from different subcultures of *Rhizophagus invermaius*. Subcultures are identified with a unique attempt and subculture numbers (Att 1646-x). Voucher specimens are identified by unique numbers (Wxxxx). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

a) A pale yellow intraradical spore, similar to those produced by species of *Rhizophagus*, from Att 1646-0 (original trap, PC) W5917.

b) Arbuscular mycorrhiza stained in blue ink, from Att 1646-60 (PC) W6177.

c) Thick-walled pale yellow intraradical spores, from Att 1646-14 (PC) W5925.

d) Extraradical spores in loose clusters, from Att1646-8 (ROC) W5927.

e) Extraradical spores develop close to the tissue-cultured roots, in ROC. Root and spores stained in blue ink.

f) Small pale yellow extraradical spore, from Att 1646-8 (ROC) W5927. The pore remains open.

g) Small pale yellow extraradical spore, from Att 1646-5 (ROC) W5921. The pore is occluded by a distal septum (arrow).

h) Small pale yellow extraradical spore, from Att 1646-5 (ROC) W5921. The pore is occluded by a basal septum (arrow).

i) Extraradical brown spore, in the same cluster as pale yellow spores, from Att 1646-33 (ROC) W6057. The subtending hypha of the brown spore is much paler than the spore walls themselves.

j) Pale yellow extraradical spore, from Att 1646-9 (HAM-P) W5929. The spore is occluded by spore wall thickening.

k) Wall structure of a pale yellow extraradical spore, from Att 1646-10 (HAM-P) W5929 showing a persistent colorless outer component and a laminated colored inner component.

l) Dense cluster of extraradical brown to reddish black spores, from Att 1646-14 (PC) W5924.

m) Extraradical orange to brown spore from a dense cluster (fascicle), from Att 1646-14 (PC) W5924.

n) Extraradical brown and colorless to pale yellow spores produced within the same fascicle, from Att 1646-42 (PC) W6176.

o) Extraradical spore and mycelium reacting to Melzer's reagent, from Att 1646-60 (PC) W6177. Both the inner surface and the laminated components (the main structural spore wall) of the spore reacted to become red.

p) Structure of the wall of an extraradical spore, from Att 1646-14 (PC) W5927. The structure appears to have three components. The outermost component is colorless and generally persistent and adherent to the main structural component of the spore, which is laminated. There is a possible third component.

q) Structure of the wall of an extraradical spore, from Att 1646-60 (PC) W6177. The third component of the wall is less easily observable in paler spores.

r) Prominent subtending hyphae of an extraradical spore, from Att1646-14 (PC) W5924. The third component of the wall is more clearly observable in mature spores.

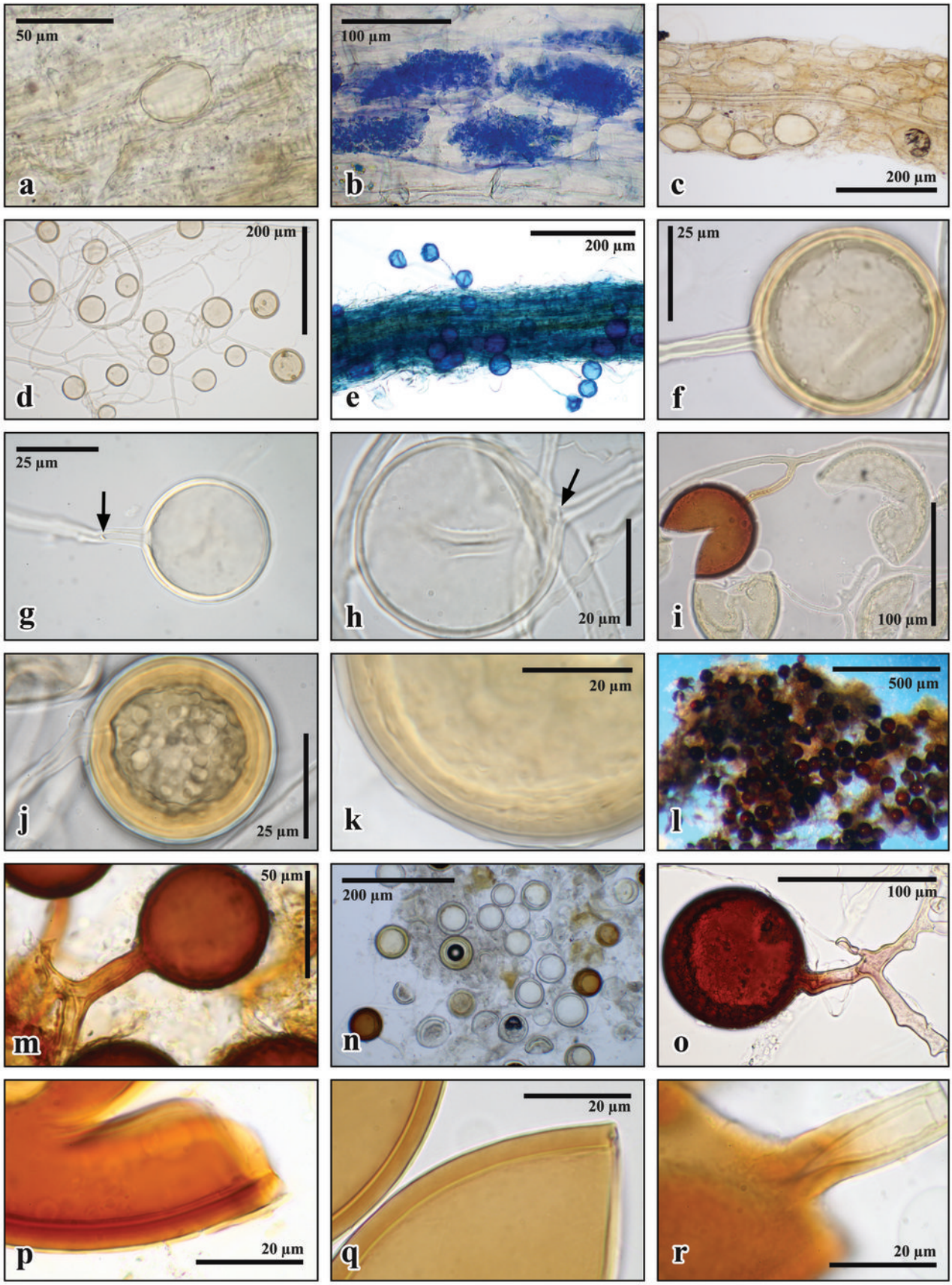
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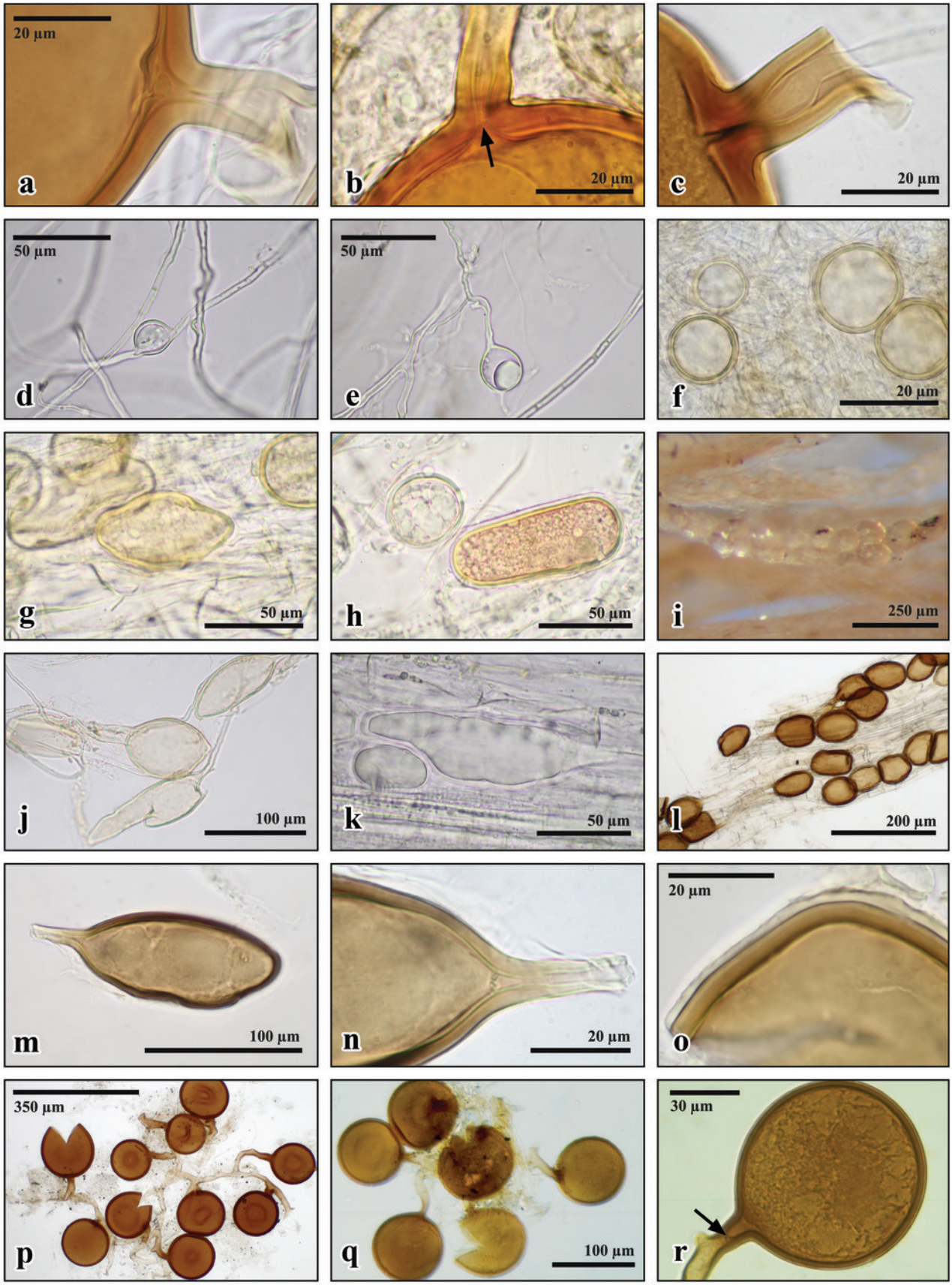
Fig. 3 - Spores of *Rhizophagus invermaius*. Images a-p were taken with material from different sub cultures which are identified with unique attempt and subculture numbers (Att 1646-x). Voucher collections are identified by unique identifiers (Wxxxx). PC=pot culture, ROC= root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.
a) Prominent subtending hypha, paler distally, of an extraradical spore from Att 1646-60 (PC) W6177 showing an innermost wall component that develops before germination.
b) Prominent colored subtending hyphae of an extraradical spore, from Att 1646-14 (PC) W5924 with a septum apparently formed *de novo* from within the spore (arrow).
c) Germination by regrowth through the subtending hypha, from Att 1646-14 (PC) W6258, showing the third wall component developed into a germination tube.
d) Intercalary colorless 'microspore' on fine colorless mycelium, from Att 1646-42 (PC) W6176.
e) Terminal colorless 'microspore' on fine colorless mycelium, from Att 1646-42 (PC) W6176.
f) Pale yellow globose intraradical spores, from Att 1646-8 (ROC) W5927.
g) Pale yellow mis-shapen intraradical spores, from Att 1646-10 (HAM-P) W5929.
h) Pink reaction to Melzer's reagent of an intraradical spore, from Att 1646-10 (HAM-P) W59279.
i) Colorless to pale yellow intraradical spores protruding through the root cortex, from Att 1646-14 (PC) W5939.
j) Very variable (irregular) shapes of pale yellow intraradical spores, from Att 1646-14 (PC) W5925.
k) Very variable (irregular) shapes of colorless intraradical spores, from Att 1646-42 (PC) W6176.
l) Brown intraradical spores with the shape affected by juxtaposition with other spores, from Att1646-59 (PC) W6249.
m) Brown somewhat mis-shapen intraradical spore from Att1646-59 (PC) W6249. The thick colorless to pale yellow outer component overlays a colored laminated component.
n) Brown intraradical spore, from Att1646-59 (PC) W6249. A septum occludes the base of the spore.
o) Structure of the wall of a brown intraradical spore, from Att1646-59 (PC) W6249. The thick colorless to pale yellow outer component overlays a colored laminated component.
p) Cluster of extraradical brown spores, from Att 1646-14 (PC) W5938 in a single fascicule.
q) Cluster (fascicle) of spores from the type of *G. invermaium*.
r) Spore from the type of *G. invermaium*. The spore is closed by a septum (arrow).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20 µm
o
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p
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100 µm
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r

Fig. 4 - Phylogenetic maximum likelihood tree computed with RAxML, based on an alignment of 1500 bp sequences covering the SSU-ITS-LSU rDNA fragment, showing that all sequences of *Rhizophagus invermaius* cluster together with 100% bootstrap support. The 19 sequences of *Rhizophagus invermaius* were obtained from extra- and intraradical spores from MUCL 54522, Att 1646-14. Other sequences are from Krüger *et al.* (2012). Support values derived from a 1,000-fold bootstrapped analysis are shown on the branches. The GTRGAMMA model was used for both bootstrapping and tree inference. Branches with < 60% bootstrap support are not shown, and a long branch was shortened by 50%, which is indicated with two diagonal slashes. The scale bar indicates the number of substitutions per site. Sequences of *Pacispora scintillans* were used as outgroup.

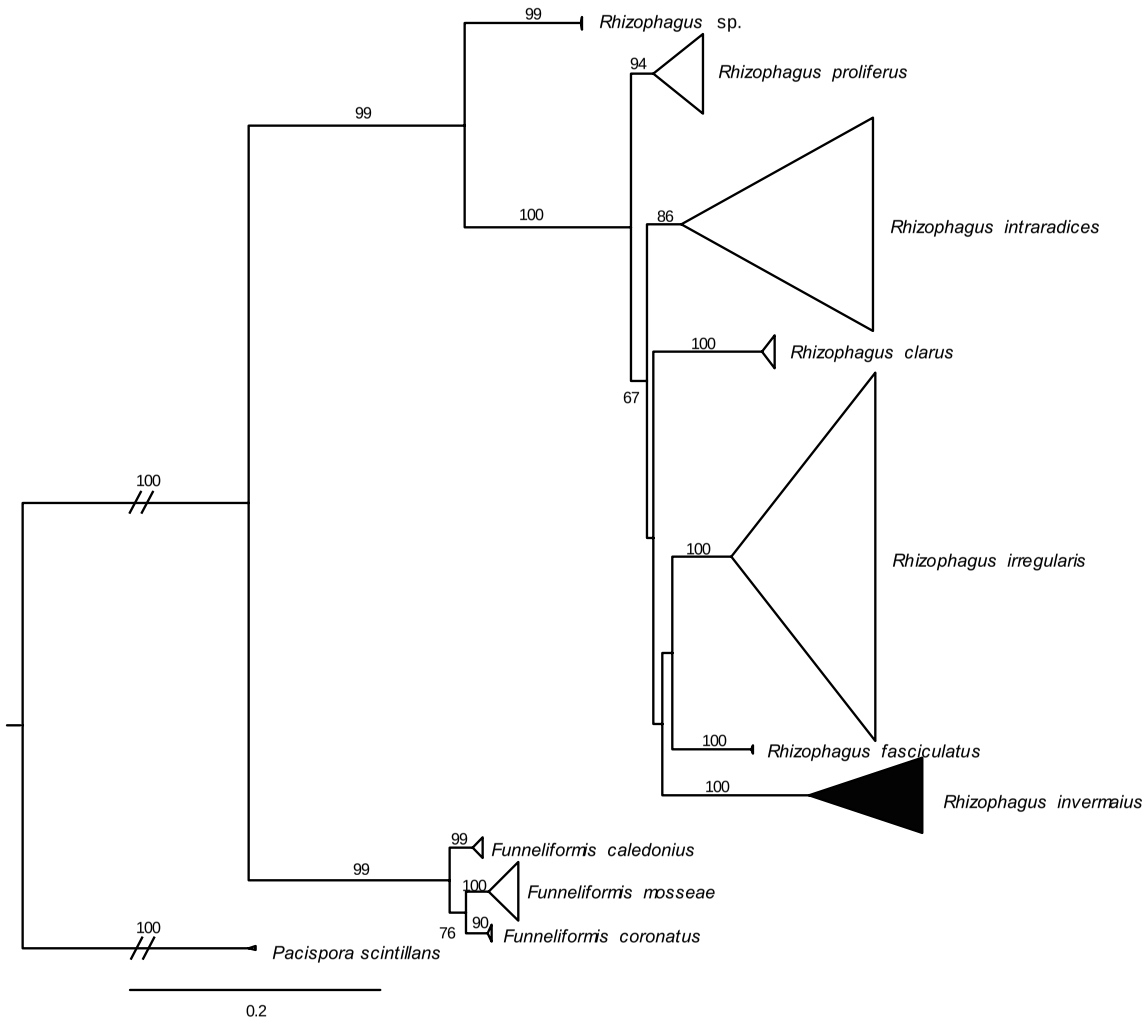
99
Rhizophagus sp.
94
Rhizophagus proliferus
99
100
86
Rhizophagus intraradices
100
Rhizophagus clarus
67
100
100
Rhizophagus irregularis
100
Rhizophagus fasciculatus
100
Rhizophagus invermaius
99
Funneliformis caledonius
99
100
Funneliformis mosseae
76
90
Funneliformis coronatus
100
Pacispora scintillans
0.2

Table I - Measurements of *Rhizophagus invermaius* spores from different subcultures identified with unique attempt number (Att 1646-x), and specimen vouchers numbers (Wxxxx). Measurements are in µm (length (L) × width [W]). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

Culture type	Spore type	Attempt #	Voucher (W no.)	Culture age	N	Min L	Max L	Min W	Max W	Mean L	Mean W	SD L	SD W
ROC	pale extraradical	5	5921	821	67	18	56	18	54	40	39	6.57	7.64
ROC	pale extraradical	7	5923	547	9	32	50	27	50	40	38	6.40	8.72
ROC	pale extraradical	8	5927a	558	50	24	69	21	67	48	46	8.94	9.87
ROC	pale extraradical	13	5937	448	19	30	45	21	45	38	36	11.01	12.11
ROC	fascicular extraradical	33	6057	258	53	40	77	38	77	58	58	8.67	8.86
HAM-P	extraradical	9	5928	451	8	30	46	24	48	35	36	4.87	7.04
HAM-P	extraradical	10	5929a	451	29	27	64	27	64	41	40	7.68	7.78
PC	fascicular extraradical	14	5924	407	100	50	141	50	96	76	72	10.64	8.15
PC	fascicular extraradical	14	none	unknown	100	52	108	52	108	79	79	7.02	7.19
PC	fascicular extraradical	14	5938	491	30	67	104	69	104	83	81	7.65	8.72
PC	fascicular extraradical	35	6179a	296	17	34	48	32	48	40	40	4.73	5.02
PC	fascicular extraradical	42	6176a	609	38	34	75	34	74	54	53	10.29	10.02
PC	fascicular extraradical	59	6248	600	65	50	85	46	82	68	67	7.53	7.44
PC	fascicular extraradical	60	6250	600	36	56	117	54	128	78	77	12.93	13.45
PC	microspores	42	6176b	609	67	18	51	10	72	27	25	7.07	8.46
PC	microspores	35	6179b	296	20	19	43	18	58	28	28	6.57	7.64
PC	microspores	59	6249a	600	25	22	53	16	50	31	27	6.39	6.46
PC	microspores	60	6177	295	27	18	59	16	37	27	23	6.69	7.12
ROC	pale intraradical	8	5927b	558	50	29	101	27	70	55	52	12.87	10.19
HAM-P	pale intraradical	10	5929b	451	100	24	120	19	109	53	47	16.69	16.24
PC	pale intraradical	0	5917	3092	31	27	96	21	50	52	32	15.52	7.38
PC	pale intraradical	14	5925	407	100	43	160	21	102	82	51	20.82	12.35
PC	pale intraradical	14	5939	491	100	34	180	18	208	80	56	27.44	23.26
PC	pale intraradical	42	6176c	609	31	27	109	21	117	73	43	22.41	19.50
PC	pale intraradical	59	6249b	600	100	34	128	16	69	64	43	13.19	10.68
PC	pale intraradical	35	6179c	296	27	32	128	34	118	77	54	22.09	19.14
PC	brown intraradical	59	6249c	600	68	53	107	42	104	82	60	11.37	11.21

Table II - Different shapes of *Rhizophagus invermaius* spores from different subcultures, identified by voucher (Wxxxx) numbers: ext (extraradical), int (intraradical) and mic (‘microspore’). PC=pot culture, ROC=root organ culture, HAM-P=half-closed arbuscular mycorrhizal plant *in vitro* culture.

Voucher	5921	5923	5927	6057	5937	5928	5929	5924	5938	0000	6176	6179	6176	6177	6179	5927	5929	5917	5925	5939	6176	6179
	ROC ext	ROC ext	ROC ext	ROC ext	ROC ext	HAM-P ext	HAM-P ext	PC ext	PC ext	PC ext	PC ext	PC ext	PC mic	PC mic	PC mic	ROC int	HAM-P int	PC int	PC int	PC int	PC int	PC int
irregular											26	6	2	1	3		5	1	36	19		8
globose	85	6	41	88	12	5	26	53	20	94	8	7	23	6	3	30	7	1	2			
subglobose	7	1	6	13	4	1	3	22	4	6			21	6	9	18	25	2	3	6	1	1
ellipsoid		1		2	1	1		1			2		2	3	1		12	12	9	21	4	6
broadly ellipsoid	4	1	1		2	1		10					12	5	3	1	15	4	3	19	4	2
oval													1			1	28	6	20	9	12	4
fusiform																	1	2		4	7	1
spatulate																			1			
bladder, pip or lemon	3							1				3		3						5		4
pyriform	1		1								2		2					2	1	2	2	
peanut-shaped			1																	1		
obovoid								7					2	2	1		4	1		3	1	
ovoid								1									1					
reniform								1					1									
subreniform								1														
ampuliform																			2			
flattened by juxtaposition								3	6										4	4		

subangular												1	1	1			2		18	7		1
subtriangular																			1			
Total	100	9	50	103	19	8	29	100	30	100	38	17	67	27	20	50	100	31	100	100	31	27

5. Potato-associated arbuscular mycorrhizal fungal communities in the Peruvian Andes

This chapter is identical to the publication:

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Mycorrhiza
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ORIGINAL PAPER

Potato-associated arbuscular mycorrhizal fungal communities in the Peruvian Andes

Carolina Senés-Guerrero · Gloria Torres-Cortés · Stefan Pfeiffer · Mercy Rojas · Arthur Schüßler

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Abstract The world's fourth largest food crop, potato, originates in the Andes. Here, the community composition of arbuscular mycorrhizal fungi (AMF) associated with potato in Andean ecosystems is described for the first time. AMF were studied in potato roots and rhizosphere soil at four different altitudes from 2,658 to 4,075 m above mean sea level (mamsl) and in three plant growth stages (emergence, flowering, and senescence). AMF species were distinguished by sequencing an approx. 1,500 bp nuclear rDNA region. Twenty species of AMF were identified, of which 12 came from potato roots and 15 from rhizosphere soil. Seven species were found in both roots and soil. Interestingly, altitude affected species composition with the highest altitude exhibiting the greatest species diversity. The three most common colonizers of potato roots detected were *Funneliformis mosseae*, an unknown *Claroideoglomus* sp., and *Rhizophagus irregularis*. Notably, the potato-associated AMF diversity observed in this Andean region is much higher than that reported for potato in other ecosystems. Potato plants were colonized by diverse species from 8 of the 11 Glomeromycota families. Identification of the AMF species is important for their potential use in sustainable management practices to improve potato production in the Andean region.

Keywords Andes · Arbuscular mycorrhizal fungi · Community analysis · Roots and rhizosphere · Nuclear rDNA · *Solanum tuberosum*

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Introduction

Arbuscular mycorrhizal fungi (AMF) are found as root symbionts in the majority of land-plant species, including the ten most important crops for human nutrition (Brundrett 2009; FAO 2012). As these fungi provide the plant with water and soil mineral nutrients (mainly phosphorus) and protection against biotic and abiotic stresses (Smith and Read 2008), they are regarded as a potential solution to increase crop yields sustainably without polluting the environment with high fertilizer and pesticide inputs. However, despite the benefits that the application of AMF inocula could have in agriculture, several limitations arise from the restricted knowledge on the complex ecological and evolutionary dynamics of plant–fungal interactions (Verbruggen et al. 2013).

Many factors play a role in the dynamics of the symbiosis between the plant and AMF. In general, AMF communities are not random assemblages (Davison et al. 2011) and both edaphic factors and the type of plants are important for successful arbuscular mycorrhiza (AM) symbioses (Powell et al. 2009). Geophysical factors (e.g., altitude) and plant-related factors such as the plant developmental stage have been reported to influence AMF community composition (Husband et al. 2002a, b; Chaurasia et al. 2005; Oehl et al. 2006; Hannulah et al. 2010). Moreover, differences exist among AMF communities detected from rhizosphere soil or roots (Renker et al. 2005; Hempel et al. 2007), which may be

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because of seasonal sporulation or plant stage-specific root colonization. Therefore, understanding the processes that shape AMF communities is still a challenge and more information regarding the drivers of AMF community composition in different agricultural systems is needed, as these are key factors for sustainable management practices. For potato, Hannula et al. (2012) showed AMF communities in the potato rhizosphere soil to be affected significantly by the plant growth stage, field site, and year-to-year variation. However, differences can exist among AMF communities from potato rhizosphere soil and roots (Cesaro et al. 2008). Thus, to identify AMF actively associated with potato at a certain time point, the analysis of AMF within roots is necessary.

Potato (*Solanum tuberosum*), with a production of 374 Mt worldwide (FAO 2012), is the world's fourth largest food crop, following maize, rice, and wheat. It is considered critical for food security in a world confronted by increasing population growth (Birch et al. 2012). Potato tubers are rich in several micronutrients and vitamin C and in many developed countries represent a secondary staple crop. In the study region, the South American Andes, potato constitutes the main staple crop, with an average family farm growing 10–12 cultivars, from the approximately 4,300 native Andean potato varieties (Brush et al. 1995). Because *S. tuberosum* is a phosphorus-demanding plant (Dechassa et al. 2003) that grows in symbiosis with AMF (Bhattarai and Mishra 1984), understanding and managing its AM association is of particular agricultural interest. Positive responses of *S. tuberosum* have been shown after inoculation with AMF (McArthur and Knowles 1993; Duffy and Cassells 2000; Davies et al. 2005b). For example, yield improved after inoculation with *Gigaspora* species in a field experiment in Cameroon (Ngakou et al. 2006). *Funneliformis mosseae* (syn. *Glomus mosseae*) demonstrated to be a good colonizer of potato plants in trap cultures in a greenhouse experiment (Bharadwaj et al. 2007) and *Rhizophagus irregularis* (syn. *Glomus irregulare*, formerly often named *Glomus intraradices*; see Stockinger et al. 2009) has been reported to be a preferential colonizer of potato in arable soils in Italy (Cesaro et al. 2008).

Landrace potato cultivars probably originated at altitudes of more than 3,000 m above sea level (mamsl) in the Andes (Spooner et al. 2005), where traditional manual labor agriculture is still practiced. The AMF communities associated with potato may be affected by increasing altitude (temperature, solar radiation, atmospheric pressure) and the correlated environmental changes (e.g., of plant communities, precipitation, seasonality). However, there is only one published report on AMF in an Andean potato field, namely of *Glomus*, *Gigaspora*, and *Scutellospora* spores in soil at 3,900 mamsl in Peru (Davies et al. 2005a). Identification at species level was not an aim of that study. To our knowledge, no further data regarding potato-associated AMF in Andean ecosystems have been published.

This study was therefore performed to characterize AMF species diversity associated with potato in the Peruvian Andes. AMF communities inhabiting the roots and also the associated rhizosphere soil were analyzed at different elevation levels and plant growth stages using molecular-biological methods that allow monitoring of AMF at species level. The aim was to elucidate the AMF diversity hosted by a potato root system and associated rhizosphere soil, to discover whether there are preferential associations between potato plants and certain AMF species, and to assess the influence of altitude and plant growth stages on such preferences.

Materials and methods

Field site and sampling

Four potato fields at different altitudes in Peru were selected. Site 1 was at 2,658 mamsl (11°16′02.2″S 75°06′56.8″W), site 2 at 3,245 mamsl (12°01′42.9″S 75°16′02.7″W), site 3 at 3,751 mamsl (11°53′14.4″S 75°25′05.1″W), and site 4 at 4,075 mamsl (12°14′40.6″S 75°03′03.9″W). Edaphic characteristics of the study sites are shown in Table 1. The field size ranged from 1,300 to 1,800 m^2. The potato variety grown was Yungay, except at site 1 (lowest altitude), where the variety Unica was grown.

For each study site (altitude), five random replicates (each from a different plant) of root systems and adjacent rhizosphere soil were collected at three plant developmental stages: emergence, flowering, and senescence, selected based on the BBCH scale according to the potato development system described by Hack et al. (1993). Plants at the emergence stage (stage 2) had one to nine basal side shoots (sampling date, December 15, 2009), at the flowering stage (stage 6), 50 % of the plants had flowers (sampling date, February 2, 2010) and at the senescence stage (stage 9), 50 % of the leaves were brown (sampling date, March 8, 2010). In total, 60 potato root samples and 60 corresponding rhizosphere soil samples were collected.

The chemical and physical properties of bulk soil from each field were determined. Rhizosphere soil (from here onwards referred to as soil) adhering to the root system was removed with a brush, sieved through a 1-mm mesh and dried for 2 h at 85 °C. The dried soil samples were placed in sterile, dry containers and kept at 4 °C until they were shipped to Europe for DNA extraction. The root systems of the corresponding plants were rinsed with water, cut into 1 cm pieces, and representative random samples were placed in 80 % ethanol in 10 ml cryovials. The root material was prepared at the field sites, immediately after harvesting the plants and later stored at −20 °C until DNA extraction.

Table 1 Edaphic characteristics of the study sites

	Site 1	Site 2	Site 3	Site 4
Region	Junin	Junin	Junin	Huancavelica
Province	Tarma	Huancayo	Huancayo	Tayacaja
District	Huasahuasi	Sicaya	Sincos	Pazos
Community	Huaya	Paraje Alpala	Ishmiqa	San José de Aymará
Elevation	2,658 mamsl	3,245 mamsl	3,751 mamsl	4,075 mamsl
Latitude south	11° 16′ 02.2″	12° 01′ 42.9″	11° 53′ 14.4.″	12° 14′ 40.6″
Longitude west	75° 06′ 56.8″	75° 16′ 02.7″	75° 25′ 05.1″	75° 03′ 03.9″
Farmer	Enrique Sueldo López	Rodrigo Santillán Quispe	Eliseo Martínez Inga	Rubén Romero Quilca
Field size	1,300 m^2 aprox	1,800 m^2 aprox.	1,400 m^2 aprox	1,500 m^2 aprox.
Field slope	0 %	0 %	15 %	30 %
Potato variety	Unica	Yungay	Yungay	Yungay
Planting date	28 October 2009	22 October 2009	21 October 2009	12 October 2009
Planting density	0.80 m×0.30 m	0.90 m×0.35 m	0.90 m×0.30 m	0.90 m×0.30 m
Fertilizer application				
Organic	Manure 7.7 t/ha	Manure 2.2 t/ha	Manure 3 t/ha	Manure 6 t/ha
Inorganic	Diammoniumphosphate, diammoniumnitrate 196 kg N/ha	Urea, diammoniumphosphate 228 kg N/ha	Urea, diammoniumphosphate, diammoniumnitrate 280 kg N/ha	Diamoniumnitrate 110 kg N/ha
	Diammoniumphosphate 177 kg P/ha	Diammoniumphosphate 255 kg P/ha	Diammoniumphosphate 164 kg P/ha	Triplesuperphosphate 150 kg P/ha
	Potassium chloride 230 kg K/ha	Potassium chloride 167 kg K/ha	Potassium chloride 214 kg K/ha	Potassium chloride 200 kg K/ha
Pesticide application	Fitoraz (Propineb+Cymoxanil)	Decis (Deltametrina)	Regent (Fipronil), Dithane (Mancozeb)	Regent (Fipronil)
Field history	2009–2010 potato	2009–2010 potato	2009–2010 potato	2009–2010 potato
	2008–2009 artichoke	2008–2009 carrot	2007–2009 potato (Canchan)	2008–2009 mixture of potato varieties
	2004–2008 fallow	2005–2008 lucerne, corn, pea	2004–2007 fallow	2003–2008 fallow
Soil analysis				
pH (1:1)	6.23	5.71	7.36	4.36
EC, dS/m	1.82	1.13	0.82	0.62
$CaCO_3$, %	0.10	0.00	19.40	0.00
Soil organic matter, %	2.34	2.98	2.28	7.00
N, %	0.18	0.28	0.14	0.41
P, ppm	61.7	56.0	50.5	64.5
K, ppm	253	268	236	418
Texture	Silt loam	Loam	Silt loam	Loam
Sand	40	40	32	46
Silt	52	40	54	46
Clay	8	20	14	8

DNA extraction

DNA was extracted from soil using a combination of a classical phenol–chloroform–isoamyl method with a modified bead-beating protocol from the FastDNA Spin Kit for soil (MP Biomedicals, Heidelberg, Germany). The modification was based on successive extractions described by Feinstein et al. (2009), who demonstrated that pooling the supernatants of successive bead-beatings results in extraction of a larger quantity of DNA from soil. For each rhizosphere soil sample, 0.5 g was transferred and repeated bead-beating was performed with the addition of 300 μl phenol–chloroform–isoamylalcohol (25:24:1, pH 8.0), in order to further increase the DNA yield from the soil samples. After pooling the supernatants in a 2-ml Eppendorf tube, two steps of classical phenol–chloroform extraction were performed (Sambrook and Russell 2006) before continuing with the protein precipitation step of the manufacturer's protocol of the FastDNA Spin Kit. The eluates of the final extraction step had a dark color, derived from substances such as humic acids and

polysaccharides, which can inhibit PCR. To further clean up the DNA, 50 μl DNA eluate was gently mixed with 50 μl of 0.32 % low-melting point agarose (Sigma-Aldrich, Vienna, Austria) in 1× TE (pH 8.0) and placed into disposable ten-well plug molds (Bio-Rad Laboratories, Vienna, Austria). After solidification, the agarose plugs were transferred to 2-ml Eppendorf tubes and washed with 1.5 ml TE buffer (pH 8.0) for 5 h. The washing step was repeated until the agarose blocks became colorless. Finally, the agarose plugs were melted at 70 °C and diluted 1:20 in H_2O to obtain a DNA solution that can be directly applied in PCR.

For DNA extraction from roots, the storage EtOH was exchanged for fresh EtOH and then 20 fragments of 1 cm length were randomly taken from each root system sample and dried for 15–30 min (depending on root thickness) at 60 °C to evaporate the ethanol. DNA was extracted using the FastDNA Spin Kit for soil following the manufacturer's instructions but using Lysing Matrix A tubes with an extra big ceramic bead instead of Lysing Matrix E, since Lysing Matrix A proved to be better for thick roots only weakly colonized by AMF (data not shown).

PCR, cloning, and RFLP

The Phusion High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt, Germany) was used for PCR with AMF-specific primers as described in Krüger et al. (2009), with the exception of using 35 cycles for the first PCR and 30 cycles for the nested PCR. Briefly, for both soil and roots, approx. 200 bp of the small subunit (SSU), the complete internal transcribed spacer (ITS) region, including the 5.8S ribosomal RNA (rRNA) gene, and approx. 800 bp of the large subunit (LSU) rRNA gene were amplified by the primers SSUmAf-LSUmAr, followed by a nested PCR using the primers SSUmCf-LSUmBr, resulting in an approx. 1.5-kb fragment. The PCR products were loaded on 1 % agarose gels with 1× Tris-acetate-EDTA buffer and run at 120 V for 30 min. DNA was visualized after ethidium bromide staining (1 μg ml^{-1}).

For soil samples, individual PCRs were performed on the five replicates per site with 1 μl DNA as template for the first PCR followed by a nested PCR, as described above. Not all replicates resulted in visible bands after PCR and gel electrophoresis: only three replicates were positive per sample for the emergence stage coming from sites 3 (3,751 m) and 4 (4,075 m) and the flowering stage coming from sites 3 (3,751 m) and 2 (3,245 m); four replicates were positive for the senescent stage coming from sites 2 (3,245 m) and 1 (2,658 m). All five replicates of the soil samples were positive for all remaining plant stages and sites. Nested PCR products of each site were pooled by taking 1 μl of each and mixing them. A total of 12 pooled PCR samples (each representing three to five replicates from one soil sample) were further analyzed.

For the root samples, volumes of 0.2–2 μl of extracted DNA were used as template (some samples contained PCR inhibitors and were only positive after reducing the amount of template). DNA from two replicates per root sample was individually PCR amplified and a total of 24 nested PCR samples (two individual replicates from 12 samples) was further analyzed. The PCR products of the root samples were not pooled, allowing the analysis of individual root samples.

The nested PCR products were used to construct clone libraries. A minimum of 40 clones with the correct length of plasmid insert for each sample were analyzed by RFLP after digestion with three restriction enzymes (Rsa I, Hinf I, Mbo I) as described by Krüger et al. (2009). A total of 1,857 clones were analyzed for both root and soil samples. Two clones were Sanger sequenced for each fragment pattern, sequences were assembled and edited in SEQASSEM (www.sequentix.de). For each sample, all distinct sequence variants were deposited at the EMBL international sequence database with accession numbers HF970195 to HF970341.

DNA sequence analyses

The new sequences were automatically aligned using MAFFT version 6 (http://mafft.cbrc.jp/alignment/software/) (Katoh et al. 2002). The alignment was then manually optimized with ALIGN (www.sequentix.de) based on the reference dataset of Krüger et al. (2012). To obtain species-level phylogenetic resolution, only sequences covering the full 1.5-kb SSU-ITS-LSU fragment were considered for phylogenetic analysis. Annotations follow the most recent systematics of the Glomeromycota (Redecker et al. 2013).

Maximum likelihood phylogenetic analyses were performed with RAxML 7.3.2. (Stamatakis et al. 2008) at the CIPRES Science Gateway V. 3.3 (http://www.phylo.org/portal2/), using 1,000 bootstraps and the GTRGAMMA model for both bootstrapping and tree inference. Phylogenetic trees were processed with FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Diversity analyses

Analysis of the community composition of AMF, in both soil and roots, was based on the number of clones (determined by analyzing the RFLP patterns) representing each AMF species (determined by phylogenetic analysis) present in a sample. Rarefaction curves, the Shannon diversity index (H′), species accumulation curves, and the estimated richness were calculated using the vegan package of R 2.15.1 (Development Core Team 2008). Rarefaction curves for soil and for roots were calculated using the rarefaction function. The curves were produced by taking into account the different species observed against the number of clones obtained per sample. The Shannon diversity index (H′) was calculated using the

diversity function by the formula $H' = -\sum p_i \log(b)\, p_i$, where p_i is the proportional abundance of species i and b is the base of the logarithm. For both soil and roots, the species accumulation curves were calculated with the specaccum function using the random method and 100 permutations; curves take into account the presence or absence of species found in the sampled sites. The estimated richness was calculated using the Chao estimator which calculates the number of observed species based on the observed number of singletons (species represented by one individual) and doubletons (species represented by two individuals).

To identify the main factors driving the community composition, we used principal coordinate analysis (PCoA), the UniFrac significance test, and the P-test on unweighted UniFrac distances between all samples using the UniFrac web interface (Lozupone et al. 2006) with 100 permutations and taking into account only data corrected for multiple comparisons. In this process, phylogenetic distances between sets of taxa in a phylogenetic tree are used to produce a distance matrix describing pairwise phylogenetic distances where standard multivariate statistics can be applied (Lozupone and Knight 2005).

Results

RFLP and sequence analyses of clone libraries

For both soil and root samples, the approx. 1.5-kb covering inserts of 1,857 clones were screened by RFLP. At least two representative clones of each RFLP pattern were sequenced and a minimum of four clones were sequenced when all clones showed identical RFLP patterns for all three of the restriction enzymes used. In total, 395 sequences of approx. 1.5 kb were characterized from the clone library. Finally, 147 AMF sequences were used as representatives for species identification by phylogenetic analysis. Of these, 112 represented unique variants and 35 were identical, but derived from different samples. BLAST analyses revealed that 471 of the 1,857 analyzed clones (25.4 %) were derived from non-AMF. Altogether, 1,386 (74.6 %) clones representing AMF were used to estimate the AMF diversity in the samples.

For the 12 pooled soil samples, 738 clones were screened, and BLAST analysis of sequenced clones indicated that 187 (25.3 %) were derived from non-AMF. From the 24 root samples, 1,119 clones were screened, and BLAST analysis showed that 284 (25.4 %) of them were derived from non-AMF. For site 4 (4,075 m), both root replicates from the senescence stage were negative for AMF (89 clones analyzed by RFLP, from which 24 were sequenced and all were from non-AMF).

General diversity of potato-associated AMF communities

In total, 212 sequences were used to construct a maximum likelihood phylogenetic tree comprising members of all the different AMF orders (Fig. 1). A full analysis (948 sequences) comprising members of all AMF genera and based on 1.5 kb sequences is shown in Supplementary Figure 1. Ninety sequences obtained from soil and 57 from root samples could be affiliated to 20 AMF clades which were interpreted as species (or putative species) in 11 genera on the basis of genetic distances and bootstrap support values. Of these, 10 (50 %) are new or unknown from published sequence data. The Peruvian sequences covered 8 of the 11 families in the Glomeromycota: *Paraglomeraceae*, *Archaeosporaceae*, *Ambisporaceae*, *Glomeraceae*, *Claroideoglomeraceae*, *Gigasporaceae*, *Acaulosporaceae* and *Diversisporaceae*. Taking into account clones derived from both soil and roots, the three most abundant species were *F. mosseae* (Sp. 12) representing 22 % of the total clones, an unknown *Claroideoglomus* sp. (Sp. 5) representing 16 % of the clones, and *R. irregularis* representing 9 % of the clones.

When comparing AMF species occurring in roots and soil, 12 AMF species were detected in roots, whereas 15 were detected in soil. Nevertheless, the Shannon diversity index was the same for both of them ($H' = 2.3$) due to the fact that only a very low number of AMF clones was obtained for some AMF species found in the soil (Table 2). Three species were more abundant in the roots: an unknown *Claroideoglomus* sp. (Sp. 5), *F. mosseae* (Sp. 12), and *R. irregularis* (Sp. 9). By far the most abundant species in the soil was *F. mosseae* (Sp. 12), followed by an unknown *Acaulospora* sp. (Sp. 15), and the unknown *Claroideoglomus* sp. (Sp. 5). Five species were detected only in roots and eight species were detected only in soil.

Sampling density and species richness

Rarefaction curves showed that the number of clones analyzed from the clone library was enough to cover the diversity for both soil and roots at four different altitudes (Fig. 2a). However, unexpectedly, only one to two AMF species could be detected in an individual root system of potato, although more AMF species have been found in the roots of other plants (e.g., tropical trees, grasses, etc.) using the same methods (unpublished data). Species accumulation curves relating to the plant and soil sampling were therefore calculated to analyze whether the total plant and soil sampling was adequate to represent all AMF species present in the studied potato fields. They revealed that sampling density was not sufficient to represent all AMF diversity present in the roots; the Chao index indicates a probable total number of 26 AMF species in roots and 22 species in rhizosphere soil (Fig. 2b).

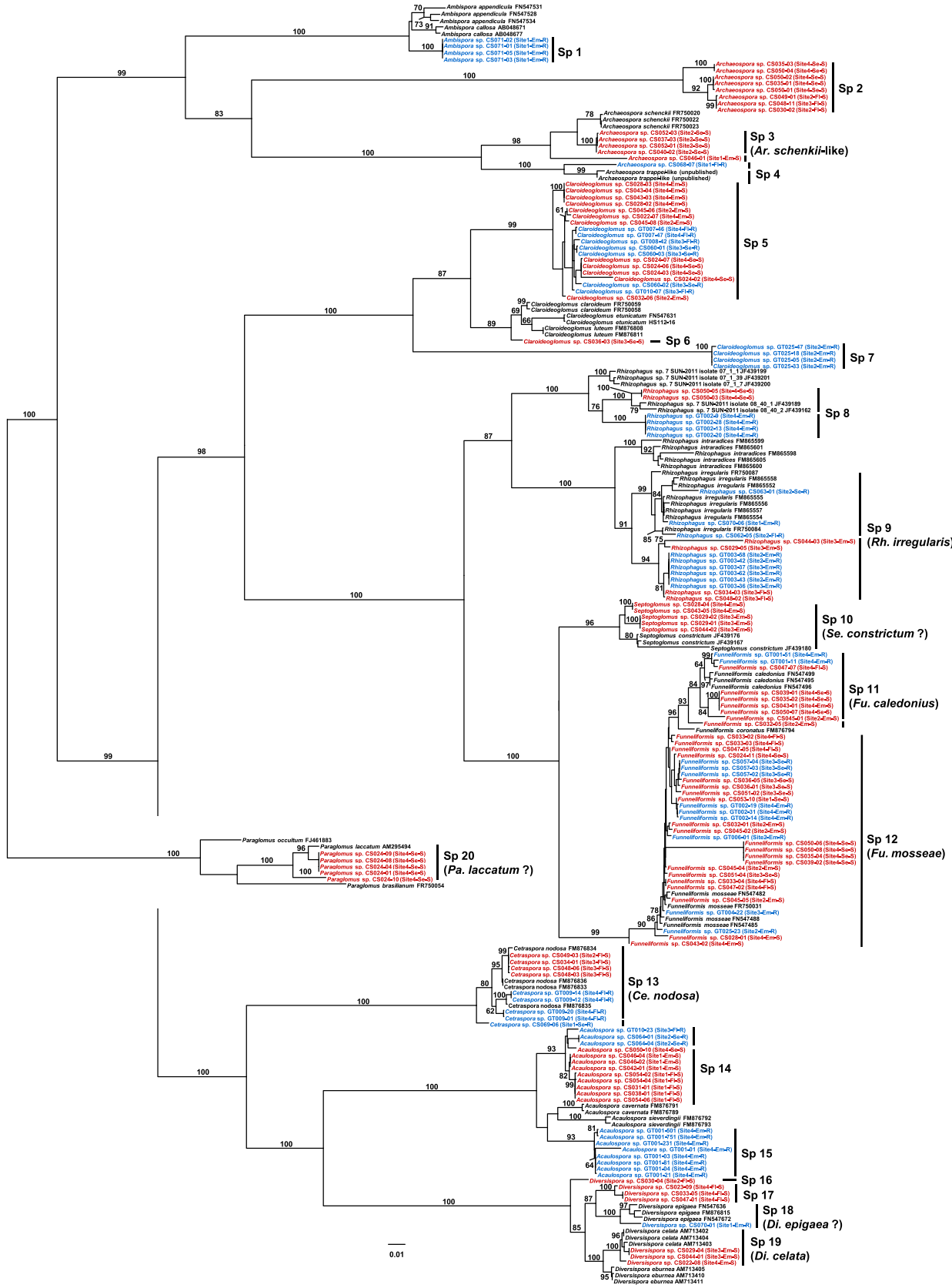

Fig. 1 Phylogenetic tree showing the species detected in the Peruvian potato fields and their close relatives (for full phylogenetic analysis see Supplementary Figure 1). *R* = roots, *S* = rhizosphere soil, *Em* = emergence, *Fl* = flowering, *Se* = senescence

Table 2 Number of clones for each AMF species in roots and rhizosphere soil at four sites (1 = 2,658 mamsl, 2 = 3,245 mamsl, 3 = 3,751 mamsl, 4 = 4,075 mamsl); (*n* roots=24; *n* soil=12 pooled samples)

		Roots				Total		Soil				Total	
		4R	3R	2R	1R	*n*	%	4S	3S	2S	1S	*n*	%
Am. sp.	Sp1	–	–	–	48	48	5.7	–	–	–	–	–	–
Ar. sp. 1	Sp2	–	–	–	–	–	–	9	3	28	–	40	7.3
Ar. *schenkii*-like	Sp3	–	–	–	–	–	–	–	–	33	13	46	8.3
Ar. sp. 2	Sp4	–	–	–	45	45	5.4	–	–	–	–	–	–
Cl. sp.	Sp5	29	139	–	–	168	20.1	49	–	8	–	57	10.3
Cl. claroideum (?)	Sp6	–	–	–	–	–	–	–	1	–	–	1	0.2
Cl. sp. 2	Sp7	–	–	39	–	39	4.7	–	–	–	–	–	–
Rh. sp.	Sp8	14		–	–	14	1.7	3	–	–	–	3	0.5
Rh. irregularis	Sp9	–	59	49	1	109	13.1	–	20	–	–	20	3.6
Se. constrictum (?)	Sp10	–	–	–	–	–	–	7	21	–	–	28	5.1
Fu. caledonius	Sp11	46	–	–	–	46	5.5	12	–	12	–	24	4.4
Fu. mosseae	Sp12	16	80	49	–	145	17.4	89	47	22	2	160	29.0
Ce. nodosa (?)	Sp13	48	–	–	48	96	11.5	–	19	4	–	23	4.2
Ac. sp. 1	Sp14	25	–	–	–	25	3.0	–	–	–	–	–	–
Ac. sp. 2	Sp15	–	5	48	–	53	6.3	4	–	–	54	58	10.5
Di. sp. 1	Sp16	–	–	–	–	–	–	–	–	15	–	15	2.7
Di. sp. 2	Sp17	–	–	–	–	–	–	18	–	–	–	18	3.3
Di. epigaea (?)	Sp18	–	–	–	47	47	5.6	–	–	–	–	–	–
Di. celata	Sp19	–	–	–	–	–	–	16	22	–	–	38	6.9
Pa. laccatum (?)	Sp20	–	–	–	–	–	–	20	–	–	–	20	3.6
Total AMF clones		178	283	185	189	835	74.6	227	133	122	69	551	74.7
Total non-AMF clones		89	0	104	91	284	25.4	88	16	18	65	187	25.3
Shannon index (AMF)		1.7	1.1	1.4	1.4	2.3		1.8	1.6	1.8	0.6	2.3	
Species number (AMF)		6	4	4	5	12		10	7	7	3	15	

Am. Ambispora, *Ar. Archaeospora*, *Cl. Claroideoglomus*, *Rh. Rhizophagus*, *Se. Septoglomus*, *Fu. Funneliformis*, *Ce. Cetraspora*, *Ac. Acaulospora*, *Di. Diversispora*, *Pa. Paraglomus*

Influence of altitude and plant stage on AMF communities

The highest AMF species diversity was found at the highest altitude (site 4, 4,075 m) for both soil (H′=1.8) and roots (H′=1.7): six species were found in the roots, the most abundant ones being *Cetraspora nodosa* (Sp. 13) and *Funneliformis caledonius* (Sp. 11). At this altitude, ten species were found in the soil, the most abundant being *F. mosseae* (Sp. 12). The lowest species diversity in roots was found at site 3 (3,751 m, H′=1.1) where the dominating species was an unknown species of *Claroideoglomus* (Sp. 5) while soil at the lowest altitude (site 1, 2,658 m, H′=0.6) showed least diversity, being dominated by an unknown *Acaulospora* sp. (Sp. 15) (Table 2).

Considering plant developmental stages (Table 3), AMF were most diverse in roots at the emergence stage (H′=2.0), whereas for soil, detected AMF communities were equally diverse at both emergence and flowering stages (H′=1.9). At the emergence stage, eight AMF species were found in soil and in roots, with *F. mosseae* (Sp. 12) being the most abundant one. At the flowering stage, AMF species were very diverse in soil (H′=1.9) but not in roots (H′=1.1) where an unknown *Claroideoglomus* sp. (Sp. 5) was the dominant fungus. In roots, four of the five AMF species found at the flowering stage could also be found at the senescence stage. At the senescence stage, the soil contained the highest number of species (nine species), *F. mosseae* (Sp. 12) being the most abundant (Table 3).

PCoA, used to determine the influence of altitude and plant developmental stages on the AMF communities, showed that communities differed significantly ($P<0.01$) between soil and roots, based both on the UniFrac significance test and the P-Test. PCoA analysis explained 65.8 % of the variation in relation to altitude; nevertheless, altitude did not have a significant effect on the AMF communities when soil and root samples were analyzed together (Fig. 3a) or separately (data not shown). Regarding the influence of plant developmental stages, PCoA analysis explained 65.3 % of the variance but, again, differences among the communities at each plant

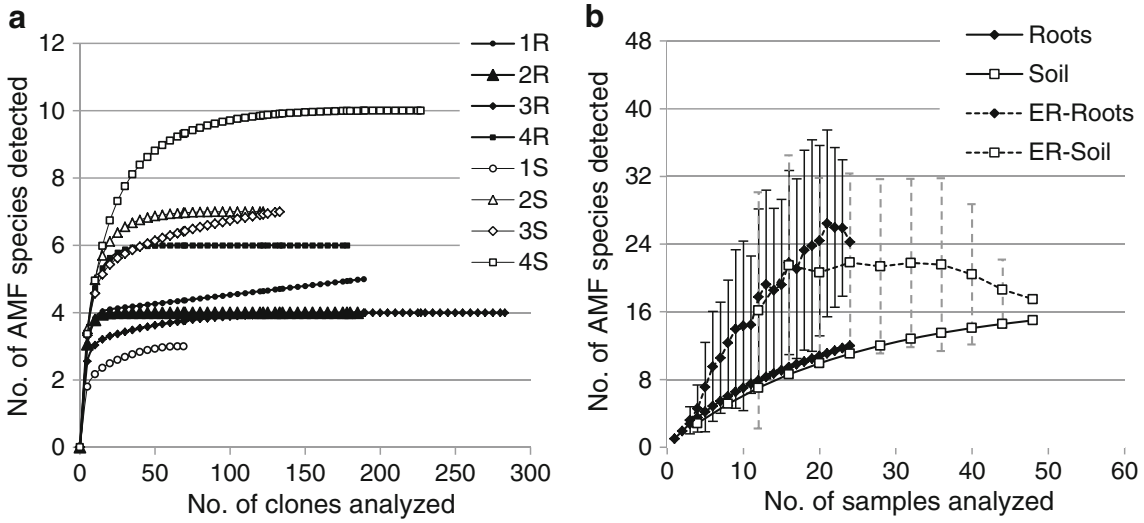

Fig. 2 **a** Rarefaction curves for the number of clones representing AMF species in the roots and rhizosphere soil at four elevations (*1* = 2,658 mamsl, *2* = 3,245 mamsl, *3* = 3,751 mamsl, 4 = 4,075 mamsl). Note that a different potato variety ('Unica') was grown at site 1 (2,658 mamsl). **b** AMF species accumulation and estimated richness curves for the total number of plants and pooled soil samples, at all four elevations and for all three plant stages. The estimated richness curves were calculated with the Chao index. *Error bars* = standard error, *R* = roots, *S* = rhizosphere soil, *ER-Roots* = estimated richness roots, *ER-Soil* = estimated richness soil

developmental stage were not significant when soil and root samples were analyzed together (Fig. 3b). However, when root samples were analyzed alone, significant differences were found between the AMF communities

Table 3 Number of clones for each AMF species in roots and rhizosphere soil at three plant developmental stages. (Em = emergence, Fl = flowering, Se = senescence); (*n* roots=24; *n* soil=12 pooled samples)

		Roots			Total		Soil			Total	
		Em	Fl	Se	*n*	%	Em	Fl	Se	*n*	%
Am. sp.	Sp1	48	–	–	48	5.7	–	–	–	–	–
Ar. sp. 1	Sp2	–	–	–	–	–	–	31	9	40	7.3
Ar. schenkii-like	Sp3	–	–	–	–	–	13	–	33	46	8.3
Ar. sp. 2	Sp4	–	45	–	45	5.4	–	–	–	–	–
Cl. sp. 1	Sp5	–	120	48	168	20.1	32	–	25	57	10.3
Cl. claroideum (?)	Sp6	–	–	–	–	–	–	–	1	1	0.2
Cl. sp. 2	Sp7	39	–	–	39	4.7	–	–	–	–	–
Rh. sp.	Sp8	14	–	–	14	1.7	–	–	3	3	0.5
Rh. irregularis	Sp9	60	1	48	109	13.1	8	12	–	20	3.6
Se. constrictum (?)	Sp10	–	–	–	–	–	28	–	–	28	5.1
Fu. caledonius	Sp11	46	–	–	46	5.5	14	1	9	24	4.4
Fu. mosseae	Sp12	97	–	48	145	17.4	53	35	72	160	29.0
Ce. nodosa	Sp13	–	48	48	96	11.5	–	23	–	23	4.2
Ac. sp. 1	Sp14	25	–	–	25	3.0	–	–	–	–	–
Ac. sp. 2	Sp15	–	5	48	53	6.3	22	32	4	58	10.5
Di. sp. 1	Sp16	–	–	–	–	–	–	15	–	15	2.7
Di. sp. 2	Sp17	–	–	–	–	–	–	18	–	18	3.3
Di. epigaea (?)	Sp18	47	–	–	47	5.6	–	–	–	–	–
Di. celata	Sp19	–	–	–	–	–	38	–	–	38	6.9
Pa. laccatum (?)	Sp20	–	–	–	–	–	–	–	20	20	3.6
Total AMF clones		376	219	240	835	74.6	208	167	176	551	74.7
Total non-AMF clones		9	146	129	284	25.4	57	74	56	187	25.3
Shannon index		2.0	1.1	1.6	2.3		1.9	1.9	1.7	2.3	
Species number		8	5	5	12		8	8	9	15	

Am. Ambispora, *Ar. Archaeospora*, *Cl. Claroideoglomus*, *Rh. Rhizophagus*, *Se. Septoglomus*, *Fu. Funneliformis*, *Ce. Cetraspora*, *Ac. Acaulospora*, *Di. Diversispora*, *Pa. Paraglomus*

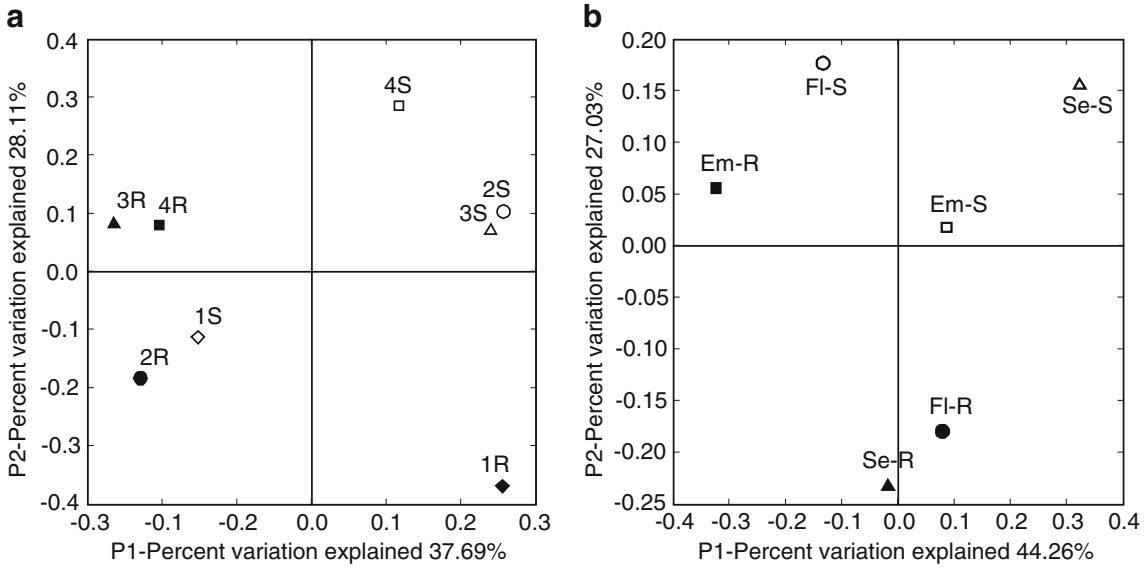

Fig. 3 Principal coordinates analysis of the AMF community in the roots (*R*) and rhizosphere soil (*S*) at **a** four elevations (*site 1* = 2,658 mamsl, *2* = 3,245 mamsl, *3* = 3,751 mamsl, *4* = 4,075 mamsl); note that a different potato variety ('Unica') was grown at site 1; **b** three plant developmental stages (*Em* = emergence, *Fl* = flowering, *Se* = senescence)

at the emergence stage compared with those at the flowering stage ($P < 0.05$; data not shown).

Discussion

Detection at the species level is an important step towards characterization of functional aspects of AMF communities and individual species in the field. However, species identification in the kingdom *Fungi* is difficult due to the unexplored diversity and lack of reliable annotated sequences (Kõljalg et al. 2013). Usually, there exists no simple sequence similarity threshold that can be used for species delimitation, and this is particularly true for AMF with their enormous intraspecific ribosomal DNA (rDNA) sequence variability (Stockinger et al. 2009, 2010). Most molecular ecological studies targeting entire AMF communities analyze an undefined taxonomic level between genus and species (e.g., Öpik et al. 2013). This allows some important comparisons at global scales but is limited in terms of AMF species diversity and community analyses because a single taxonomic unit may cover more than one species while, at the same time, several distinct taxonomic units may represent sequence variants from a single species. It has been shown that a 1.5-kb fragment covering the SSU-ITS-LSU rDNA region is suitable for members of the Glomeromycota as an extended DNA barcode providing species-level resolution, also in field studies (Krüger et al. 2009; Stockinger et al. 2010; Schoch et al. 2012). In the present study of potato-associated AMF in the Peruvian Andes, an RFLP and Sanger sequencing approach were chosen to analyze this 1.5-kb rDNA fragment because if many "unknowns", not represented by phylogenetically informative long sequences, were found in such an ecosystem, application of next generation sequencing methods would not improve species level resolution. This approach has proved reasonable, as half of the detected AMF species were previously not characterized at the DNA level, and the relative abundance of AMF species could be determined by relating RFLP patterns to sequence data. Although this approach might include some wrongly related RFLP patterns, this error appears to be minimal as there was no indication of any misinterpretation of the RFLP patterns from the obtained sequence data. Even though field samples were analyzed from the Andean ecosystems, only a low number of non-AMF clones (25 %) were obtained when compared to other primers used in molecular ecological studies (e.g., Alguacil et al. 2009; Sánchez-Castro et al. 2012).

The analyses of AMF associated with potato in the Peruvian Andes show a relatively high fungal diversity. Twenty clades, interpreted as species, were detected in roots and rhizosphere soil at the four study sites from 2,658 to 4,075 mamsl. Interestingly, this is comparable with the level of AMF diversity reported for the central (Liu et al. 2011) and southern Tibet Plateau (Gai et al. 2009). However, direct comparisons are difficult because different studies use variable means to describe the AMF communities. In the present study, where obtained sequence types were characterized at species level, half of the species represent new ones or species previously not characterized by sequence data, which indicates the existence of a large number of unknown AMF species in the Andean ecosystems.

Although potato appears to be colonized by a wide variety of AMF in the Andean ecosystems, species from *Archaeospora*, *Ambispora*, and *Diversispora* were rarely

detected in roots and only at site 1 (2,658 m), the lowest altitude studied. A lack or infrequent detection of *Paraglomeraceae* and *Archaeosporaceae* species in roots has also been reported for some plants in Central Europe (Hempel et al. 2007), whereas Gosling et al. (2013) recently reported that a species related to, or representing, *Paraglomus laccatum* is widespread in organically managed agricultural soils in England. On the other hand, *F. mosseae*, considered as a "generalist" AMF that can cope with soil disturbance, was found in potato roots under varying environmental conditions at the different Andean locations, and most abundantly at the plant emergence stage. This concurs with previous observations describing this AMF species as an early-stage colonizer (Sýkorová et al. 2007). Bharadwaj et al. (2007) detected only *F. mosseae* in the roots of potato plants inoculated with soil from an oxeye daisy (*Leucanthemum vulgare* Lam) field from Sweden. However, *F. mosseae* was not found in potato roots in the field study by Cesaro et al. (2008) where *R. irregularis* dominated instead. Both these species were found in the Andean potato roots. Also noticeable is the frequent detection of *Acaulospora* spp. at different potato-growing sites in the Peruvian Andes. Members of this genus have been reported at altitudes up to the limit of vegetation (3,000 m) in the Alps (Oehl et al. 2006). Some discrepancies were found in the AMF communities between potato rhizosphere soil and roots, which is in agreement with the observations reported by Cesaro et al. (2008). Even though the diversity index is the same for both (H′=2.3), the number of species in the soil was higher. In total, from the 15 species in soil and 12 in roots, 7 were detected in both. The present data on AMF in Andean potato ecosystems support the concept that different AMF species or taxa can be host or habitat generalists, or specialists (e.g., Oehl et al. 2010; Öpik and Moora 2012). However, clearly more detailed information is needed to draw conclusions about the specialists and the drivers of their occurrence.

In the present study, AMF communities were not fully represented by the sampling strategy used. Although sampling density was comparable to that in many published studies, the species accumulation curves based on plant and soil sampling indicated that the sampling was not dense enough to cover all the diversity. Thus, although rarefaction curves based on the clone libraries indicated sufficient sampling, the low number and high variability of AMF species found in individual root systems resulted in incomplete coverage of the root-associated diversity. Consequently, the approach used did not cover all the potato-associated AMF diversity. The expected total number of potato-associated AMF for all studied samples would be in the range of 31±8 species, based on the expected numbers for roots (24±8 species) and soil (19±4 species) and an expected overlap of little more than 50 % of the species in roots and soil. It should be kept in mind that in general, when interpreting many published studies, rarefaction curves describing the representation in clone libraries do not necessarily reflect the situation in the primary samples.

The AMF associated with potato varied in the different Andean ecosystems. In contrast to many other studies, *R. irregularis* was not a dominating species in potato; it was detected mostly in roots at site 2 (3,245 m) and site 3 (3,751 m). This species is usually described to be ubiquitous (Sýkorová et al. 2007) and occurring frequently in, or dominating, agricultural systems. It was also reported as a preferential colonizer of potato plants in an agricultural area at 85 mamsl around Castelnuovo, Italy (as "*G. intraradices*"; Cesaro et al. 2008). In the Andean potato roots, *F. mosseae* was found to be much more widespread and frequent than *R. irregularis*, colonizing plants at three different sites, whereas in the study of Cesaro et al. (2008) it was detected only in soil. Nevertheless, both fungi were found in both the Italian and Peruvian fields, although very different from each other (climate, soil, potato variety, etc.). A *Claroideoglomus* sp. (Sp. 5) was also found to be abundant in potato roots at sites 3 and 4, but despite the high phylogenetic diversity present in the fields, *Gigaspora* species were not detected, in contrast to a previous report for a Peruvian potato field (Davies et al. 2005a). By re-analyzing LSU sequences, to properly compare with the data published by Cesaro et al. (2008) from Italy, seven AMF species were annotated (see Supplementary Fig. 1). Most of them were from rhizosphere soil, only two from roots. Interestingly, the latter were also detected in the Peruvian potato roots: an unknown *Rhizophagus* sp. (Sp. 8) and *R. irregularis* (Sp. 9). *R. irregularis* was more dominant in potato roots compared to soil in both the Peruvian and Italian fields. This species was detected abundantly in this and many other studies, possibly relating to its extensive sporulation within roots.

AMF diversity was affected by altitude in the Andean potato fields. Schmidt et al. (2008) reported that roots of native plants in the Peruvian Andes were colonized by AMF only below 5,300 mamsl, and different plants were found to be colonized by AMF in the Bolivian Andean highlands (Urcelay et al. 2011). In general, the majority of altitude-related studies show that there is a decrease in species richness with increasing altitude, with highest diversity at mid-elevations (between 1,000 and 2,000 m) (Rahbek 1995; Sanders and Rahbek 2012). Chaurasia et al. (2005) showed that AMF diversity decreases in the rhizosphere soil with increasing altitude, concurring with observations by Lugo et al. (2008, 2012) in which AMF richness, diversity, and colonization levels in grasses of the Puna region in South America were negatively correlated with increase in altitude. Interestingly, the opposite was found in Andean potato fields; the highest AMF diversity occurred at the highest altitude (site 4, 4,075 m) in both soil and root samples. However, even though the PCoA (which is based on phylogenetic distances, thus being independent of taxonomic nomenclature) showed that the AMF communities found at the

lowest altitude (site 1, 2,658 m) separated from the others, pairwise comparisons using the UniFrac significance test and the P-test indicated that there are no significant differences in the fungal communities at the different sites. According to the diversity index, the soil (but not the roots) from site 1 has a much lower diversity compared to the other sites but it is difficult to interpret which factors are causal. It must be noted that a different potato variety was grown in this site.

The composition of the AMF community was also affected by potato plant developmental stages. The pairwise comparisons of the phylogenetic distances among the different AMF communities showed that plant developmental stages are significantly different in their AMF composition when analyzing the rhizosphere soil and root sample data together. In general, the soil maintains a similar number of species and diversity index, whereas the roots have more significant differences in the AMF community at different plant developmental stages. In contrast, Hannula et al. (2012) showed that the plant growth stage significantly affects AMF communities in rhizosphere soil of potato plants, regardless of the plant variety studied, and that the senescence stage in the rhizosphere soil hosted the most diverse fungal community.

It is interesting that most of the AMF species detected in potato roots at the emergence stage did not persist during different plant growth stages. We could not find a *Claroideoglomus* sp. (Sp. 5) that clearly dominated in roots in the flowering stage at the emergence stage, where roots hosted the highest AMF diversity. Plant community succession and plant growth stage can strongly influence the AMF-associated community. For example, early successional soils in sand dunes contained the most phylogenetically diverse AMF communities (Sikes et al. 2012), and AMF types dominating in newly germinated seedlings of tropical trees were almost entirely replaced by previously rare types in the surviving seedlings the following year (Husband et al. 2002a, b).

In conclusion, the number of AMF species found in Peruvian potato fields is high and similar to that found in some other high altitude ecosystems that appear to harbor a high AMF diversity. Surprisingly, no more than two AMF species could be detected in an individual root system. This might indicate that the number of AMF species simultaneously colonizing an individual potato root system is limited or that there is a bias in the adopted sampling strategy, PCR, or cloning. AMF that are frequently associated with potato at different sites and at different plant stages could be identified, in particular *F. mosseae* (Sp. 12), an unknown *Claroideoglomus* sp. (Sp. 5), and *R. irregularis* (Sp. 9). These easily cultivable generalists which also colonized early plant developmental stages are promising candidates for AMF application in sustainable potato agriculture. The AMF community composition did not vary significantly among sites but differences in the Shannon's diversity index existed. A deeper analysis will be needed to monitor the AMF communities more in detail and to provide better insight into preferential AMF–potato associations under different environmental conditions. However, as most of the AMF species detected were not yet present in DNA sequence databases, long sequences such as the ones published here are needed for deeper analyses of larger sample numbers at the species-level by 454-pyrosequencing of the ITS and/or LSU region.

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6. A conserved AM fungal core-species community colonizes potato roots in the Andes

This chapter is identical to the publication:

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Abstract

Plant-symbiotic arbuscular mycorrhizal fungi (AMF) are of high global ecological and economic impact, but describing environmental communities of AMF at the species level remains a challenge, although such knowledge is needed to understand AMF-plant preferences and also to apply AMF in sustainable agriculture.

Here, the potato-associated AMF species community composition was assessed for three Andean countries along an altitudinal gradient and at different plant stages, by using 454 GS-FLX+ sequencing of a 760 bp LSU rRNA gene PCR amplicon. Two methods for analyzing the AMF community were compared: defining OTUs based on a simple sequence similarity threshold, or affiliating reference sequences to species based on a high throughput phylogenetic annotation approach using an evolutionary placement algorithm. The later approach was not only more precise, but also fundamental to robustly unveil the AMF community composition and for meaningful conclusions. The principal advantage of this approach was also demonstrated by using artificially constructed datasets based on validated public database sequences.

The affiliation of sequence reads to species using phylogenetic annotation revealed a surprisingly conserved AMF core-species community structure in Andean potatoes, regardless of different plant stages and environmental factors. In total, 41 species were detected and in some cases more than 25 species were found colonizing an individual root system. *Acaulospora* species were identified as dominant colonizers, co-occurring with *Cetraspora nodosa* and certain *Claroideoglomus* and *Rhizophagus* species in most potato root samples.

Keywords: 454 pyrosequencing/ Andean ecosystems/ arbuscular mycorrhizal fungi species communities/ DNA based species identification/ evolutionary placement algorithm/ *Solanum tuberosum* (potato)

Introduction

In a world confronted with an increasing human population, one of the main challenges is sustainable food production without negative impacts on valuable natural resources. While intensive agriculture promoted during the green revolution secured food demand over the last decades, it was also accompanied by high environmental costs that are nowadays recognized as public health and food production threats (Tilman et al. 2002). As an alternative, a more sustainable

agriculture respecting the positive impact of many soil microorganisms, including their potential utilization, has received significant attention (van Loon 2007; van der Heijden et al. 2008). One of the most relevant groups of such soil microbes are arbuscular mycorrhizal fungi (AMF), obligate symbionts of the vast majority of land plants, including the ten most important human food crops (Smith and Read 2008; Brundrett 2009; FAO 2012). In exchange for photosynthesis derived carbohydrates, AMF transport inorganic nutrients and water from the soil to the plants and can strongly increase the utilization efficiency of fertilizers, especially the nonrenewable phosphorus (P) (Tawaraya et al. 2012). Therefore the use of AMF as inoculum for agricultural purposes is promising and may be most important for P-demanding crops such as potato (*Solanum tuberosum*) (Dechassa et al. 2003).

Potato has a worldwide increasing value (Birch et al. 2012) and is currently the 4th largest food crop with a production of 365 Mt, following maize, rice and wheat (FAO 2012). Cultivated potato originated in the Andean region (Spooner et al. 2005) where nowadays is a staple crop, therefore increasing the yield of native potato cultivars by using AMF is a topic of interest (Davies et al. 2005). However, although AMF have been found colonizing plants at high altitudes in the Andes, at the Bolivian Altiplano (Urcelay et al. 2011) and at up to 5,250 meters (Schmidt et al. 2008), so far there are no studies analyzing the AMF community composition of potato in Andean ecosystems. Moreover, the general knowledge about AMF species colonizing potato roots is scarce, even though a better understanding of preferential AMF-host associations would facilitate the specific selection of AMF to be used as inoculum in sustainable agricultural practices. The identification of AMF in plant roots can only be obtained by using molecular markers. Although DNA-based detection is frequently used for field samples, the taxonomic level of resolution usually is undefined and concise information of the AMF species community composition remains unknown. For molecular ecological studies, the small subunit (SSU) (Öpik et al. 2013) and/or internal transcribed spacer (ITS) (Redecker 2000) and/or the large subunit (LSU) rDNA regions (Mummey and Rillig 2007) were frequently used. Yet, due to the low variability in the SSU, an extremely high intraspecific variability in the ITS or the use of relatively short LSU fragments, most analyses using these markers led to phylogenetic resolution at an undefined taxonomic level in-between species and genus (Stockinger et al. 2009).

At present 454 GS-FLX+ amplicon-sequencing provides read lengths of up to approx. 1 kb and is the high throughput sequencing method providing the best phylogenetic resolution power to monitor AMF in the field. We therefore developed a 454-sequencing based process to monitor AMF species and applied it to analyze potato-associated AMF communities. The SSU-ITS-LSU rRNA gene region used was defined as an extended DNA barcode resolving closely related AMF species (Stockinger et al. 2010; Schoch et al. 2012). Importantly, it can be amplified from field samples using AMF specific PCR primers (Krüger et al. 2009), which recently were confirmed to have the broadest taxonomic coverage among other PCR primers frequently applied for AMF detection (Kohout et al. 2014). The primers amplify approx. 1500 bp sequences, which serve to compute a robust reference sequences phylogenetic tree functioning as a "phylogenetic backbone" (Krüger et al. 2012) for the placement of the shorter, approx. 760 bp long 454 sequences by a maximum-likelihood evolutionary placement algorithm (EPA).

The principal problem in such approach is that a large number of unknown AMF species must be expected, in uncharacterized, putatively highly diverse ecosystems. The lacking sequence information for those AMF would result in deep sequencing data impossible to be robustly affiliated to species. Therefore, we recently analyzed the potato associated AMF in the Peruvian Andes by a clone library and Sanger sequencing based approach, characterizing the above noted 1500 bp extended DNA barcode (Senés-Guerrero et al. 2014). It turned out that approximately half of the AMF species in this ecosystem were unknown. Here, the 1500 bp sequences of these formerly uncharacterized AMF now allow the computation of a phylogenetic backbone for deep sequencing analyses.

For the first time the AMF species community of a crop plant from Andean ecosystems is analyzed in depth. The main goal was to describe, at species level, the composition of the AMF community colonizing potato roots, and to determine putative main players involved in potato AM in the ecosystems studied. We wanted to i) validate our new approach by studying whether the interpretation of the AMF community structure is principally influenced by the methods used, ii) determine how many and which AMF species live in an individual root system and which AMF co-occur, and iii) analyze whether the AMF species community composition is influenced by altitude and/or plant developmental stage.

Materials and Methods

Sample collection

In each of the three studied countries, Bolivia, Ecuador and Peru, potato roots were sampled at three plant developmental stages (emergence, flowering and senescence; according to the potato development system described by Hack et al. 1993), from four potato fields which were located at four different altitude ranges (from 2,658 to 4,075 mamsl). Five replicate samples were collected at each sampling. The five replicates from the senescent stage from one Ecuadorian field could not be sampled due to early harvesting by the field owner without notice. In total, 175 samples were collected, from these 105 were analyzed by 454 sequencing (3 replicates per location per stage). In total, six different potato varieties were grown at the different locations (Bolivia: Waycha; Ecuador: Superchola, Guata, Fripapa; Peru: Yungay, Unica) (Online Resource 1). Directly after sampling the individual root systems were washed with water and cut into 1 cm pieces. Representative samples for each root system were placed in 80% ethanol in 10 ml cryovials. The root material was immediately prepared like this at the field sites and samples were later stored at -20°C until DNA extraction.

454-pyrosequencing

DNA was extracted from the root samples using the FastDNA Spin Kit for soil following the manufacturer's instructions but using Lysing Matrix A tubes with an extra big ceramic bead as described in Senés-Guerrero et al. (2014). The first PCR was performed as described by Krüger et al. (2009) with the AMF specific primers SSUmAf-LSUmAr using 35 cycles and 0.5 µl of the total DNA as template. The primers target a 1.8 kb region covering part of the SSU rRNA gene, the complete ITS region (including the 5.8S rRNA gene) and approx. 900 bp of the LSU rRNA gene. The product of the first PCR served as template for a nested PCR amplifying an approx. 760 bp fragment targeting the LSU rRNA gene. A fusion-primer amplicon strategy was used. The forward primer LSU-D1f (5´-TAAGCGGAGGAAAAGAAAMTAAC-3´) was synthesized together with the 454 adaptor A and different multiplex identifiers (MIDs). The reverse primer LSUmBr (Krüger et al. 2009) was synthesized with the 454 adaptor B (Eurofins MWG Operon, Ebersberg, Germany). One 20 µl PCR reaction contained 10 µl of the Phusion High-Fidelity DNA Polymerase Mastermix (New England Biolabs, Frankfurt, Germany), 0.5 µM of each primer and 0.2 µl of the first PCR amplicon. The cycling conditions were

99°C for 5 min, followed by 25 x (99°C for 10 s, 60°C for 30 s, 72°C for 1 min), followed by 72°C for 10 min. For each sample, three individual PCRs were performed and the products were observed by gel electrophoresis. After confirming a visible band, PCR replicates were pooled.

The pooled samples were sent to the company IMGM Laboratories (Martinsried, Germany) where each amplicon was separately purified using solid phase reversible immobilization (SPRI) paramagnetic bead-based technology (AMPure XP beads; Beckman Coulter, Krefeld, Germany) and quantified using PicoGreen dsDNA Assay Kit (Invitrogen, Darmstadt, Germany). Three libraries were generated containing the amplicon samples (pooled equimolarly) each with different MIDs. Each library was purified three times applying two different methods. First a gel extraction followed by a size selection step (> 250 bp) performed twice using the SPRI paramagnetic beads. Sequencing was done by using the GS FLX+ Titanium Sequencing Kit (Roche, Basel, Switzerland).

The sequencing run has been stored at the Sequence Read Archive at the NCBI with accession number PRJNA242351.

Bioinformatic analyses

Image and signal raw pyrosequencing data were processed by the Roche 454 GS-FLX+ inherent software packages applying the LongAmplicon3 processing pipeline which allows for 3´end trimming, recommended for processing long amplicon reads. Further downstream analyses were carried out by using the QIIME pipeline (Caporaso et al. 2010). The following parameters were used to select reads: no more than 15 ambiguous bases, maximum length of homopolymer run of 15, a maximum number of 5 primer mismatches and sequences with a minimum length of 500 bp including the primers. Sequences which did not fulfill these requirements were discarded. Sets of sequences were divided into clusters using UCLUST (Edgar 2010) at a similarity threshold of 98%. Representative sequences (RS) were used for downstream analyses either as OTUs or as input sequences for EPA based species affiliation. The 98% similarity threshold was empirically determined to always separate RS from different species into different clusters, by analyzing the approx. 760 bp target region sequences from 1,167 well defined AMF reference sequences. After clustering, singletons were removed and the remaining RS were blasted against the NCBI database to identify and remove non-AMF sequences.

Species delimitation

Briefly, species delimitation consisted of two steps: in the first, a reference phylogenetic tree based on 1.5 kb sequences was computed and in the second, each RS of a 98% similarity cluster was individually placed into this reference tree and annotated to species.

For the first step, sequence alignments were done using MAFFT version 6 (Katoh et al. 2002) and manually optimized and merged with the reference alignment of Krüger et al. (2012) using ALIGN (www.sequentix.de) as described in Senés-Guerrero et al. (2014). A maximum-likelihood phylogenetic tree was calculated with RAxML-HPC2 at the CIPRES Science Gateway (http://www.phylo.org/portal2/) with 1,000 bootstraps and the GTRGAMMA model by using 1,078 unique 1.5 kb reference sequences from defined AMF species (Krüger et al. 2012) and from sequences obtained from a clone library constructed for Peruvian root samples (Senés-Guerrero et al. 2014). Additionally, new clone library sequencing data from Bolivia and Ecuador (accession numbers HG969311-HG969373; published here) were included. This reference phylogenetic tree was composed of only 1.5 kb sequences and used as a "phylogenetic backbone".

In the second step, each RS was aligned to the reference sequence alignment with MAFFT using a progressive method. The alignment containing the reference sequences plus the individual RS together with the reference tree were the input data for the phylogenetic placement of each RS. For this, the RAxML Evolutionary Placement Algorithm (EPA) with the GTRGAMMA model was used through the web interface (Berger et al. 2011; Berger and Stamatakis 2011). EPA individually assigns each sequence (in our case the RS) to the branches of the reference phylogenetic tree by using a maximum-likelihood model. The result consists of an interactive reference phylogenetic tree on which, by selecting tree branches, the affiliated sequences are displayed. A table with tree branches and affiliated sequences is also provided by EPA, which has been rigorously tested by its authors, proving its accuracy in placing short reads into a phylogenetic tree (for detailed information see Berger et al. 2011). Archaeopteryx Treeviewer 0.970 beta X was used to visualize the phylogenetic tree (Han and Zmasek 2009) and taxonomic annotations, following the most recent systematics of the *Glomeromycota* (Redecker et al. 2013), were manually done. Like this, each RS was affiliated to a described or yet unnamed species in the phylogenetic reference tree.

To further analyze our species annotation method, we compared our previously published clone library Sanger-sequencing data obtained from Peruvian potato rhizosphere soil and root samples (Senés-Guerrero et al. 2014) with the 454 sequences obtained from the same root samples (rhizosphere soil was not included). To allow comparisons, species previously detected with the Sanger sequencing approach were annotated with the same species number in the present 454 sequencing analyses. 454-read relative abundance (RA) and the frequency of occurrence (FO) were the criteria to compare the samples.

Validating taxa annotation: comparing 97%-OTU, monophyletic clade and EPA based methods

We compared two methods that are commonly used to delimitate AMF taxa against EPA, by using an artificially constructed AMF community sequence dataset. This was done to demonstrate the principle problems associated to the frequently used 97%-OTU or simple monophyletic clade approaches. The artificial community consisted on 228 defined sequences comprising 7 closely related species from the genus *Rhizophagus* (see Krüger et al. 2012). Sequences from *Rhizophagus irregularis* were shortened to the same 760 bp LSU rDNA fragment as used in the 454-sequencing approach (from here onwards referred to as query sequences). These query sequences, derived from public database sequences, were annotated by three approaches: a 97% similarity threshold (97%-OTU), a monophyletic clade approach and species-affiliation using EPA.

Two maximum-likelihood phylogenetic trees were computed, one for the monophyletic clade and one for the EPA approach. For the 97% similarity threshold, the query sequences were clustered by using UCLUST and results displayed as OTUs in the tree computed for the monophyletic clade approach. For the monophyletic clade approach, the tree was computed from the 760 bp target region only, to reproduce the strategy usually applied in pyrosequencing studies. For this, 228 *Rhizophagus* sequences, 176 query sequences and 14 *Paraglomus* sequences (as outgroup) were automatically aligned by using MAFFT and a maximum-likelihood phylogenetic tree was calculated using RAxML-HPC2 at the CIPRES Science Gateway with the GTRGAMMA model.

For the EPA approach the steps previously described were conducted. Briefly, a reference maximum-likelihood phylogenetic tree based on 1.5 kb sequences was calculated. The same sequences as used for the monophyletic clade approach were

analyzed, except that some identical sequences were removed. The phylogenetic backbone reference tree contained 225 *Rhizophagus* sequences and 12 *Paraglomus* sequences. In the second step, 146 query sequences of approx. 760 bp were individually placed into the branches of the reference tree by EPA and annotated to species.

Networks

To display putative major AMF players associated with potato, two main sets of networks were analyzed and directly compared: OTUs (corresponding to 98% similarity threshold 454 representative sequences) and species (EPA based affiliation) networks. These sets of networks were created to visualize shared OTUs or species among different altitudes, plant developmental stages and plant varieties. Networks were produced following the QIIME pipeline and visualized using Cytoscape 3.0.1 (Cline et al. 2007). Dominant species were identified by analyzing RA (relative read abundance) and FO (frequency of occurrence).

Data analysis and statistics

All analyses related to AMF community composition were performed using the vegan package (Oksanen et al. 2011) in R 3.1.0 (R Core Team 2014), unless stated otherwise.

To analyze and compare whether the method used to delimitate AMF taxa influences the interpretation of the community structure, OTU and species data matrixes were resampled to 200 reads and the Bray-Curtis index was used as a dissimilarity measure among the different sites. As it turned out that the EPA based species affiliation method was clearly superior to the similarity threshold based OTU approach, only EPA derived data were analyzed by nonmetric multidimensional scaling (NMDS) using the metaMDS function with the Bray-Curtis index. We used the envfit function to determine the relationship of altitude and plant stage with the AMF species scores in the NMDS by using 999 permutations. Furthermore, only for the EPA derived species data, changes on the beta-diversity were evaluated by permutational MANOVA (PerMANOVA) using the Arrhenius dissimilarity index with the adonis function. The dissimilarities for altitude and plant stage were partitioned and their significance analyzed by using 999 permutations. In this case, beta-diversity is calculated taking into account the number of species shared between two sites and the number of species unique to each site.

For both, the NMDS and beta-diversity analyses, two matrixes were the input. One matrix contained the normalized read counts of the species found per sample and the second matrix contained the plant stage or altitude for each sample. Raw read counts were normalized using the package DESeq2 (Anders and Huber 2010) in R, as it was shown that other classical methods for read count normalization are not appropriate to detect differentially abundant species (McMurdie and Holmes 2014). To analyze altitude, we grouped the samples into 4 different altitude categories: 4 = ≥ 4,001 m, 3 = 3,561 – 4,000 m, 2 = 3,001 – 3,560 m and 1 = ≤ 3,000 m (Online Resource 2).

Results

Comparison among taxa annotation methods

For our test dataset, based on publicly available validated sequences, the EPA approach resulted in all 146 *R. irregularis* 760 bp query sequences being correctly placed in the *R. irregularis* clade. None of the diverse sequences was misplaced into branches belonging to other *Rhizophagus* species (Fig. 1a and Online Resource 3). Thus, the correct result of the analysis was that all sequences belonged to one AMF species, *R. irregularis*.

When clustering the *R. irregularis* query sequences at a 97% similarity threshold, this resulted in 18 OTUs (Online Resource 4). Clustering the same sequences at 98% resulted in 32 OTUs (data not shown). The monophyletic clade approach resulted in a tree with very low bootstrap support values for many clades and it was difficult to delimit the different *Rhizophagus* species from one another (Figs. 1b-c, Online Resource 4). Even though the query sequences from *R. irregularis* were not clustering with sequences from other species, delimiting monophyletic clades to define species was not possible. To highlight the inaccuracy of taxa delimitation when using both, 97%-OTUs and a monophyletic clade approach, we marked different sequences belonging to 97%-OTUs in the tree computed for the monophyletic clade approach. Sequences from the same OTU were spread over different monophyletic clades and one monophyletic clade could contain different OTUs (Fig. 1b, Online Resource 4). Furthermore, to indicate that intraspecific sequence variability leads to misinterpretation of phylotypes as different taxa or taxonomic units, we also labelled different sequence variants obtained from the well-studied culture *R. irregularis* DAOM197198 or from one single spore DNA extract of *R. intraradices* FL208 (Online Resource 4). The full reference phylogenetic tree used for the EPA approach and the

placement of the query sequences are shown in the Online Resource 3. The full phylogenetic analysis of the monophyletic clade approach, also displaying the placement of 97%-OTUs, is shown in the Online Resource 4.

Processing of pyrosequencing data for AMF species affiliation

From a total of 105 samples (12 samples each for Bolivia and Peru, 11 samples for Ecuador, from 4 altitudes and 3 plant developmental stages, in 3 replicates), PCR on 102 samples resulted in visible products. All three PCR-negative replicates were from the same sample (Peru, altitude 1, senescence stage) which was excluded in downstream analyses. The initial amount of reads was 698,297. After quality filtering and removing reads below 500 bp, 366,088 reads of at least 500 bp length were clustered using a 98% similarity threshold into 4,943 representative sequences (RS). This was done because for the region analyzed, the frequently used similarity threshold of 97% led to a number of clusters containing sequences from different species (e.g., sequences of *Gigaspora rosea* with *G. margarita*, *Funneliformis mosseae* with *F. coronatus*, *Claroideoglomus luteum* with *C. claroideum* and *Acaulospura scrobiculata* with *A. spinosa*; data not shown). This problem could be avoided by using a 98% similarity threshold for clustering.

After singleton removal, 3,218 RS were left, of which 956 (29.7%) were non-AMF. From these, 96% were from fungi, 3.6% from plants and 0.4% from protozoa. Finally, 2,262 RS containing 255,740 reads with an average read length of 718 bp remained for AMF. By using EPA we annotated these to 41 species from 12 AMF genera (Figs. 2 and 3; for read abundance see Online Resource 5).

Comparing OTUs against EPA based species affiliation for potato associated AMF

To demonstrate how the annotation of OTUs or species can influence our understanding of the AMF community composition, we used the identical dataset to visualize networks containing either OTUs or species. The OTU networks always showed a similar trend in which the majority of the OTUs was specific to one variable (one altitude, plant stage or potato variety). On the contrary, species networks indicated that most of the AMF species were shared and only few were specific to a certain condition (Fig. 4).

Furthermore, we analyzed the similarities of the AMF communities among the sampling sites for both, OTUs and species by using the Bray-Curtis index. For comparison, we did a sub-sampling step; we excluded the samples E3Sc and E4Sc

from the species data and E1Sc and E2Sc from the OTUs data because of having fewer than 200 reads. The Bray-Curtis similarity for OTUs indicated a community composition represented by two major clusters (Fig. 5a) whereas when analyzing species, the AMF community composition of the sites appeared less structured (Fig. 5b). Importantly, the clustering topology showed that the OTU-defined community composition is totally inconsistent with the species-defined community (Fig. 5). As EPA species affiliation must be interpreted more robust than 97%-OTU and also basic monophyletic clade approaches, the community composition based on OTUs analyses appears unreliable.

AMF species diversity in potato roots

We proved that the EPA annotation of species is more robust compared to other frequently used methods and that conclusions on the community composition would strongly rely on the method used for taxa definition. We therefore, based on published knowledge (Stockinger et al. 2010) and our empirical data, analyzed EPA based species-affiliation data for further interpretation.

Rarefaction curves showed that plateau levels were reached for most of the samples when analyzing at the species level (Online Resource 5b). Based on relative read abundance (RA), the five most abundant species (from lowest to highest) were an unknown *Claroideoglomus* sp. (Sp. 5), *Claroideoglomus claroideum* (Sp. 39), *Cetraspora nodosa* (syn. *Scutellospora nodosa*) (Sp. 13), an unknown *Acaulospora* sp. (Sp. 23), and another unknown *Acaulospora* sp. (Sp. 14) (Online Resource 6). Based on frequency of occurrence (FO), the vast majority of potato plants (85%, 87 plants) were colonized by the most abundant *Acaulospora* Sp. 14 (Fig. 2 and Online Resource 7).

Preferential potato root colonizers

We considered that the species colonizing the plants at most altitudes, plant stages and potato varieties, were preferential colonizers of potato, based on FO. No species was present in all of the samples, nevertheless the overall most abundant (based on RA) unknown *Acaulospora* sp. (Sp. 14) appeared in 85% of the samples, followed by *C. nodosa* (Sp. 13; 3rd most abundant species based on RA) and *Claroideoglomus* sp. (Sp. 5; 5th most abundant species) each appearing in 78% of the samples (see Fig. 2 for FO values and Online Resource 6 for RA values). *Acaulospora* sp. (Sp. 23; 2nd most abundant species) appeared in 71% and *C. claroideum* (Sp. 39; 4th most

abundant species) appeared in 56% of the samples. These species therefore can be interpreted as a conserved core-species community of potato in the Andes.

Regarding coexistence of species, from 87 plants that hosted *Acaulospora* sp. (Sp. 14), 75 plants (86%) also contained *C. nodosa* (Sp. 13) and a *Claroideoglomus* sp. (Sp. 5). Regarding genera, *Cetraspora*, *Rhizophagus*, *Acaulospora* and *Claroideoglomus* colonized 68 plants (67%) simultaneously.

Among the six different potato varieties, 20 (49%) from the 41 total AMF species are shared among all of them. For the plant developmental stages, 31 species (76%) are shared among the three stages. For altitude, 26 species (63%) are shared among the four altitude groups (Fig. 4 and Online Resource 7).

Number of species colonizing an individual potato root system

The AMF species number in a single root system was displayed as different size groups (zero species, 1 to 5 species, 6 to 10 species, etc.; Fig. 6). 102 individual root systems contained 1 to 25 species in relatively similar amounts. 2% of the root samples contained more than 25 species (Fig. 6). The amount of species found in a single root sample was not related to the plant stage, e.g. for some plants in the emergence stage more than 20 species could be detected while for others only less than five.

Influence of altitude and plant stage on AMF communities

Nonmetric multidimensional scaling analysis of the species data resulted in a two-dimensional solution with a total stress value of 0.28. The NMDS plot revealed no structure in the community composition when we marked the 41 species based on their genus (Fig. 7). Moreover, altitude and plant stage had no significant influence on the AMF community (Table 1). However, the PerMANOVA conducted to test whether altitude or plant stage were influencing the beta-diversity of the different sites showed that altitude was a significant factor ($P < 0.05$) (Online Resource 8).

Comparison with a clone library Sanger sequencing approach

From clone libraries derived from Peruvian samples (24 root and 12 pooled rhizosphere soil samples) we annotated 20 species, some of them found only in rhizosphere soil (see Senés-Guerrero et al. 2014). For these samples a probable number of 31 ± 8 species was indicated by the Chao index. From the 454 sequences derived from the same root samples of Peru (35 samples) we annotated 37 species, including 10 species (out of 12) found in the root samples of the clone library. An *Ambispora* sp. and an *Archaeospora* sp. found in roots and previously annotated in

the clone library as “Sp1” and “Sp4” were not found by 454 sequencing. Other species (6, 16, 17 and 19) that were found only in rhizosphere soil with the Sanger sequencing approach were also not detected by 454 sequencing of the root samples.

Discussion

Methods for AMF species delimitation

Here, for the first time, we used 454 GS-FLX+ pyrosequencing of an approx. 760 bp LSU rDNA amplicon together with a high throughput, maximum-likelihood based phylogenetic annotation approach (EPA) to monitor AMF in the field at species level. This approach is based on an aligned reference sequence database and a maximum-likelihood phylogenetic reference tree. We used EPA because it provides more accurate species annotation than other methods used for high throughput monitoring and community studies of AMF, considering theoretical assumptions, published data (Stockinger et al. 2010; Berger and Stamatakis 2011) and our empirical tests.

In general, pyrosequencing data analysis pipelines can have a strong impact on the biological conclusions (Bakker et al. 2012). Using a precise method is crucial to obtain ecologically meaningful data and for fungi some specific considerations have been highlighted (Lindahl et al. 2013). For AMF, the difficulty starts from selecting the genetic marker region, continues with the similarity threshold used to cluster sequence reads and increases when trying to assign sequences into taxonomic groups. Moreover, every type of analysis depends on the quality of the baseline data used to compare with. In this context one problem for AMF (and also fungi in general) is, that many species have not yet been described or characterized by DNA sequences, which is especially problematic in unexplored ecosystems harboring high diversity. In such regions, many unknown AMF species are expected to exist (Kivlin et al. 2011; Liu et al. 2011), as turned out for the Andean region studied here with about 50% of the AMF species detected being uncharacterized by DNA sequences (Senés-Guerrero et al. 2014).

It is not possible to discriminate closely related AMF species by using simple sequence similarity thresholds and/or phylogenetic cluster analyses based on limited phylogenetic signal, for example when analyzing short average reads lengths of 200-400 bp as obtained with the previous 454 GS-FLX technologies. Thus, until now, a robust tracing of AMF at the species level has not been possible with 454

sequencing, but data regarding basic AMF community patterns and the putative factors driving such communities have been obtained (Öpik et al. 2009; Dumbrell et al. 2011; Davison et al. 2012; Lekberg et al. 2012; Öpik et al. 2013). Most of the earlier 454 sequencing based AMF community studies analyzed data by clustering reads into 97%-OTUs and matching them using BLAST. Some of the drawbacks of this approach are that many fungal sequences in public databases have not been correctly annotated or have quality issues (Kõljalg et al. 2013), leading to incorrect species descriptions and also, that whenever a taxon is not present in the reference sequences, the BLAST based assignment can be misleading (Berger et al. 2011). Another approach for the analysis of 454 sequencing data is the definition of OTUs based on clades in phylogenetic trees including known sequences (Horn et al. 2014). The latter approach is called a monophyletic clade approach and may, besides the formation of clades, also use the support values for the respective clades to interpret OTUs. Nevertheless, calculating a phylogenetic tree using short read length sequences, even of 760 bp lengths, leads to low resolution and support for clades and is prone to misinterpretations (see Online Resource 4). Lekberg et al. (2014) suggested that defining OTUs by using either a similarity threshold of 97% or by using a monophyletic clade approach did not affect interpretations of the general AMF community patterns. However, this assumption depends on the level of interpretation. Using a monophyletic clade approach based on short sequences does not provide species resolution and the taxonomic level of OTUs is undefined (see Online Resource 4). The high intraspecific variability of the rDNA sequences of AMF may cause the consequent splitting of individual species into many OTUs when using %-thresholds, as well when using monophyletic clades. Beside the noise of such data, this may cause OTU undersampling and consequent misinterpretations.

Thus, when circumscribing 454 sequences to AMF taxonomic units many factors, which are unfortunately often ignored, can lead to misinterpretations, including primer selection, BLAST-based annotation, similarity thresholds, method of OTU definition, and ecosystems with many unknown species. We tried to reduce the afore mentioned problems by i) selecting a region that previously was discussed to provide species resolution when using 454-sequencing (Stockinger et al. 2010), ii) testing similarity thresholds for sequence clustering using reference sequences as an empiric dataset to avoid sequences from different species falling into the same cluster and iii) using an EPA based approach for species annotation, in which

sequences are individually placed into a phylogenetic reference tree. Even though such annotation may also be biased (e.g., due to misalignment of sequences, see Berger and Stamatakis 2011), it appears to be significantly better for AMF community structure analyses than any of the other commonly used methods for high throughput sequencing data analysis. We could robustly annotate the representative sequences to unknown as well as to described species

Taxonomic coverage and species diversity

Interpretations of AMF diversity are strongly influenced by the PCR primer choice. Some primer combinations discriminate against certain AMF lineages (Gamper et al. 2009), while others result in high non-specific amplification (Alguacil et al. 2009). The region that we amplified in the first PCR offers species resolution power (Stockinger et al. 2010) and the primers used (Krüger et al. 2009) allow the widest taxon coverage compared to other commonly used primers targeting a single nuclear rDNA marker (Kohout et al. 2014). It may be mentioned that Kohout et al. (2014) reported chimera formation when using these primers. However, this was interpreted based on the use of *Taq* DNA polymerase, whereas the use of a high-fidelity enzyme with a fused DNA-binding domain, as the Phusion DNA polymerase recommended by Krüger et al. (2009), prevents most of the processes leading to chimera formation.

Here we annotated 41 species, 15 of them (37 %) unknown or previously not described in sequence databases and 5 of them (12 %) closely related to but separated at the species level from known species. Other studies using 454 sequencing have reported 70 AMF OTUs in an area of approximately 7 m^2, analyzing samples at summer and winter seasons (Dumbrell et al. 2011), 32 AMF OTUs in grassland plots (Lekberg et al. 2012), and 37 (Davison et al. 2012) and 48 (Öpik et al. 2009) AMF OTUs in forest plots. However, interpreting and comparing richness and species diversity is difficult from afore mentioned studies, for example because the number of OTUs is strongly dependent on the variability of the marker region and the threshold value used. We demonstrated this by the analysis of published LSU sequences of *R. irregularis*, resulting in 18 OTUs at 97% or 32 OTUs at 98% similarity, for one species only (see Online Resource 4).

AMF species associated with potato

To determine AMF preferentially colonizing potato, we used proportional read abundance as a semi-quantitative measure of abundance. Despite known biases when using 454 reads to determine biological abundance (e.g., rRNA gene copy

numbers, primer bias, varying PCR efficiency in different samples), comparing proportional abundance of one species with itself across samples and replicate 454 runs is reliable (Amend et al. 2010; Kauserud et al. 2012). Interestingly our data show that most of the samples are colonized by a conserved group of AMF species (67% of the plants from 12 studied sites were colonized by certain species from the genera *Acaulospora*, *Cetraspora*, *Claroideoglomus* and *Rhizophagus* simultaneously) which appear to be main players in potato AM in the Andean region. For the potato plants it is both surprising and noticeable that this core AMF species community is relatively conserved throughout the studied region since the sampling sites are from remote areas, e.g., a distance of 3,169 km from Loja (Ecuador) to Cochabamba (Bolivia), and from a wide variety of climatic conditions, e.g., in Cochabamba the average day temperature is approx. 24ºC during summer and mild during winter, whereas at study sites above 4,000 mamsl even in summer frost may occur periodically at night and strong frosts are frequent during winter.

In previous studies, *F. mosseae* was the only species found colonizing potato roots in soil-trap cultures (Bharadwaj et al. 2007) and *R. irregularis* (as "*Glomus intraradices*") was reported as the preferential colonizer of potatoes in an Italian agricultural field at low altitude (85 mamsl) (Cesaro et al. 2008). In the Andean region, surprisingly the potato roots colonizers detected in highest relative abundance and frequency were two unknown *Acaulospora* spp. (Sp. 14 and Sp. 23) found individually or together at all conditions and in 91 (89%) of the samples analyzed, perhaps indicating host preferences. The very frequent appearance of *Acaulospora* spp. in the Andes might directly or indirectly be related to altitude, since members of this genus were also frequently reported at altitudes around 3,000 m in the Alps and in the Chilean Andes (Oehl et al. 2006; Oehl et al. 2011), as well as at up to 3,520 m in the South American Puna grassland (Lugo et al. 2008) and at the Tibetan Plateau (Li et al. 2014; Gai et al. 2012). Due to their high abundance (RA) and frequency of occurrence (FO), we considered these *Acaulospora* spp. as main potato colonizers in the Andes. We cannot yet state about the biogeography of these species because of insufficient availability of data for comparison.

AMF species diversity in individual potato root systems

An individual plant root system is usually colonized by several AMF species. For potato plants high levels of AMF colonization were reported (McArthur and Knowles 1992; Davies et al. 2005) but surprisingly only one or two species per root system

were detected by Sanger sequencing of clone libraries from Peruvian ecosystems (Senés-Guerrero et al. 2014). In contrast, 454 sequencing from the same samples as analyzed in that study revealed that more than 25 AMF species can be present in a single root system, indicating the limitations of the clone library based approach. The joint presence of certain species may be related to phylogenetically distinct AMF having different complementarity functions (Maherali and Klironomos 2007). For potato, the frequency of occurrence of AMF in individual root systems strongly indicates functional complementarity beneficial for the host, based on the observation that an *Acaulospora* sp. (Sp. 14), *C. nodosa* (Sp. 13), and an *Claroideoglomus* sp. (Sp. 5) were found coexisting in 74% of all samples (75 plants). 67% of all samples (68 plants) additionally contained one of the eight detected *Rhizophagus* species.

Influence of plant stage and altitude on AMF species communities

Analyzes of OTU networks indicate that most of the OTUs are specific to either an altitude, plant stage or plant variety, in agreement with previous studies showing habitat or seasonal differences and host preferences in the AMF communities (Husband et al. 2002; Öpik et al. 2009; Dumbrell et al. 2011; Kivlin et al. 2011). Contrary, the EPA-based species networks based on the same data showed that most of the AMF species appeared at all altitudes, plant stages and plant varieties. Also when using the Bray-Curtis index the results obtained for OTU and species datasets were contradictory. Bray-Curtis similarities for AMF communities were in general higher when analyzed by their OTUs than when analyzed as species, but most important is that the analysis of the OTUs resulted in a completely different topology of clustering compared to that of the species. Because the EPA based species affiliation method must be considered as much superior to the 98% or 97% similarity-based OTU affiliation, we conclude that results derived from such OTUs must be interpreted with caution.

The contradictory results among OTUs and species obtained with the networks and the Bray-Curtis index can partly be explained by different OTUs being detected in different samples, but representing the same species. Such OTUs seem to be unique for that sample, yet, this uniqueness may in many cases be based on undersampled highly variable DNA phylotypes.

Surprisingly, even though there were many diverse factors inherent to the samples, the AMF species community revealed a similar structure in the NMDS plot. Plant stages or altitude showed no significant influence in the AMF community confirming

this observation. However, the beta-diversity of the sites was significantly influenced by altitude according to the perMANOVA analysis. The different dissimilarity indexes used should be interpreted in different ways. Altitude influences the species that the sites share but it is not a significant factor driving the general AMF community structure. Horn et al. (2014) demonstrated that the AMF community composition in a small area harboring high plant diversity and different abiotic soil conditions was not affected by environmental effects, concluding that biotic factors such as AMF-plant interactions were more influential. Because the core-species community of potato was conserved over a wide range of environmental conditions, our study also supports that the host plant has strong influence on the AMF community. On the other hand, Lugo et al. (2008, 2012) reported that increasing altitude had a negative impact on AMF richness and diversity, which is in contrast to reports from the Tibetan Plateau, where Gai et al. (2012) showed that AMF diversity did not change with increasing elevation. Different stages in plant age and succession also influence the AMF communities (Husband et al. 2002; Aldrich-Wolfe 2007). For example, Zangaro et al. (2008, 2013) showed that root colonization and spore density decreased among successional stages from grasslands to mature forests. Other studies indicate host preferences (Scheublin et al. 2004; Sýkorová et al. 2007; Torrecillas et al. 2012; Yang et al. 2012). In general, standardized analyses, possibly based on the methods presented here, may allow a more robust interpretation and comparison of such data.

Comparison with a clone library Sanger sequencing approach

From the clone library previously established from the same Peruvian DNA extracts as used here, the most abundant potato root colonizers were an unknown *Claroideoglomus* sp. (Sp. 5), *F. mosseae* (Sp. 12) and *R. irregularis* (Sp. 9). From the 454 relative abundance data for Peru, the most abundant colonizers (from lowest to highest) were *Claroideoglomus* sp. (Sp. 5), *Rhizophagus invermaius* comb. ined. (Sp. 35) (=*Glomus invermaius* (Hall), Potten et al. 2014, submitted for publication; not previously found in the clone library) and the previously found *Acaulospora* sp. (Sp. 14). Two species, *Ambispora* sp. (Sp1) and *Archaeospora* sp. (Sp4) annotated from the clone library were not found by 454 sequencing. Because we could annotate other *Ambispora* and *Archaeospora* species, we hypothesize that these species perhaps were present but their sequences incorrectly affiliated to very closely related species after the automatic alignment of the 454 sequences. Using different approaches and targeting different fungal communities, Kauserud et al. (2012) and

Tedersoo et al. (2010) observed that some of the most abundant OTUs obtained in 454 sequencing were not recovered in the clone library or found in low amounts. In our case, it became clear that there are strong limitations when using a clone-based Sanger sequencing approach but nevertheless, the utility of long sequences, especially for unknown species, should not be overlooked as they provide the phylogenetic context for 454 sequence taxonomic affiliation.

In conclusion, for a better understanding of the dynamics of AMF communities the characterization of their species is necessary. This is especially important to compare among different studies by ecologically meaningful diversity patterns. The improvement of pyrosequencing methods with the goal of obtaining longer reads will eventually permit accurate species annotation. Using ~760 bp reads combined with the EPA approach allowed us to annotate sequences to both unknown and described species with high resolution. Even though the analyzed samples were from sites with highly variable environmental conditions, we could identify the members of a conserved potato AMF core species community, composed of two *Acaulospora* spp., *C. nodosa* and an unknown *Claroideoglomus* sp., usually accompanied by one or more *Rhizophagus* species. The identification and characterization of the yet unnamed AMF species associated with food crops will facilitate a selective design of AMF inoculum and application schemes with the purpose of improving sustainable agricultural practices.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Fig 1 Maximum-likelihood phylogenetic trees showing different AMF taxa delimitation methods. Sequences from *R. irregularis* were used as query sequences and were annotated to species in the genus *Rhizophagus* by using EPA (a), clustering at 97% similarity threshold (97%-OTUs) (b), and a monophyletic clade approach (b and c). 97%-OTUs are marked in blue and sequences used as query sequences in red. Reference sequences are in black. Using EPA species affiliation, all query sequences were correctly placed in the *R. irregularis* clade (a). Problems when annotating 454 reads by using 97%-OTUs are shown: two OTUs in a single monophyletic clade (OTU6 and 12), spread OTUs partly clustering with other OTUs (OTU10) (b). Problems when annotating species using ~760 bp sequences and a monophyletic clade approach are shown: low phylogenetic resolution not allowing species definition (c).

Fig 2 Frequency of occurrence (FO) of 41 annotated AMF species and the number of individual root samples (total root samples analyzed = 102) in which they were found. A conserved species pattern can be observed in the three countries. The same conserved patterns can be observed when analyzing different altitudes or plant stages shown in Online Resource 7.

Fig 3 454-read abundance of the 12 AMF genera found at 12 field sites (B = Bolivia, E = Ecuador, P = Peru; 1 = site 1, 2 = site 2, 3 = site 3, 4 = site 4; Em = emergence, Fl = flowering, Sc = senescence; A1 = altitude group 1 (≤ 3,000 m), A2 = altitude group 2 (3,001 – 3,560 m), A3 = altitude group 3 (3,561 – 4,000 m), A4 = altitude group 4 (≥ 4,001 m); W = Waycha, S = Superchola, G = Guata, F = Fripapa, Y = Yungay, U = Unica. Inset shows the relative abundance in percentage for each genus.

Fig 4 Networks showing the distribution of AMF OTUs and species, based on the identical representative sequences (RS) of 98% similarity clusters. Nodes correspond to either OTUs or species and connecting edges indicate different conditions. a) OTU and b) species networks at different altitude ranges; 1 (green) = altitude 1 (≤ 3,000 m), 2 (blue) = altitude 2 (3,001 – 3,560 m), 3 (purple) = altitude 3 (3,561 – 4,000 m), 4 (orange) = altitude 4 (≥ 4,001 m). c) OTU and d) species networks of AMF colonizing different plant stages; Em = emergence (green), Fl = flowering (orange), Sc = senescence (purple). e) OTU and f) species networks of AMF colonizing different potato varieties; W = Waycha (orange), U = Unica (violet), F= Fripapa (green), G =

Guata (blue), S = Superchola (purple), Y = Yungay (red). In the species networks, nodes show the species number and nodes highlighted in yellow represent species shared by all the different variables.

Fig 5 Bray-Curtis similarity of the sampled sites when using OTUs (a) or species (b) to delimit the AMF community. Example sites that are identical when analyzing OTUs and different when analyzing species are highlighted in red. B = Bolivia, E = Ecuador, P = Peru; 1 = site 1, 2 = site 2, 3 = site 3, 4 = site 4; Em = emergence, Fl = flowering, Sc = senescence.

Fig 6 Number of AMF species, in percentage, detected in individual root system samples. Zero to more than 25 species were separated in seven size groups (0, 1 to 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, > 25 species). The percentage of AMF species found (in a single root system) within these groups is shown.

Fig 7 Nonmetric multidimensional scaling (NMDS) ordination plot for the 41 AMF annotated species, color-coded by their genus. Vectors show the tested variables, altitude and plant stage.

Online Resources

Online Resource 1: Description of edapho-climatic conditions of the study sites.

Online Resource 2: Description of the altitude groups and the sites that belong to each group.

Online Resource 3: Maximum-likelihood phylogenetic tree showing the EPA approach by using reference *Rhizophagus* sequences. Individual *R. irregularis* query sequences (approx. 760 bp) were placed in the tree branches by using EPA (shown by arrows). Query sequences that were placed in terminal nodes are marked with arrows starting at the name of the reference sequence. Reference numbers of the *R. irregularis* query sequences are shown in a box. *Paraglomus* sequences were used as outgroup.

Online Resource 4: Maximum-likelihood phylogenetic tree showing results of the 97%-OTUs and the monophyletic clade approach. *Rhizophagus* together with the query sequences were used to compute the tree. A sequence similarity threshold of 97% was used to cluster the query sequences into 18 OTUs, marked in blue. Query sequences and their reference number are marked in red. Sequence variants belonging to isolates of *R. irregularis* DAOM 197198 or a single spore of *R.*

intraradices FL208 are marked by colored squares. *Paraglomus* sequences were used as outgroup.

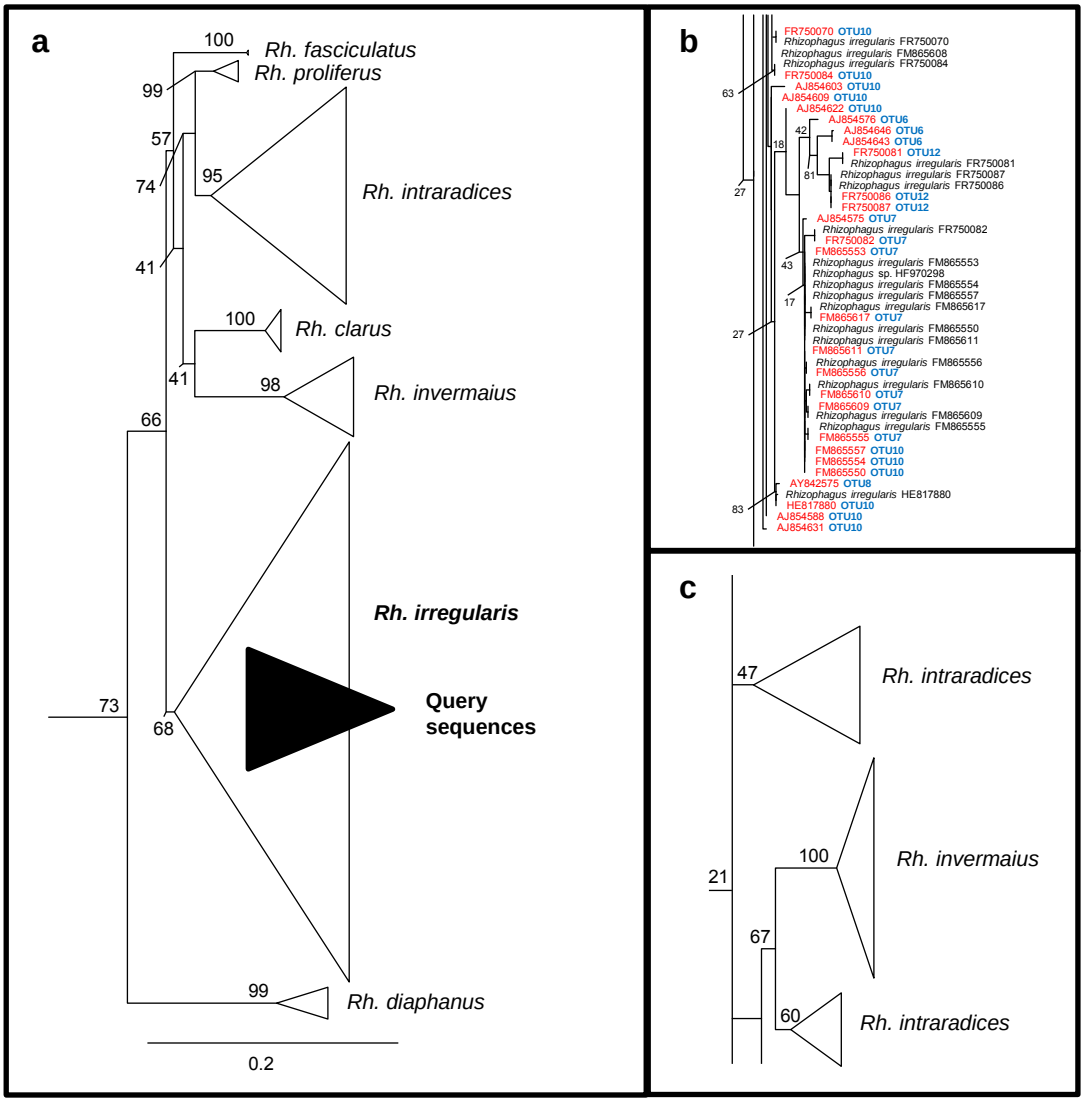
Online Resource 5: Rarefaction curves of the amount of AMF OTUs (a) and species (b).

Online Resource 6: Read abundance of species, genus and OTUs.

Online Resource 7: Number of individual root samples in which 41 annotated AMF species were found at different altitudes categories and plant stages.

Online Resource 8: Influence of altitude and plant stage on the beta-diversity of the AMF communities annotated as species. *P*-values are based on 999 permutations.

Fig 1 Maximum-likelihood phylogenetic trees showing different AMF taxa delimitation methods. Sequences from *R. irregularis* were used as query sequences and were annotated to species in the genus *Rhizophagus* by using EPA (a), clustering at 97% similarity threshold (97%-OTUs) (b), and a monophyletic clade approach (b and c). 97%-OTUs are marked in blue and sequences used as query sequences in red. Reference sequences are in black. Using EPA species affiliation, all query sequences were correctly placed in the *R. irregularis* clade (a). Problems when annotating 454 reads by using 97%-OTUs are shown: two OTUs in a single monophyletic clade (OTU6 and 12), spread OTUs partly clustering with other OTUs (OTU10) (b). Problems when annotating species using ~760 bp sequences and a monophyletic clade approach are shown: low phylogenetic resolution not allowing species definition (c).


a
100
Rh. fasciculatus
Rh. proliferus
99
57
74
95
Rh. intraradices
41
100
Rh. clarus
41
98
Rh. invermaius
66
Rh. irregularis
73
68
Query sequences
99
Rh. diaphanus
0.2
b
FR750070 OTU10
Rhizophagus irregularis FR750070
Rhizophagus irregularis FM865608
Rhizophagus irregularis FR750084
FR750084 OTU10
63
AJ854603 OTU10
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AJ854622 OTU10
AJ854576 OTU6
42
AJ854646 OTU6
AJ854643 OTU6
18
FR750081 OTU12
Rhizophagus irregularis FR750081
81
Rhizophagus irregularis FR750087
27
Rhizophagus irregularis FR750086
FR750086 OTU12
FR750087 OTU12
AJ854575 OTU7
Rhizophagus irregularis FR750082
FR750082 OTU7
FM865553 OTU7
43
Rhizophagus irregularis FM865553
Rhizophagus sp. HF970298
Rhizophagus irregularis FM865554
17
Rhizophagus irregularis FM865557
Rhizophagus irregularis FM865617
FM865617 OTU7
27
Rhizophagus irregularis FM865550
Rhizophagus irregularis FM865611
FM865611 OTU7
Rhizophagus irregularis FM865556
FM865556 OTU7
Rhizophagus irregularis FM865610
FM865610 OTU7
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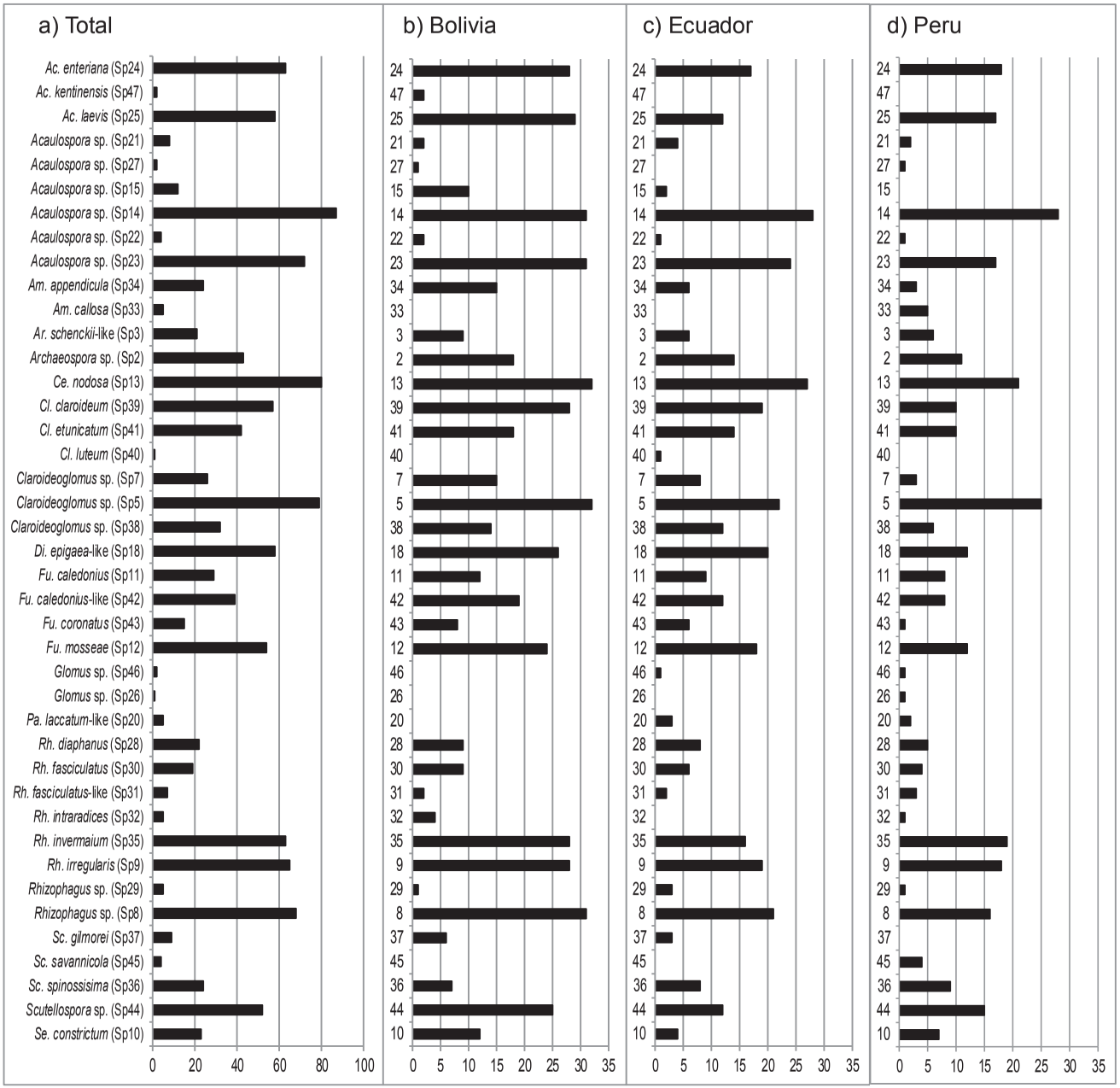
Rhizophagus irregularis FM865609
Rhizophagus irregularis FM865555
FM865555 OTU7
FM865557 OTU10
FM865554 OTU10
FM865550 OTU10
AY842575 OTU8
Rhizophagus irregularis HE817880
83
HE817880 OTU10
AJ854588 OTU10
AJ854631 OTU10
c
47
Rh. intraradices
100
Rh. invermaius
21
67
60
Rh. intraradices

Fig 2 Frequency of occurrence (FO) of 41 annotated AMF species and the number of individual root samples (total root samples analyzed = 102) in which they were found. A conserved species pattern can be observed in the three countries. The same conserved patterns can be observed when analyzing different altitudes or plant stages shown in Online Resource 7.


a) Total
b) Bolivia
c) Ecuador
d) Peru
Ac. enteriana (Sp24)
Ac. kentinensis (Sp47)
Ac. laevis (Sp25)
Acaulospora sp. (Sp21)
Acaulospora sp. (Sp27)
Acaulospora sp. (Sp15)
Acaulospora sp. (Sp14)
Acaulospora sp. (Sp22)
Acaulospora sp. (Sp23)
Am. appendicula (Sp34)
Am. callosa (Sp33)
Ar. schenckii-like (Sp3)
Archaeospora sp. (Sp2)
Ce. nodosa (Sp13)
Cl. claroideum (Sp39)
Cl. etunicatum (Sp41)
Cl. luteum (Sp40)
Claroideoglomus sp. (Sp7)
Claroideoglomus sp. (Sp5)
Claroideoglomus sp. (Sp38)
Di. epigaea-like (Sp18)
Fu. caledonius (Sp11)
Fu. caledonius-like (Sp42)
Fu. coronatus (Sp43)
Fu. mosseae (Sp12)
Glomus sp. (Sp46)
Glomus sp. (Sp26)
Pa. laccatum-like (Sp20)
Rh. diaphanus (Sp28)
Rh. fasciculatus (Sp30)
Rh. fasciculatus-like (Sp31)
Rh. intraradices (Sp32)
Rh. invermaium (Sp35)
Rh. irregularis (Sp9)
Rhizophagus sp. (Sp29)
Rhizophagus sp. (Sp8)
Sc. gilmorei (Sp37)
Sc. savannicola (Sp45)
Sc. spinossisima (Sp36)
Scutellospora sp. (Sp44)
Se. constrictum (Sp10)
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0 5 10 15 20 25 30 35
0 5 10 15 20 25 30 35
No. of plant replicates

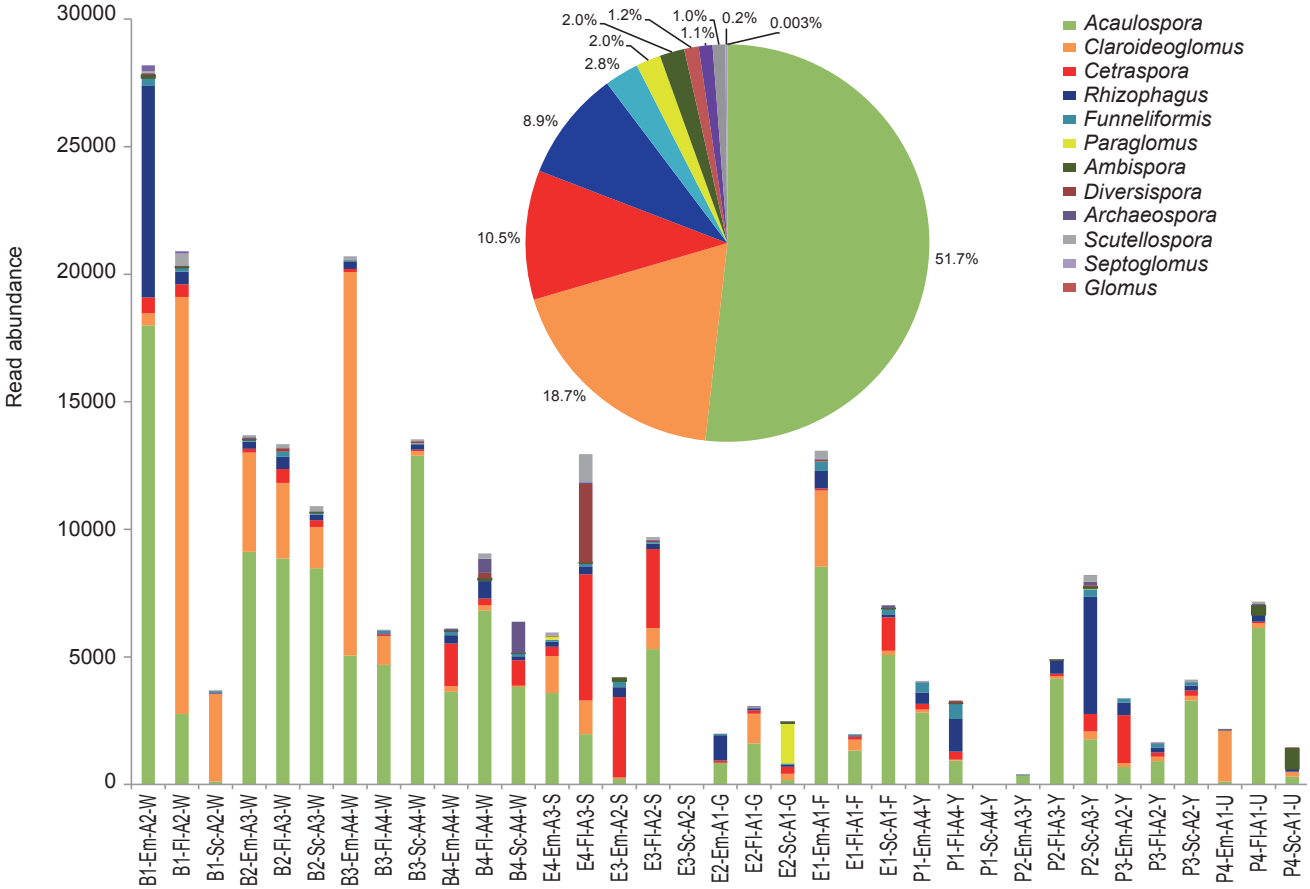

Fig 3 454-read abundance of the 12 AMF genera found at 12 field sites (B = Bolivia, E = Ecuador, P = Peru; 1 = site 1, 2 = site 2, 3 = site 3, 4 = site 4; Em = emergence, Fl = flowering, Sc = senescence; A1 = altitude group 1 (≤ 3,000 m), A2 = altitude group 2 (3,001 – 3,560 m), A3 = altitude group 3 (3,561 – 4,000 m), A4 = altitude group 4 (≥ 4,001 m); W = Waycha, S = Superchola, G = Guata, F = Fripapa, Y = Yungay, U = Unica. Inset shows the relative abundance in percentage for each genus.

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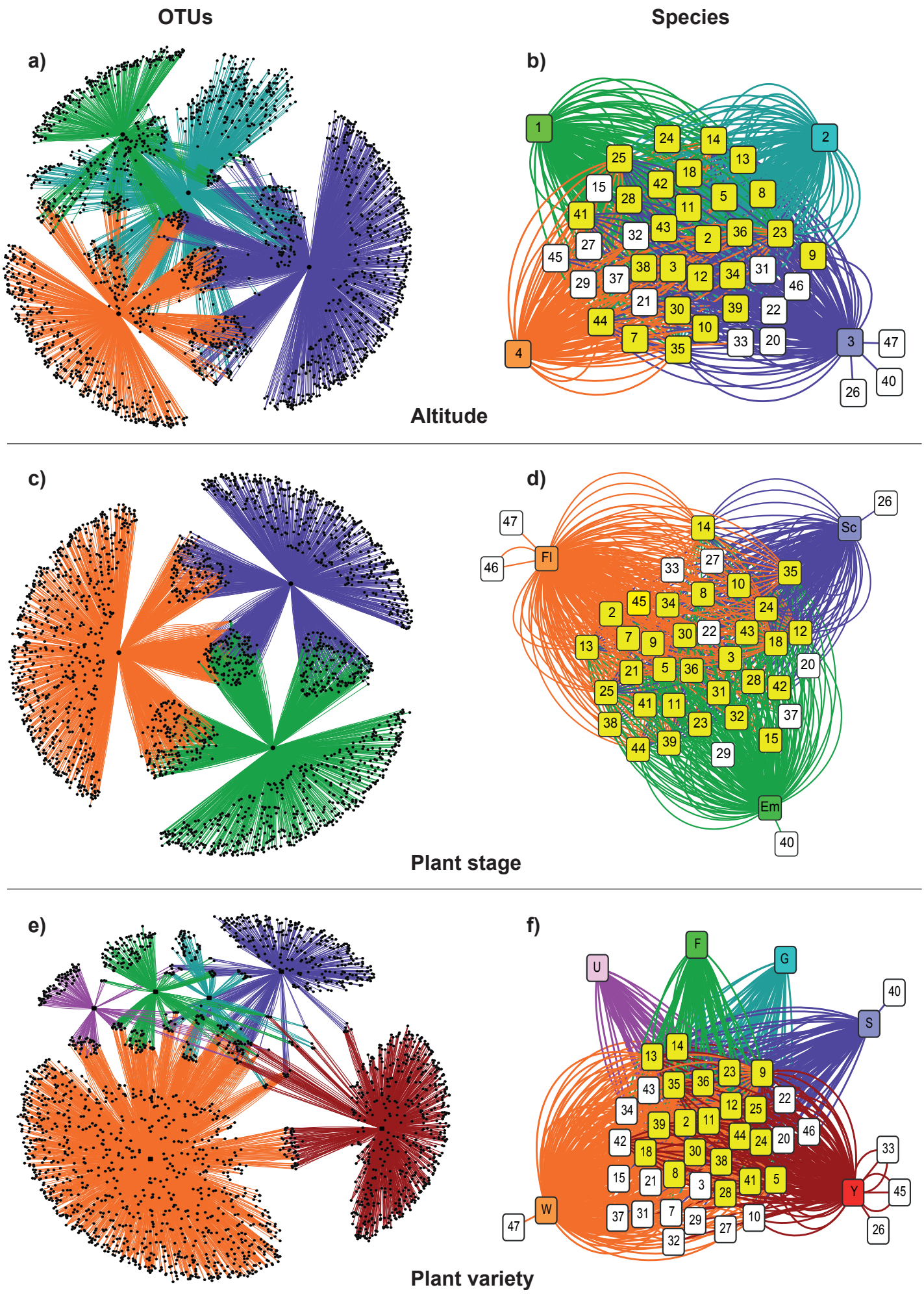
OTUs
Species
a)
b)
1
2
3
4
Altitude
c)
d)
Fl
Sc
Em
Plant stage
e)
f)
F
G
S
U
W
Y
Plant variety

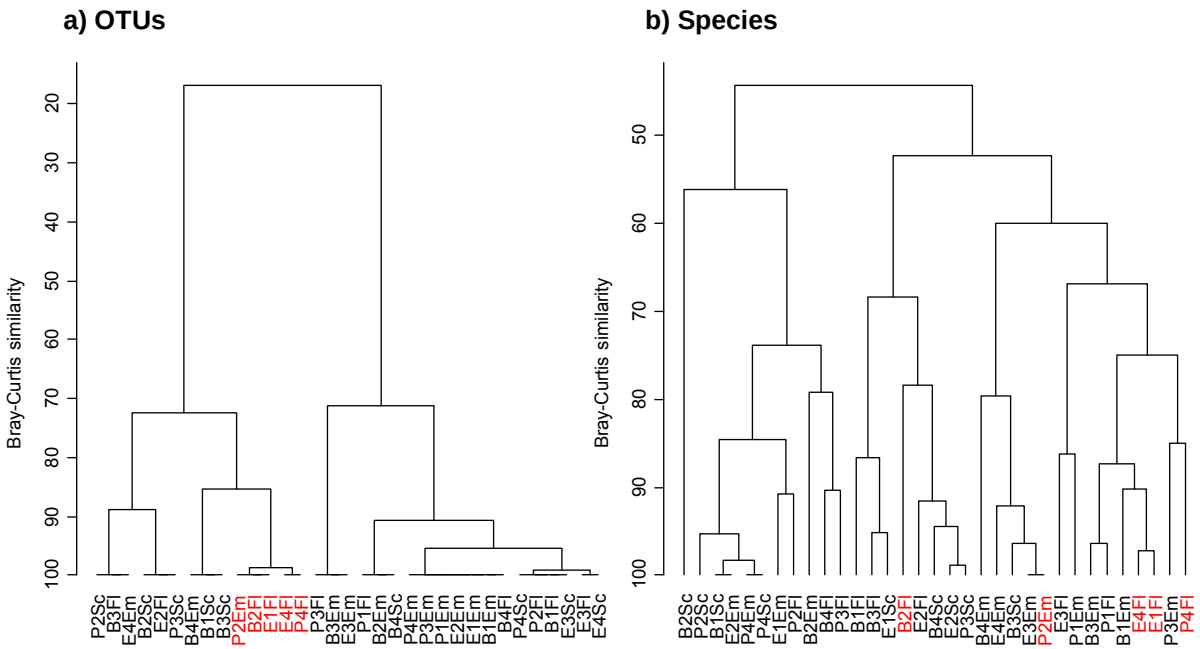

Fig 5 Bray-Curtis similarity of the sampled sites when using OTUs (a) or species (b) to delimit the AMF community. Example sites that are identical when analyzing OTUs and different when analyzing species are highlighted in red. B = Bolivia, E = Ecuador, P = Peru; 1 = site 1, 2 = site 2, 3 = site 3, 4 = site 4; Em = emergence, Fl = flowering, Sc = senescence.

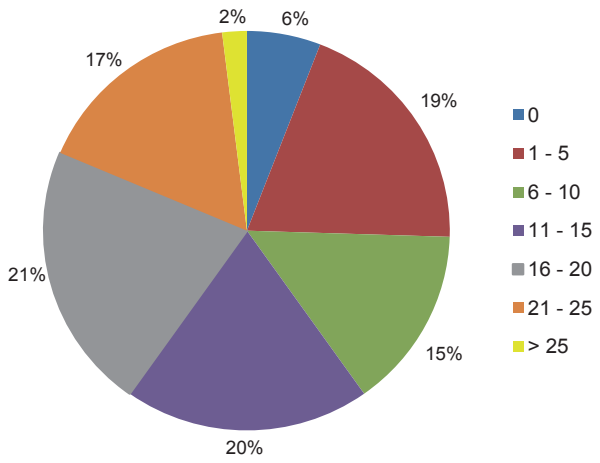

Fig 6 Number of AMF species, in percentage, detected in individual root system samples. Zero to more than 25 species were separated in seven size groups (0, 1 to 5, 6 to 10, 11 to 15, 16 to 20, 21 to 25, > 25 species). The percentage of AMF species found (in a single root system) within

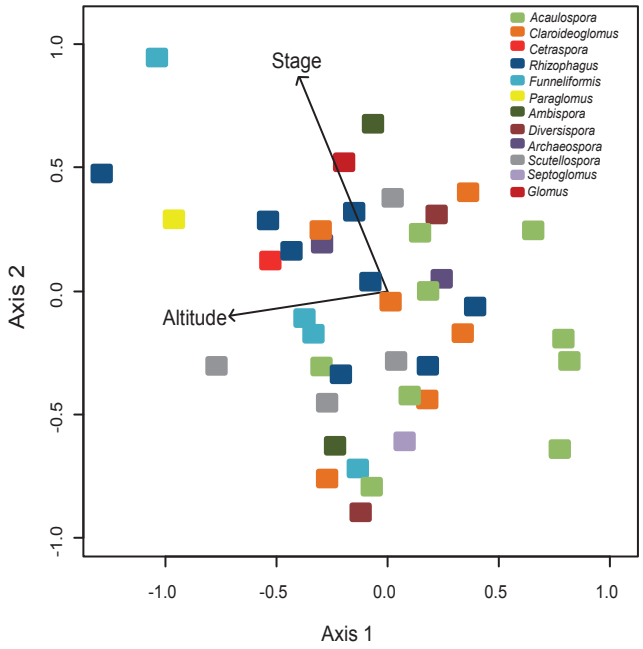

Fig 7 Nonmetric multidimensional scaling (NMDS) ordination plot for the 41 AMF annotated species, color-coded by their genus. Vectors show the tested variables, altitude and plant stage.

7. Discussion

7.1 General discussion

The use of new sequencing technologies has made an enormous amount of environmental DNA sequences available, providing new insights but also challenges in the field of molecular ecology. For AMF, one such challenge is the affiliation of sequences into taxonomic units that provide meaningful information about preferences in the AMF-plant interactions and the driving forces of this symbiosis. However, currently there is no consensus among mycorrhizal ecologist regarding the affiliation of DNA sequences to AMF species, even though this would improve our understanding of the AMF diversity and the design of more effective schemes for AMF inocula application.

Thus, the aim of my doctoral thesis was to characterize the members of AMF communities associated with potato roots from the Andean region at the species level by using 454 pyrosequencing, allowing for the first time the interpretation of potentially existing preferential AMF associations of an important food crop, potato.

7.2 Fungal sequence databases

The improvement in sequencing technologies has contributed greatly to facilitate fungal identification. With the purpose of documenting research findings and sharing them with the scientific community, several sequence databases have been created. These databases such as GenBank and its partners in the International Nucleotide Sequence Databases Collaboration (INSDC) are publicly available and are an open source of nucleotide information. However, they rely on the user taxonomic annotations, which have resulted in many wrongly named sequences (Nilsson et al., 2008). The lack of reliable reference sequences and curated databases prompted efforts from mycologists to establish specialized fungal databases that offered taxonomical accuracy (Kõljalg et al., 2013 – Chapter 3). Recently, a large scale effort to re-assess fungal ITS sequences was made by the National Center for Biotechnology Information (NCBI) Taxonomy database, where a separate curated database, the RefSeq Targeted Loci (RTL), was expanded to include Fungi (Schoch et al., 2014). This database currently holds 104 ITS reference sequences from *Glomeromycota*, being the genus *Acaulospora* the one with more

ITS curated records compared to the other AMF within this database. From the number of sequences in this database and considering that there are approx. 250 AMF species described, it is clear that more efforts are necessary to produce good-quality and well-annotated reference AMF sequences. Moreover, this has to be extended to other loci, because the ITS alone does not provide species resolution for closely related AMF (Stockinger et al., 2009).

7.3 AMF species delimitation

The difficulty to delimit AMF species arises from the lack of a biological species concept. Spore morphology has therefore been used to define species, providing information that could be misleading due to the variability of spore characteristics and convergent similar characters among divergent AMF taxa (Walker et al., 2007). Moreover, different spore morphs occur at variable growth conditions, making it difficult to characterize species based only on microscopic observations (Potten et al., 2014 – Chapter 4). The problem further increases when original type material is unavailable, lost or in a bad condition, which makes it impossible to link species-defining biological material to its corresponding sequences. As an example, for *Rhizophagus invermaius* (syn. *Glomus invermaium* – Chapter 4) the original type specimen was collected from field material and no culture was established (Hall 1977) eliminating the possibility of comparing its DNA to the one from other samples found in environmental ecological studies.

Inaccurate taxonomical classifications of AMF species based on spore morphology were later in conflict with molecular evidence, such is the case of the model fungus DAOM197198 which was used as *Glomus intraradices* but afterwards re-classified, based on molecular evidence, as *R. irregularis* (syn. *Glomus irregularis*; Stockinger et al., 2009).

In addition to problematic classification, many AMF species are not yet described and it is expected that unexplored ecosystems harbor a high diversity (Kivlin et al., 2011; Liu et al., 2011). Both of these issues have interrelated consequences for the analysis of high throughput environmental DNA sequences because firstly, environmental sequences often can´t be matched to described taxa and secondly, the lack of reference sequences could lead to false sequence-based annotation, because many unknown sequences could affiliate (probably with low phylogenetic signal) to sequences that are not an accurate match.

Since the Andean region remains an unexplored ecosystem regarding AMF, for my thesis I started analyzing samples using a Sanger sequencing and clone library based approach (Senés-Guerrero et al., 2014 – Chapter 5) to characterize long sequences for the unknown species in the samples. The extended DNA barcode for AMF is a 1.5 kb fragment covering partly the SSU region, the full ITS and a part of the LSU rDNA region (Schoch et al., 2012; Stockinger et al., 2010). Because of its length it cannot be directly sequenced by next generation sequencing (NGS) techniques. Half of the sequences obtained represented new species or species previously not characterized by DNA sequence data, stressing the importance of setting up a reference sequence dataset that is needed to robustly annotate high throughput sequencing data. Nonetheless, the used method has strong limitations regarding the amount of sequences that can be obtained. For example, in potato roots a maximum of 2 AMF species were detected in a single root system compared to more than 25 that were detected by using 454 pyrosequencing (Senés-Guerrero & Schüßler, 2014 – Chapter 6), from identical DNA-extraction samples.

At present, many molecular ecological studies regarding AMF are done by using 454 pyrosequencing. This method can provide up to ~ 1 million reads per run and 1 kb read length when using the recently improved GS-FLX+ chemistry. The previous chemistry, GS-FLX, has successfully been used to describe AMF community patterns in several ecosystems (Öpik et al., 2009, Dumbrell et al., 2011, Davison et al., 2012; Lekberg et al., 2012) but because of its sequencing read length average of ~400 bp, it is not suited to delimit closely related AMF species.

Most AMF community studies analyze 454 sequencing data by clustering reads into operational taxonomic units (OTUs) at a certain similarity threshold (usually 97%) and match them to reference sequences by using BLAST. Few studies analyze 454 sequencing reads by constructing phylogenetic trees and delimiting AMF taxa by analyzing monophyletic clades (Sýkorová et al., 2007; Horn et al., 2014). Recently both approaches were compared by Lekberg et al. (2014) who showed that delineating OTUs by using either a similarity threshold of 97% or by using a monophyletic clade approach does not affect interpretations of the AMF community patterns. However, this interpretation does not take into account that the chosen monophyletic clade approach did not circumvent the problem of having many OTUs (phylotypes) per species. These OTUs (phylotypes) are the result of high intraspecific variability which often results in artificially formed clades that are interpreted as

different genus and/or species, providing misleading information about community patterns.

In the frame of my thesis, we used a method to taxonomically annotate high throughput sequence data that involved the individual affiliation of each representative sequence into a phylogenetic tree. The tree itself is based on sequences that were either from characterized AMF species and/or environmental species characterized for the Andean region (Chapter 5). The results showed that fundamentally different conclusions can be obtained when analyzing the AMF community composition by either using a similarity threshold based analysis or by using phylogenetic annotation, which implies that the latter approach provides a higher and more robust phylogenetic resolution, needed to annotate OTUs to species.

The diversity found in samples coming from Peru, Bolivia and Ecuador was high, 41 AMF species from divergent lineages were annotated (Chapter 6). However, comparisons with other ecological studies are difficult to make, mostly because of the different methods used to define and annotate species or OTUs. Therefore, reaching a consensus on how to delimitate AMF species when using 454 sequencing reads is necessary, as it would improve our understanding of the mechanisms of assembly of AMF communities, the biotic and abiotic factors that influence them and of AMF-plant preferences.

7.4 AMF associated with potato plants

Potato agriculture is becoming more important worldwide. Potatoes are among the 20 most important food and agricultural commodities around the world (FAO 2012). Thus, increasing potato productivity as well as the efficient use of resources for its production are essential. Potato has a sparse and shallow root system, therefore is very sensitive to drought stress (Jefferies 1993) and the efficiency of acquiring a large amount of nutrients from the surrounding soil is limited. Moreover, potato faces several biotic threats like pests and diseases having a high negative impact on potato yield (Birch et al., 2012 and citations therein). Pest and pathogen control are usually done by using chemicals and a high amount of fertilizer is normally used in potato fields (FAO 2008). Hence, a sustainable solution for both potato production and pest/pathogen control is actively searched.

AMF have shown positive results when inoculated to potato plants. Yield and tuber size distribution are the most important characteristics that determine success of potato production and both of these were increased by using a commercial AMF inoculum (Duffy & Cassells, 2000), however in the same study, it was also reported that the use of a single AMF species (*R. irregularis*, as "*Glomus intraradices*") had unfavorable effects compared to the control. Therefore, preferential associations among AMF isolates and potato cultivars that can lead to either positive or negative outcomes were shown. Indeed, preferential associations have been reported for potato plants in Italian fields, where *R. irregularis* (as *G. intraradices*) was reported as the main colonizer (Cesaro et al., 2008).

The AMF species *G. intraradices* did not have a clear species definition until it was shown in Stockinger et al. (2009) that two main cultures used and identified as DAOM197198 and BEG195 and believed to be *G. intraradices* were in fact *G. irregularis*, which was placed in the genus *Rhizophagus* as *R. irregularis* (Schüßler & Walker, 2010; Redecker et al., 2013). We demonstrated that this was also the case for the fungus named *G. intraradices* and described as dominating potato colonizer in the study of Cesaro et al. (2008). By analyzing their published DNA sequences and affiliating them to our phylogenetic reference tree (Chapter 5) we showed that their sequences represent *R. irregularis* and not *G. intraradices* as it was reported. For the study of Duffy & Cassells (2000) there are no available sequences because they only analyzed AMF by spore morphology. Since this study reported a negative effect of "*G. intraradices*" on potato (cv. Golden Wonder), it would have been interesting to determine whether their "*G. intraradices*" belonged indeed to that species. As virtually all fungi named *G. intraradices* in publications before the year 2010 in fact were *R. irregularis*, it is likely that also the Duffy & Cassells fungus was from this species. The results presented in Cesaro et al. (2008) and Duffy & Cassells (2000) highlight the importance of accurately identifying AMF species to determine which AMF are preferentially associated to potato roots.

Based on relative abundance relating RFLP patterns and Sanger sequencing data (Chapter 5) together with read relative abundance and frequency of occurrence obtained with 454 sequencing (Chapter 6), AMF species that are putatively main players in the Andean ecosystem and in potato plants were identified.

Although a conserved group of AMF species was identified (67% of the plants from 12 studied sites were colonized by *Cetraspora nodosa*, *Acaulospora* spp.,

Rhizophagus spp. and *Claroideoglomus* spp. simultaneously), it became obvious that several unknown *Acaulospora* spp. were the most dominant species colonizing potato roots, making them candidates for AMF inoculum application. It is not clear whether these species are habitat specialists. They were not reported from other ecosystems, but this could just be an effect of insufficient available data. However, at the moment only one of the six *Acaulospora* spp. identified by sequencing methods has been isolated from the field and established in culture. For the other unknown *Acaulospora* spp. isolation has not been possible, preventing their immediate use to establish cultures or to perform experiments.

Surprisingly, even though the samples used for my thesis came from different Andean countries (Peru, Bolivia and Ecuador), climatic conditions and various plant developmental stages, a conserved AMF species community was observed. Multivariate statistics showed that altitude, unexpectedly, was not influencing the AMF community composition. At first by using principal coordinates analysis (PCoA; Chapter 5) and later by using permutational manova (PerManova; Chapter 6), contradicting previously reported results showing that increasing altitude was negatively correlated to AMF diversity (Chaurasia et al., 2005; Lugo et al., 2008, 2012). When a Sanger sequencing based study was conducted on Peruvian root samples (Chapter 5), plant developmental stages were shown to have an influence on the AMF community composition, which was not the case when analyzing 454 sequencing data (Chapter 6). This may be because of the low amount of AMF species that could be obtained with Sanger sequencing, emphasizing the importance of using deep sequencing technologies for molecular ecological studies.

7.5 Co-occurrence of AMF species in individual root systems

An individual root system is usually colonized by several AMF species, however their identification has always been complicated. There have been some studies analyzing the co-existence of AMF lineages which showed that phylogenetically distinct AMF have different functions that complement each other, decreasing competition among them (Maherali & Klironomos, 2007). In this context, some functional traits have been identified. Members of the *Gigasporaceae* were discussed as limited root colonizers with high levels of hyphal growth within the soil, members of *Rhizophagus* and *Claroideoglomus* as extensively colonizing roots, and members of the *Acaulosporaceae* as poor colonizers from both roots and soil (Hart &

Reader, 2002; Maherali & Klironomos, 2007; Powell et al., 2009). However, analyzing the co-occurrence of AMF at the species level under natural circumstances has not been previously done.

In my thesis, we observed that *Cetraspora nodosa*, an *Acaulospora* sp. and *Claroideoglomus* sp. were coexisting in most samples (74%, 75 plants) and that 68 plants (67%) additionally contained one of eight detected *Rhizophagus* species (Chapter 6). Contrary to what was previously reported, these data show that *Acaulospora* spp. are able to abundantly colonize potato roots, whereas it is uncertain if this is due to host preference or a biogeographical specificity for the Andean ecosystem.

The species found in individual root systems belong to divergent phylogenetic lineages, therefore their co-occurrence suggests that they have functional complementarity which can be beneficial for the host. The identification of species that are simultaneously colonizing individual root systems regardless of plant developmental stage or environmental conditions has important consequences on our understanding of how an AMF community is assembled and it could be useful in the future when planning the application of AMF as inoculum in the field. In such case, complementary species which are also preferential colonizers could be used in a mixed-inoculum approach instead of single-species inoculum using generalist AMF colonizers.

7.6 AMF, bacteria and potato plants from the Andes

Synergistic interactions of AMF with bacteria that result in positive effects for plant growth have been reported. However, the underlying mechanisms of such interactions are still largely unknown. The possible mechanisms involve: i) a direct effect of the bacteria over the AMF, exerting thus an indirect effect over the plant; ii) a direct influence of the bacteria over the physiology of the plant or iii) an indirect synergism that improves nutrient acquisition for both partners (Artursson et al., 2006 and citations therein). AMF also have an indirect effect on bacteria communities by changing the composition of root exudates and by exudating carbohydrates and glycoproteins. There is no doubt that understanding how bacteria, AMF and plants interact has important consequences for the future use of microbial inocula application in sustainable agricultural practices. Therefore, within the frame of the VALORAM project, other research Institutions explored the topic of potato-associated

bacteria in the Andean region (Ghyselinck et al., 2013). Some bacterial isolates obtained from the Andes (*Pseudomonas koreensis*, *Pseudomonas corrugata* and *Enterobacter*) showed growth-promotion effects on potato plantlets under controlled laboratory conditions. These isolates were obtained from the same sampled material that was used to analyze AMF communities and because positive interactions between bacteria and AMF have been described, we speculate that this could be also the case for potato plants. However, so far we have not been able to find correlations among the presence/absence of groups of bacteria with AMF (S. Pfeiffer, personal communication), even though this was our expected scenario. In future experiments, it would be interesting to mix dominant AMF potato colonizers together with bacteria strains with a demonstrated growth-promotion effect and assess their combined outcome on plant performance, as well as tracing of the inoculants.

7.7 AMF used as inoculum in the field

Due to the negative consequences that intensive agriculture has imposed on the environment (Tilman et al., 2002), improving sustainable practices in agriculture is a current concern. It has been shown that AMF can decrease fertilizer use and improve or maintain plant yield and biomass. Therefore, AMF have been used as inocula in different field experiments (Pellegrino et al., 2011; 2012, Ceballos 2013). Usually AMF inoculation consists on the introduction of a “generalist” species such as *R. irregularis* or *F. mosseae* in the field. However, these species are not always successful at colonizing plant roots or are not the best candidates to obtain a high yielding AMF-plant combination.

From analyzing more than 200 samples of potato plants, it became clear that unknown *Acaulospora* spp. were the dominant colonizers in the Andes. However, because these species are not available as cultures, an experiment was carried out using *R. irregularis* as inoculum (Lojan et al., unpublished). The results showed that neither in plant yield nor in plant performance the inocula were successful. Moreover, 454 sequencing revealed that, again, the dominant colonizers were *Acaulospora* spp. and that even though *R. irregularis* was inoculated, it was not abundantly found. The experiment only lasted 6 months and important questions remain: does *R. irregularis* need more time to successfully colonize root samples, and which were the factors influencing the colonization or the lack of it? It was reported that it took 18 months for an haplotype of *R. irregularis* to be frequently found in field samples (Sýkorová et al.,

2012), yet the inocula concentrations used in our experiment are high and recommended for field inoculation, which should be sufficient to obtain rapid colonization. It can also be hypothesized that *R. irregularis* is not a preferential colonizer of potato plants, because it has not been abundantly found in the potato root samples previously analyzed by 454 sequencing. However, it cannot be ruled out that this species is not compatible with the native AMF community or that environmental factors were restraining its propagation.

It is clear that more experiments would be needed to assess whether is better using native AMF or rapid generalist colonizers. Nevertheless, even though the use of preferential colonizers could be advised, the lack of or the difficulty to obtain pure cultures for many AMF species found in molecular studies makes this impossible.

For the future, joint efforts of culturing and describing new AMF species, along with the use of deep sequencing techniques would allow a more effective use of AMF inocula with the goal of improving sustainable agricultural practices.

8. Outlook

Many of the molecular ecological studies on AMF nowadays use high throughput sequencing and a vast amount of information is being analyzed and published. However, there is no consensus among mycorrhizal ecologists on how to best delimit sequences to AMF species. Even though there is many data regarding AMF in natural ecosystems, it is not possible to compare among studies because of the variable means used to define and annotate sequences. It is clear that a standardized manner of analyzing sequences is needed. However, approaches like delimiting OTUs with unknown and within-dataset variable resolution between species and genus (sometimes maybe even above-genus) is of little help to such standardization, in particular if such OTUs are interpreted as species, which is unfortunately often done. Molecular identification of AMF did not yet use a common language to describe organisms in a comparable manner. The method that we used, in which individual 454 sequencing reads are placed into a reference phylogenetic tree, is able to place sequences at the species level at a better phylogenetic resolution than what has been previously published.

To better understand the underlying mechanisms of the AMF-plant interactions, several aspects can and should be improved, e.g., DNA barcoding of AMF species which are available in culture, increasing reference datasets which can be deposited in curated web sites (such as the workbench Pluto F) and characterizing high throughput sequences by using a standardized approach.

Analyzing 454 sequencing data to the strain level would provide very important information when performing field experiments. From an applied point of view, knowing which strains improve plant performance and persist in the field would make inocula definition and application more efficient and consequently, improve sustainable agricultural practices. However, there is no applicable system available yet that could be used to analyze AMF strains in the field. Maybe mitochondrial genome data will change this in future, but there is still a long way to go and first of all comprehensive datasets for all major AMF lineages need to be established, before any specific and defined system could be elaborated. Using the molecular tools presented here we were able to identify AMF by high throughput sequencing data to the species level. Similar systems could be used in the future to identify AMF strains, but specific PCR primers or sequence reference datasets are not yet existing.

At the moment it is often unknown whether individual AMF species or a mixed inoculum provide better results in field application. Nevertheless, the situation found in the field indicates strongly that there is functional complementarity of the AMF communities associated with potato roots, which indicates that at least for certain conditions an inoculation with a suited, crop-specific mixture of AMF will be more efficient than using a single fungus. By identifying preferential AMF-plant associations and the conditions under which they function, crop-specific inoculum mixtures could be designed for application with specific plants or environmental conditions. The results of the studies presented here indicate that the development of broadly applicable inocula based on three to four main player AMF species could be straightforward to improve application, at least for potato where a conserved set of highly abundant “core AMF” was found for diverse environmental conditions. Using similar approaches for other crops could identify the AMF most promising for use in future crop-specific sustainable agricultural management practices.

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10. Appendix

10.1 Supplementary data – Chapter 3

The following data are supplementary material for the publication **Towards a unified paradigm for sequence-based identification of fungi.**

Fig S1: Generation of the global key: technical description

Two distinct sequence datasets were used to generate global key clusters:

1. sequences from the UNITE database (UNITE dataset: 12 667 sequences);
2. fungal rDNA ITS sequences retrieved from GenBank (INSD dataset: 323 513 sequences) using the following search string

```
((("Fungi"[ORGN] AND (140[SLEN] : 3000[SLEN])) AND
(((ITS1[titl] OR ITS2[titl]) OR 5.8S[titl]) OR "internal
transcribed spacer"[titl] OR "internal transcribed
spacers"[titl] OR "ITS 1" [titl] OR "ITS 2"[titl])) NOT
"Uncultured Neocallimastigales"[ORGN]
```

Step 1: quality filtering

Initial quality filtering (sequences flagged as "low quality" or "chimeric" on the PlutoF workbench (Abarenkov et al., 2010b)) discarded 64 and 9 365 sequences from the UNITE (Abarenkov et al., 2010a) and INSD (Benson et al., 2006) datasets respectively.

Step 2: fungal ITS extractor

For the remaining 326 751 sequences ITS1 and ITS2 were separated using the fungal ITS extractor (Nilsson et al. 2010). Sequences without ITS2 region (61 475), sequences containing more than 3 ambiguous (N) nucleotides in the ITS2 region (2 591), and sequences with questionable suitability for the global key by manual inspection (62) were excluded from further analysis.

Step 3: USEARCH clustering (clustering step 1)

ITS2 regions for the 262 623 sequences surviving the cleaning step were submitted to USEARCH v6.0.307 (Edgar, 2010) analysis for clustering on 80% similarity threshold with the following command

```
usearch –clusterfast infile.fasta –id 0.80 –centroids
centroids_out.fasta –uc clusters_out.uc
```

Clustering produced 7 470 clusters and 4 902 singletons, 1 046 sequences having length < 32 nucleotides were discarded by the program.

Step 4: aligning clusters

All clusters were aligned using the multiple sequence alignment program MAFFT v6.833b (Katoh et al., 2002) with the following parameters

number of sequences in cluster <= 200: `mafft-linsi`
200 < number of sequences in cluster <= 750: `mafft --retree 2 --maxiterate 3`
number of sequences in cluster > 750: `mafft`

Sequence alignment was carried out separately for full-length ITS and ITS2 sequences. Sequence ordering in mafft alignment is stored in the database for viewing purposes.

Step 5: blastclust clustering (clustering step 2)

Both full-length ITS and ITS2 sequence clusters from clustering step 1 (UCL clusters) were clustered further using blastclust version 2.2.22 (Altschul et al. 1997) on different similarity tresholds (97-99%) using the following program parameters

```
blastclust -i infile.fasta -S 97 [97.5, 98, 98.5, 99] -L 0.85 -a 8 -e
F -o outfile -p F
```

Step 6: choosing representative sequences for clustering step 2 (SH) clusters

Representative sequences for all SH clusters (full-length ITS and ITS2 region on different similarity tresholds) were calculated using the following procedure

1. consensus sequence for each SH cluster was generated by USEARCH program with the following command

   ```
   usearch -cluster_fast infile.fasta -consout consensus.fasta -id
   0.80
   ```

2. consensus sequence was blasted against all sequences in the same cluster for finding out best match among “true” sequences using megaBLAST version 2.2.23 (Zhang et al., 2000) with the following parameters

   ```
   megablast -W 8 -r 2 -q -3 -G 5 -E 2 -v 1 -b 1 -m 8 -i
   consensus.fasta -d cluster_db
   ```

NB! This is a separate paragraph of the PlutoF manual. Please use current manual at http://unite.ut.ee/temp/plutof2/files/PlutoF_2.5_Manual_small.pdf if needed or cited in this document.

6. Global key annotations

6.1. Finding the clusters of sequences

- 6.1.1. Using menu Search and edit => All sequences
- 6.1.2. Using menu Global Key annotations => GK annotations

6.2. Working with clusters

6.3. Working with species

6.4 Guidelines for the choosing reference sequence

6. Global key annotations

Terms

Biological sample: Any physical sample, which includes DNA of organism(s). For example, living or collection specimen, soil, water, air, blood, tissue, etc.

Reference sequence (RefS) serves as a name anchor for the species hypothesis and is chosen by the expert. It may originate from any biological sample, viz. herbarium specimen, living culture, soil, water, air, tissue of other organism, etc. RefS is utilised in the scientific communication where identification of organism is based on DNA sequences.

Representative sequence (RepS) serves as a name anchor for the species. It is chosen automatically for all species hypothesis in all clusters based on identical criteria. RepS allows to name and communicate species until RefS becomes available for given species.

Name of the reference sequence. Reference sequence maybe identified on species, genus, family or higher level. The name of the reference sequence is a combination of taxon name and unique INSDC or UNITE accession code.

Example 1: The INSDC sequence EU668254 originate from the plant mycorrhizal root and identified as a Pseudotomentella sp. in UNITE database. If it is selected as a reference sequence then its name is "Pseudotomentella sp. EU668254".

Example 2: The INSDC sequence EU668254 is originate from the sporocarp and identified as a Pseudotomentella mucidula in UNITE database. If it is selected as a reference sequence then its name is "Pseudotomentella mucidula EU668254".

Name of the representative sequence is formed the same way as for reference sequences (see examples above).

Currently PlutoF cloud supports only fungal ITS based annotations and key which is based on UNITE database (unite.ut.ee). The UNITE database includes core data set of ITS sequences which originate from the fruitbodies identified by experts as well as all INSDC fungal ITS sequences with sufficient quality. Technical description of the selection of fungal ITS sequences and subsequent clustering is available in the end of this manual.

6.1. Finding the clusters

There are two basic ways how to find clusters of the specific taxon.

6.1.1. Using menu Search and edit => all sequences

Please consult paragraph 3.7 for the searching sequences of particular taxa. In Figure 6-1 is shown search results for the species *Tomentella sublilacina*. The direct link to the Global Key cluster is shown in the end of each sequence. We recommend to use Qview option which is much faster because the alignment of the cluster is displayed in black and grey instead of colours. UCL4 and UCL5 are acronyms for the Fungal Global Key versions four and five respectively. Clicking on cluster name (eg. UCL4_000977) will display this cluster with alignment in full colours.

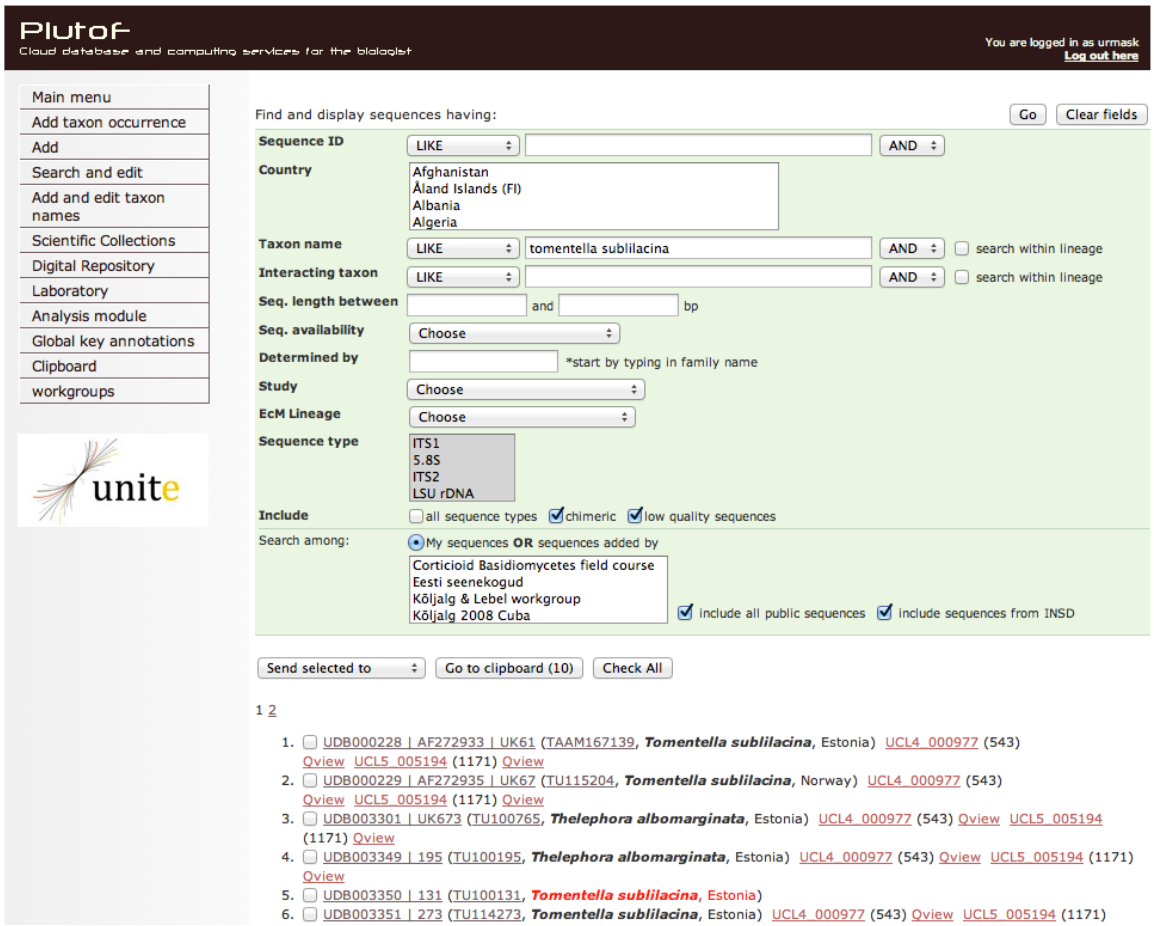


Figure 6-1. Search results for the *Tomentella sublilacina* sequences.

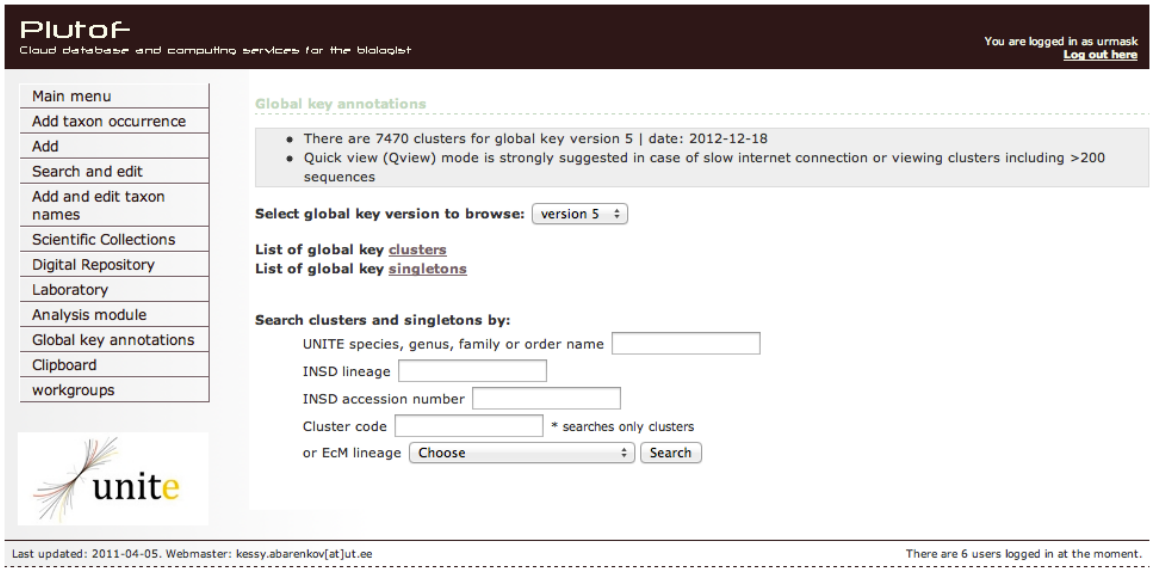


Figure 6-2. Global Key annotations => GK annotations window.

6.1.2. Using menu Global Key annotations => GK annotations

In the Figure 6-2 is shown Global Key annotations => GK annotations window. Here you can **Select global key version to browse**; **List of global key singletons** (sequences which didn't fall into any cluster); **List of global key clusters**; **Search clusters and singletons by** UNITE or INSD taxon names, INSD accession number, cluster code or by ectomycorrhizal (EcM) lineage.

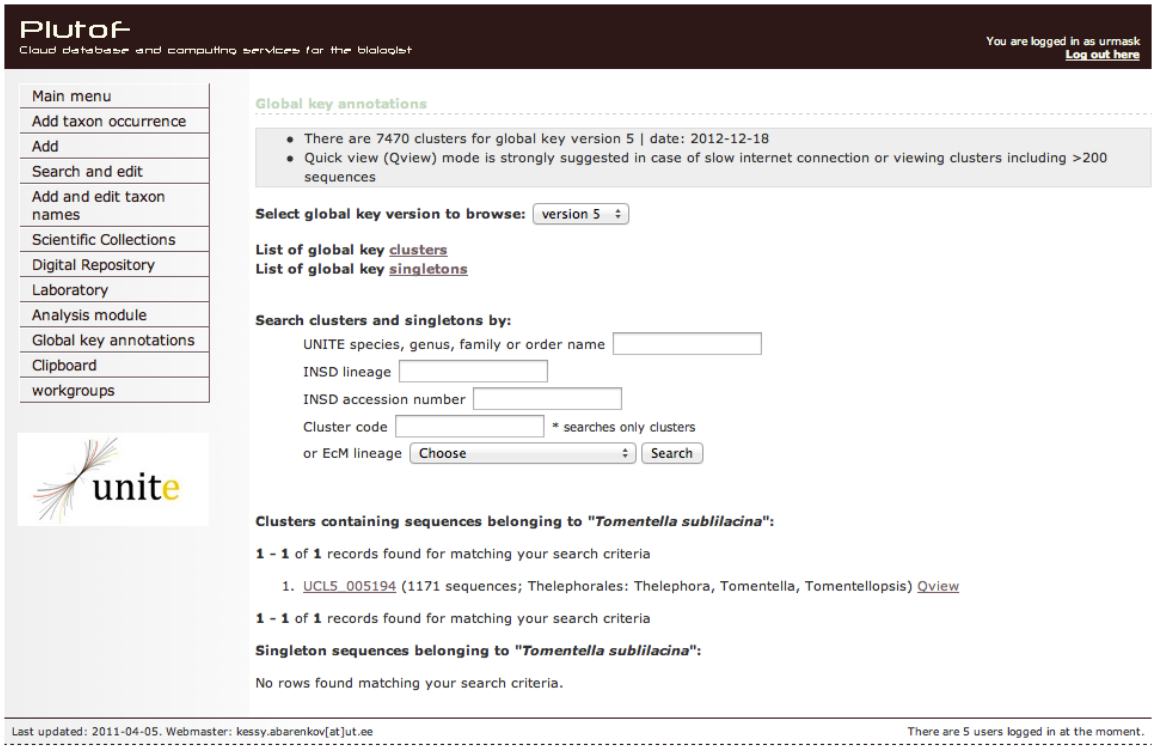


Figure 6-3. Result of the search "Tomentella sublilacina".

In Figure 6-3 is shown result of the **Search clusters and singletons by** UNITE name "Tomentella sublilacina". To display cluster you can preferably click on link "Qview" or on cluster code "UCL5_005194". Figure 6-4 shows the cluster UCL5_005194 window which is opened when clicking on "Qview".

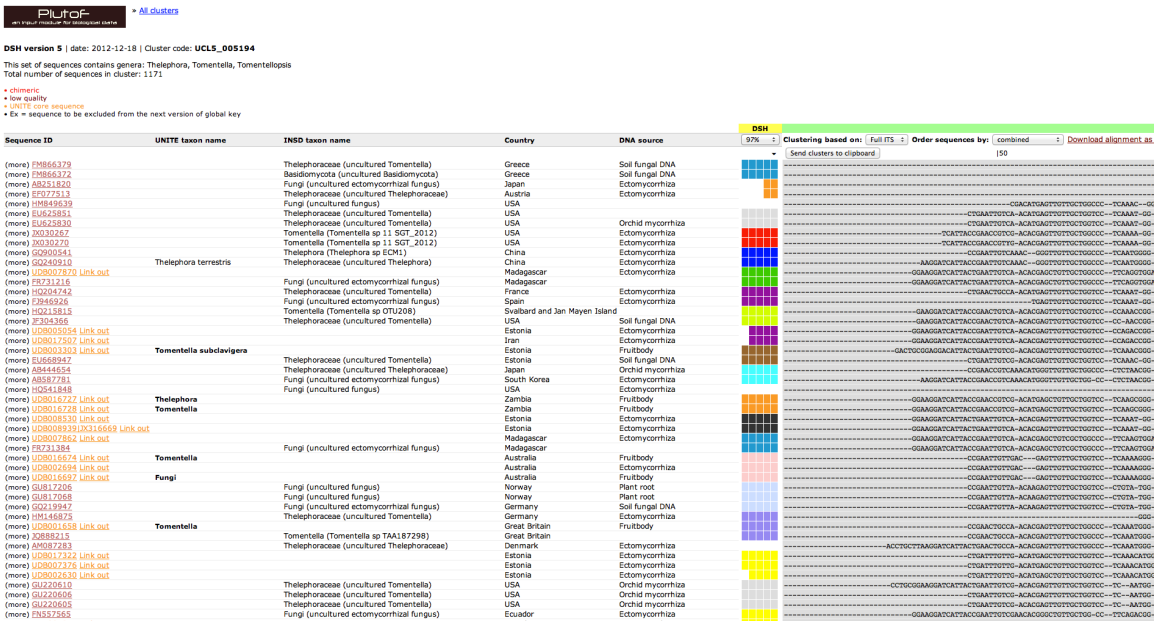


Figure 6-4. View of the cluster UCL5_005194.

6.2 Working with clusters

On Fig 6.4 is shown cluster UCL5_005194. The header of the window displays information on version and cluster ID. Next lines list genera (UNITE names only), which appear in this cluster and number of sequences in cluster. The sequence is likely chimeric if its ID and other text in this line is shown in red and it will be removed from the next version. If the text is brown then the sequence is low quality and will be removed in next version as well. UNITE core sequence ID-s are shown in yellow. Ex in the front of Sequence ID shows that expert decided that it will be removed from the next version of the key. Each cluster has following columns: 1) Sequence ID displays UNITE or/and INSD accession code which is hyperlink to the original as well as annotated data which can be edited by expert (see paragraph 3.8 in PlutoF manual). Clicking on "more" will open a small window below the line which displays all alternative identifications if present and allows to add data on specimen as well as mark sequence if it should be removed from next version; 2) UNITE taxon name is name given to the specimen or to the sequence from any other biological sample; 3) INSD taxon name displays name of the sequence in the INSD original data; 4) Country shows the name of the country from where the sequence originates; 5) DNA source shows the type of biological sample from where the sequence originates; 6) Next column allows to choose threshold value for the species discrimination (see also 6.3); 7) Clustering based on allows to switch between full ITS and ITS2 based alignments and species; 8) Order sequences allows to reorder sequences based on mafft alignment or blastclust outputs (default is combined approach); 9) Download alignment as a FASTA file.

6.3 Working with species

Column “DSH” on Figure 6-4 is divided into five strips based on ITS sequence similarity threshold values 99, 98.5, 98, 97.5 and 97% (from left to right). Strip cells of the sequences, which cluster together based on specific threshold value have the same colour. If sequence is not clustering with any sequence then the strip cell is colourless. For example 3rd and 4th sequences on Figure 6-4 are not clustering with 99, 98.5 and 98% threshold values, but do with 97.5 and 97%. Figure 6-5 shows the middle part of the same cluster (UCL5_005194). The cursor is on the left edge of the left strip (99% threshold value based clustering). Clicking on it will display new window shown on Figure 6-6.

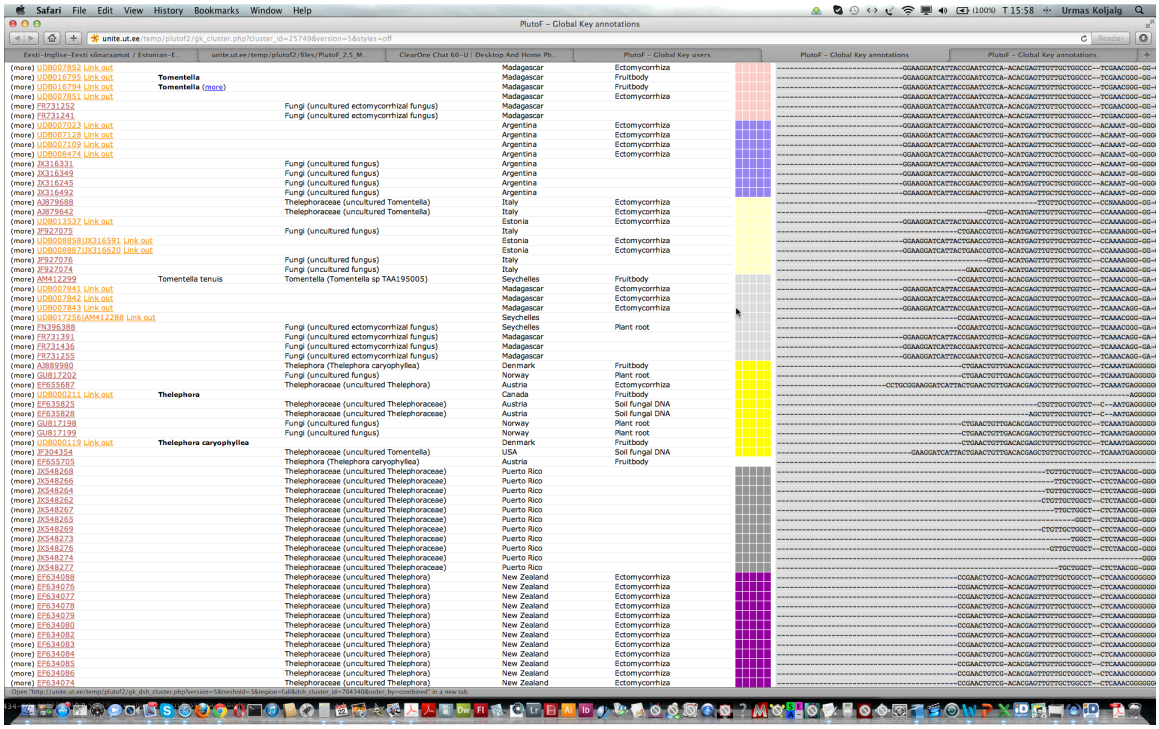
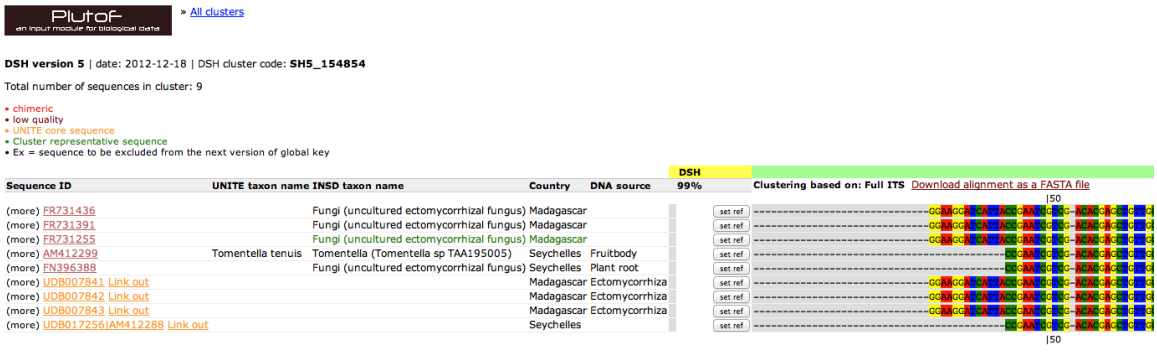


Figure 6-5. Middle part of the cluster UCL5_005194.



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» All clusters

DSH version 5 | date: 2012-12-18 | DSH cluster code: **SH5_154854**

Total number of sequences in cluster: 9

- chimeric
- low quality
- UNITE core sequence
- Cluster representative sequence
- Ex = sequence to be excluded from the next version of global key

Sequence ID	UNITE taxon name	INSD taxon name	Country	DNA source	DSH 99%	Clustering based on: Full ITS Download alignment as a FASTA file
						|50
(more) FR731436		Fungi (uncultured ectomycorrhizal fungus)	Madagascar		set ref	
(more) FR731391		Fungi (uncultured ectomycorrhizal fungus)	Madagascar		set ref	
(more) FR731255		Fungi (uncultured ectomycorrhizal fungus)	Madagascar		set ref	
(more) AM412299	Tomentella tenuis	Tomentella (Tomentella sp TAA195005)	Seychelles	Fruitbody	set ref	
(more) FN396388		Fungi (uncultured ectomycorrhizal fungus)	Seychelles	Plant root	set ref	
(more) UDB007841 Link out			Madagascar	Ectomycorrhiza	set ref	
(more) UDB007842 Link out			Madagascar	Ectomycorrhiza	set ref	
(more) UDB007843 Link out			Madagascar	Ectomycorrhiza	set ref	
(more) UDB017256|AM412288 Link out			Seychelles		set ref	
						|50

Figure 6-6. Cluster of nine sequences based on 99% similarity threshold value.

In this window (Figure 6-6) expert can set reference sequence of the species by clicking on button “set ref”. See paragraph “Reference sequence” for the guidelines how to choose it. Clicking on “set ref” of the *Tomentella tenuis* sequence AM412299 will reload this page as shown on Figure 6-7. Reference sequence can be unset by clicking on button “unset ref”. In this widow automatically chosen representative sequence is shown in green colour.

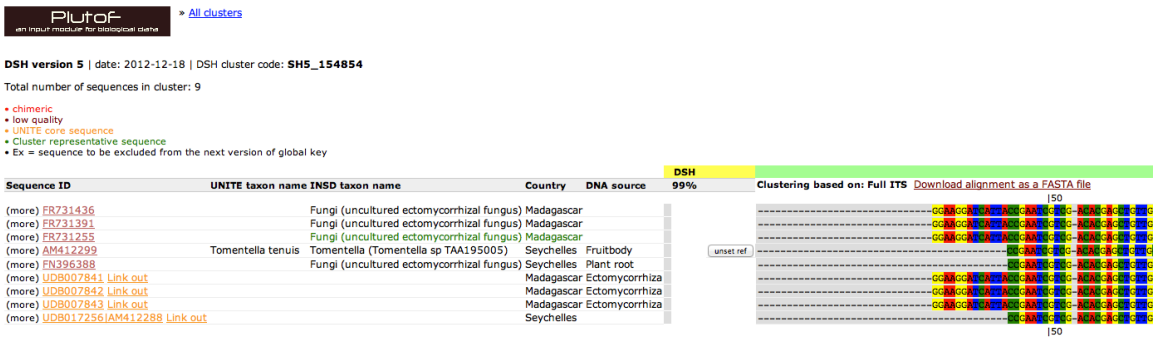

Figure 6-7. *Tomentella tenuis* sequence AM412299 is set as a reference sequence.

Reloaded window shown on Figure 6-5 will display reference sequence for all strips (Figure 6-8)

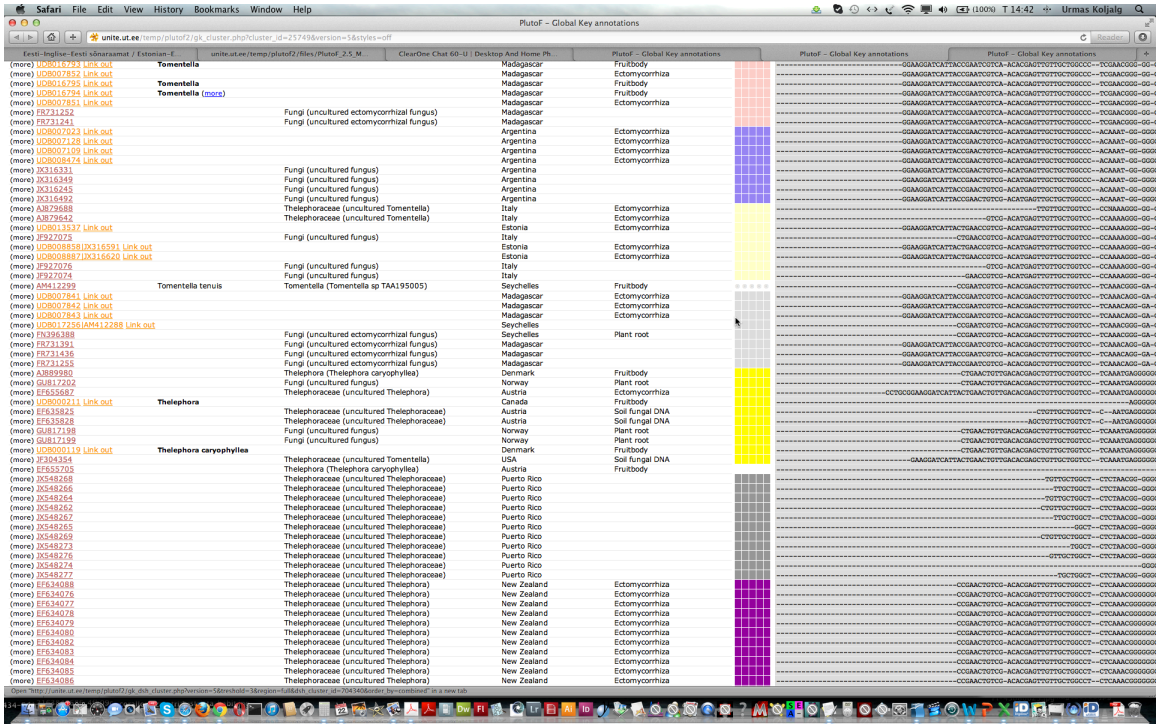

Figure 6-8. Middle part of the cluster UCL5_005194 with reference sequence of *Tomentella tenuis* marked for all five strips.

6.4 Guidelines for the choosing reference sequence

Basic guidelines

I. Sequence from type material has priority
Sequence of the type material has no priority if it is short or of low quality.

II. One reference sequence per species hypothesis.
Example 1: Species hypothesis (SH) based on 97% similarity threshold value includes one reference sequence X. If this SH is divided into two species by 98% similarity threshold value then one SH will include reference sequence X, but second SH should receive new reference sequence Y.

Example 2: If two SH which have reference sequences X and Y are lumped together then one of them will become reference sequence of the new SH. Currently PlutoF will automatically select reference sequence, which was chosen first. This decision can be amended by expert.

III. Reference sequence can be replaced.
Reference sequence X can be replaced by a new sequence Y if its source stands higher in "Reference sequence selection priority list" (see below).

Example: Reference sequence X is derived from soil sample but later sequence Y from living culture becomes available. It falls inside the same SH as reference sequence X and therefore may replace it.

Remark: Current version of the PlutoF needs that expert will make the replacement. The alarming system that potentially better reference sequence is available will be implemented in future version.

Practical recommendations for the selection of reference sequence

Reference sequence selection priority list
The selection priority in decreasing order is as follows (by assuming that sequences are of high quality): type material, specimen in public collection, living culture in public collection, and sequence from any other biological sample.

1. If type specimen is sequenced then it is also reference sequence of this species. It carries the species name.

If the sequence of type specimen is not in the species cluster or if it is low quality then we recommend following selection procedures:

2. The sequence from authentic herbarium specimen or living culture which is identified by expert should be chosen. The species name of the specimen is also the name of the

reference sequence. The locality of the reference sequence should be as close as possible to the type material locality.

3. If species cluster includes only sequences from biological samples like soil, water, air, tissue of other organism, etc. then sequence available in INSD should be chosen. If there are no sequences from INSD then sequence submitted into other public databases like UNITE should be chosen. The name of the reference sequence is accession code accompanied by genus name if available.

4. Cloned sequences are not recommended as a reference sequences except cases when well grounded SH includes cloned sequences only.

Fig. S3 Format of the UNITE reference sequences FASTA file available for download at unite.ut.ee and used by QIIME

HEADER:

```
; UNITE fungal identifier reference dataset 27.03.2013
; This UNITE dataset should be cited as Abarenkov K, Nilsson RH, Larsson KH, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Ursing BM, Vrålstad T, Liimatainen K, Peintner U, Kõljalg U (2010). The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytologist, 186(2), 281 - 285.
; This UNITE dataset is licensed under a Creative Commons Attribution-ShareAlike 3.0 Unported License.
; FASTA sequence header line format: SHxxxxxx.05FU|cl|refs|accno|UNITE lineage
; Where:
; Column 1: the UNITE version and accession number of the species hypothesis
; Column 2: specifies whether the sequence belongs to cluster (cl), is singleton in cluster (cls) or is singleton outside any cluster (s)
; Column 3: specifies whether the sequence is representative (“reps”) or reference (“refs”)
; Column 4: INSD accession number [or UNITE accession number for sequences that are not in INSD]
; Column 5: UNITE taxon name together with hierarchical classification (essentially Index Fungorum as implemented in UNITE) including any taxonomic re-annotation given by a third-party annotator
; FASTA header line example:
; >SH104007.05FU|cl|refs|UDB016438|k__Fungi; p__Ascomycota; c__Leotiomycetes; o__Helotiales; f__Helotiaceae; g__Hymenoscyphus; s__Hymenoscyphus albidus
; UNITE name of the species hypotheses format (formed from reference or representative sequences only): |UNITE name|accno|SHxxxxxx.05FU
; UNITE name of the species hypotheses example: Hymenoscyphus pseudoalbidus|GU586904|SH133781.05FU
; External URLs to species hypotheses and UNITE sequences should be formed as followed:
; 1. Species hypotheses - http://unite.ut.ee/gk/sh.php?name=[species hypotheses name]
; 2. UNITE sequence - http://unite.ut.ee/bl_forw.php?nimi=[UNITE accession number]
```

SEQUENCES:

```
>SH114093.05FU|cl|refs|GU586876|k__Fungi; p__Ascomycota; c__Leotiomycetes; o__Helotiales; f__Helotiaceae; g__Hymenoscyphus; s__Hymenoscyphus albidus
CATTACAGAGTTCCTGCCCTCACGGGTAGAAACCCCACCCTTGTGTATATTATATTGTTGCTTTAGCAGGTCGCCCCCCG
GGGCGTTGGCCTCGGCTGACCGTGCCTGCTAGAGGATCCTAAACTTTGAAATACAGTGTCGTCTGAGTACTATTTAATAG
TTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATT
TAGACCAACTCCCGCTCCGGCGGGGTCTTGGGCTCCGCCTCTGGGCGGGCCTTAAAACCAGTGGCGGTGCCCTAAGGCTC
TACGCGTAGTAATTCTTCTCGCGATAGGGTCCTTGAGGTGTCTTGCCAGAAACCCCCAACTCTCTAGGGTTGACCTCGGA
T

>SH133781.05FU|cl|refs|GU586904|k__Fungi; p__Ascomycota; c__Leotiomycetes; o__Helotiales; f__Helotiaceae; g__Hymenoscyphus; s__Hymenoscyphus pseudoalbidus
CATTACAGAGTTCCTGCCCTCACGGGTAGAAACCCCACCCTTGTGTATATTATATTGTTGCTTTAGCAGGTCGCCCTCTG
GGCGTCGGCCTCGGCTGACTGTGCCTGCTAGAGGACCCTAAATTTTGAAATACAGTGTCGTCTGAGTACTATTTAATAGT
```

TAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTT
AGACCAACTCCCGCTCCGGCGGGGTCTTGGGCTGCGCCTTTGGGCGGGCCTTAAAACCAGTGGCGGTGCCCTAAGGCTCT
ACGCGTAGTAATTCTTCTCGCGATAGGGTCCTTGCGGTGTCTTGCCAGCAACCCCCAACTCTCTAGGGTTGACCTCGGAT

Remark: The sequences in FASTA format shown here are selected in order to reflect the content of the paper.

Fig. S4. Screenshot of the UNITE global key workbench depicting the cluster UCL5_005639.

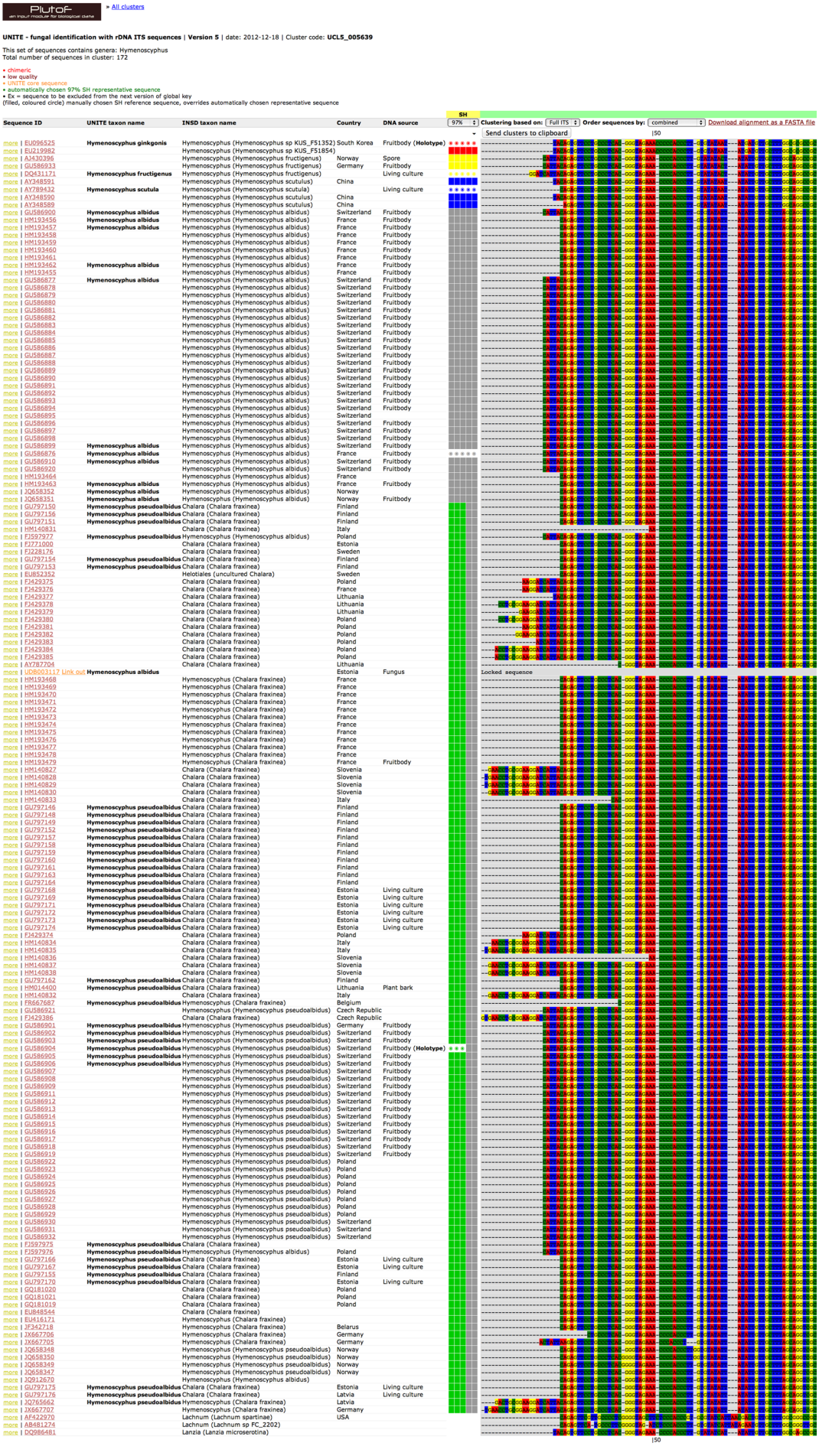

Fig. S5. Screenshot of the UNITE global key workbench depicting the species hypotheses SH5155686.05FU. This workbench allows to choose reference sequences.

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» All clusters

UNITE - fungal identification with rDNA ITS sequences | **Version 5** | date: 2012-12-18 | SH cluster code: **SH155686.05FU**

Total number of sequences in cluster: 39

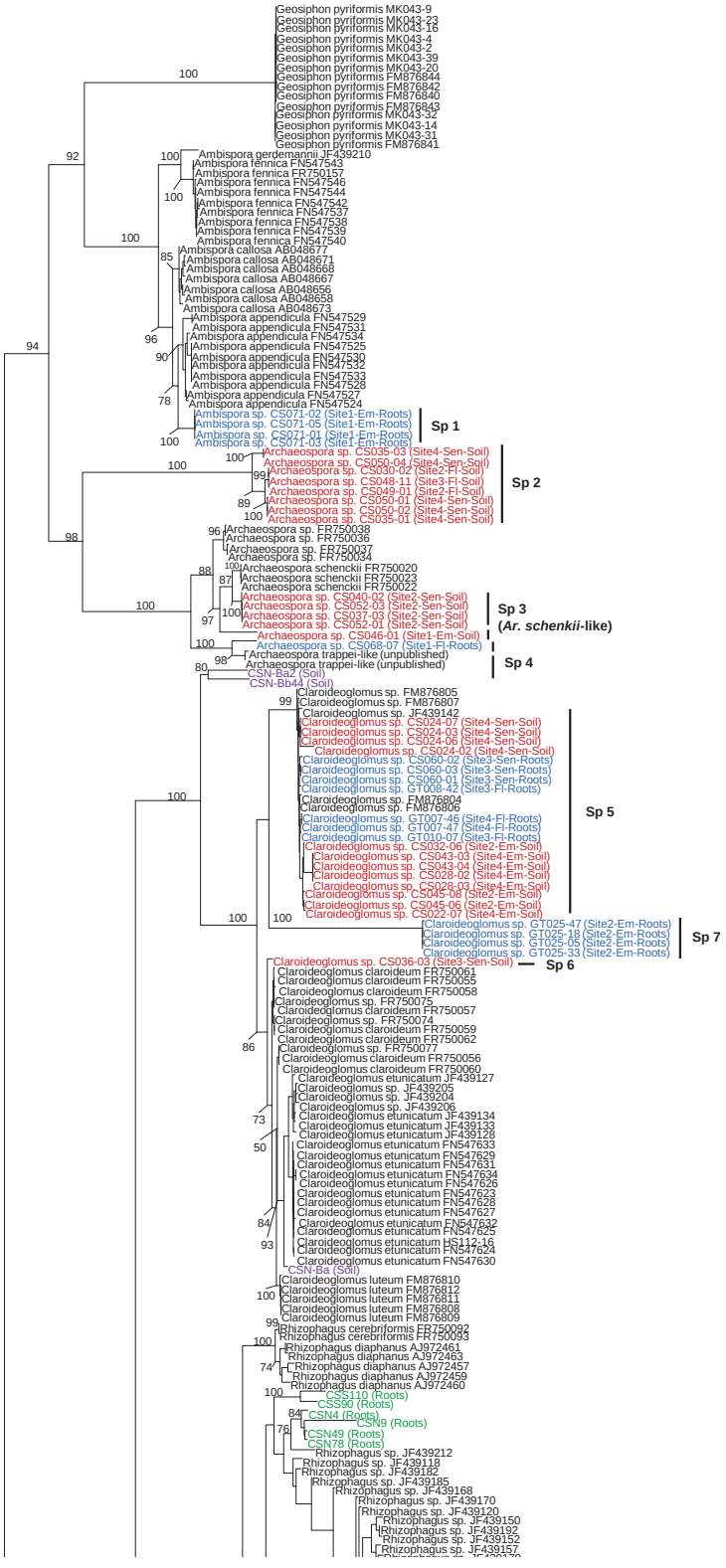
- chimeric
- low quality
- UNITE core sequence
- automatically chosen SH representative sequence
- Ex = sequence to be excluded from the next version of global key

					SH	
Sequence ID	**UNITE taxon name**	**INSD taxon name**	**Country**	**DNA source**	**99%**	**Clustering based on: Full ITS** Download alignment as a FASTA file
						|50
more | GU586920		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | JQ658352	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Norway			
more | JQ658351	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Norway	Fruitbody		
more | GU586910	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586900	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586899	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586898		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586897		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586896		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586895		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland			
more | GU586894		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586893		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586892		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586891		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586890		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586889		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586888		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586887		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586886		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586885		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586884		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586883		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586882		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586881		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586880		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586879		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586878		Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586877	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	Switzerland	Fruitbody		
more | GU586876	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody	RefSeq	
more | HM193462	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193461		Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193460		Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193459		Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193458		Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193457	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193456	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193455		Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		
more | HM193464		Hymenoscyphus (Hymenoscyphus albidus)	France			
more | HM193463	**Hymenoscyphus albidus**	Hymenoscyphus (Hymenoscyphus albidus)	France	Fruitbody		

10.2 Supplementary data – Chapter 5

The following data are supplementary material for the publication **Potato-associated arbuscular mycorrhizal fungal communities in the Peruvian Andes.**

Fig. S1. Full Phylogenetic analysis showing the species detected in the Peruvian potato fields and AMF representatives from all orders of the *Glomeromycota*. Sequences obtained by Cesaro et al. (2008) from two Italian potato fields are included. Red = Peruvian rhizosphere soil derived sequences; Blue = Peruvian root derived sequences; Green = Italian root derived sequences; Purple = Italian soil derived sequences.




Rhizophagus sp. JF439179
Rhizophagus sp. JF439166
Rhizophagus sp. JF439172
Rhizophagus sp. JF439153
Rhizophagus sp. JF439158
Rhizophagus sp. JF439208
Rhizophagus sp. JF439151
Rhizophagus sp. JF439207
Rhizophagus sp. JF439154
Rhizophagus sp. JF439181
Rhizophagus sp. JF439104
Rhizophagus sp. JF439098
Rhizophagus sp. JF439115
Rhizophagus sp. JF439105
Rhizophagus sp. JF439156
Rhizophagus sp. JF439164
Rhizophagus sp. JF439116
Rhizophagus sp. JF439117
Rhizophagus sp. JF439113
Rhizophagus sp. JF439102
Rhizophagus sp. JF439121
Rhizophagus sp. JF439099
Rhizophagus sp. JF439103
Rhizophagus sp. JF439186
Rhizophagus sp. 10_SUN_2011_JF439139
Rhizophagus sp. JF439114
Rhizophagus sp. JF439100
Rhizophagus sp. JF439097
Rhizophagus sp. JF439193
Rhizophagus sp. JF439110
Rhizophagus sp. JF439187
CSS-Ba5 (Soil)
Rhizophagus sp. JF439201
Rhizophagus sp. JF439199
Rhizophagus sp. JF439200
Rhizophagus sp. JF439189
Rhizophagus sp. JF439162
Rhizophagus sp. CS050-05 (Site-4-Sen-Soil)
Rhizophagus sp. CS050-03 (Site-4-Sen-Soil)
Rhizophagus sp. GT002-20 (Site4-Em-Roots)
Rhizophagus sp. GT002-9 (Site4-Em-Roots)
Rhizophagus sp. GT002-13 (Site4-Em-Roots)
Rhizophagus sp. GT002-28 (Site4-Em-Roots)
Sp 8
Rhizophagus sp. JF439202
Rhizophagus sp. JF439191
CSN-Bb1 (Soil)
Rhizophagus sp. JF439123
Rhizophagus sp. JF439211
Rhizophagus sp. JF439122
Rhizophagus proliferus FM992400
Rhizophagus proliferus FM992401
Rhizophagus proliferus FM992391
Rhizophagus proliferus FM992388
Rhizophagus proliferus FM992397
Rhizophagus proliferus FN547500
Rhizophagus intraradices JF439108
Rhizophagus intraradices FM865601
Rhizophagus intraradices FM865562
Rhizophagus intraradices FM865597
Rhizophagus intraradices FM865586
Rhizophagus intraradices epitype_HE817879
Rhizophagus intraradices FM865602
Rhizophagus intraradices FM865575
Rhizophagus intraradices FM865581
Rhizophagus intraradices FM865572
Rhizophagus intraradices FM865583
Rhizophagus intraradices FM865580
Rhizophagus intraradices FM865559
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Rhizophagus intraradices FM865546
Rhizophagus intraradices FM865568
Rhizophagus intraradices HE817871
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Rhizophagus intraradices FM865570
Rhizophagus intraradices FM865566
Rhizophagus intraradices FM865607
Rhizophagus intraradices FR750372
Rhizophagus intraradices JF439173
Rhizophagus clarus FM865537
Rhizophagus clarus FM865541
Rhizophagus clarus FM865539
Rhizophagus clarus FM865540
Rhizophagus clarus FM865538
Rhizophagus clarus FM865543
Rhizophagus clarus FM865542
Rhizophagus clarus FM865536
Rhizophagus clarus FM865544
Rhizophagus fasciculatus FR750073
Rhizophagus fasciculatus FR750072
CSN114 (Roots)
Rhizophagus irregularis JF439183
CSS62 (Roots)
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Rhizophagus sp. GT003-37 (Site3-Em-Roots)
Rhizophagus sp. GT003-62 (Site3-Em-Roots)
Rhizophagus sp. GT003-42 (Site2-Em-Roots)
Rhizophagus sp. GT003-36 (Site3-Em-Roots)
Rhizophagus sp. GT003-43 (Site2-Em-Roots)
Rhizophagus sp. CS034-03 (Site3-Fl-Soil)
Rhizophagus sp. CS048-02 (Site3-Fl-Soil)
Rhizophagus sp. CS044-03 (Site3-Em-Soil)
Rhizophagus sp. CS029-05 (Site3-Em-Soil)
Sp 9
(Rh. irregularis)
Rhizophagus irregularis (unpublished)
CSS7 (Roots)
CSS33 (Roots)
CSS10 (Roots)
CSN67 (Roots)
CSS5 (Roots)
CSN7 (Roots)
CSN38 (Roots)
CSN99 (Roots)
CSN60 (Roots)
CSS8 (Roots)
CSS92 (Roots)
CSS8 (Roots)
CSS67 (Roots)
Rhizophagus irregularis FR750218
Rhizophagus irregularis FR750068
Rhizophagus irregularis FR750064
Rhizophagus irregularis FR750098
Rhizophagus irregularis FM865552
Rhizophagus irregularis FR750096
Rhizophagus irregularis HE817882
Rhizophagus irregularis FM865551
Rhizophagus irregularis FR750065
Rhizophagus irregularis FR750069
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Rhizophagus irregularis FM865558
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Rhizophagus irregularis FR750099
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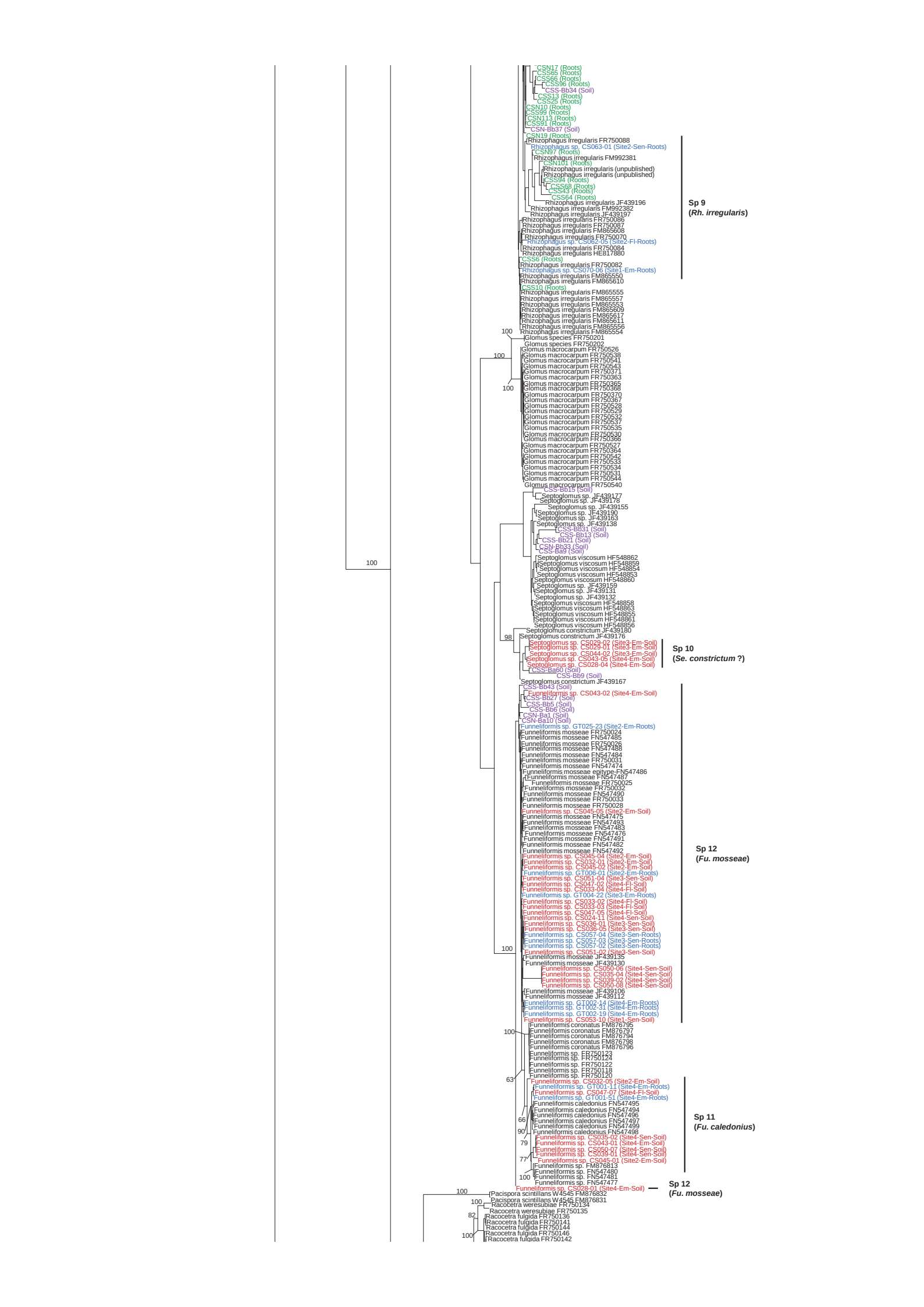
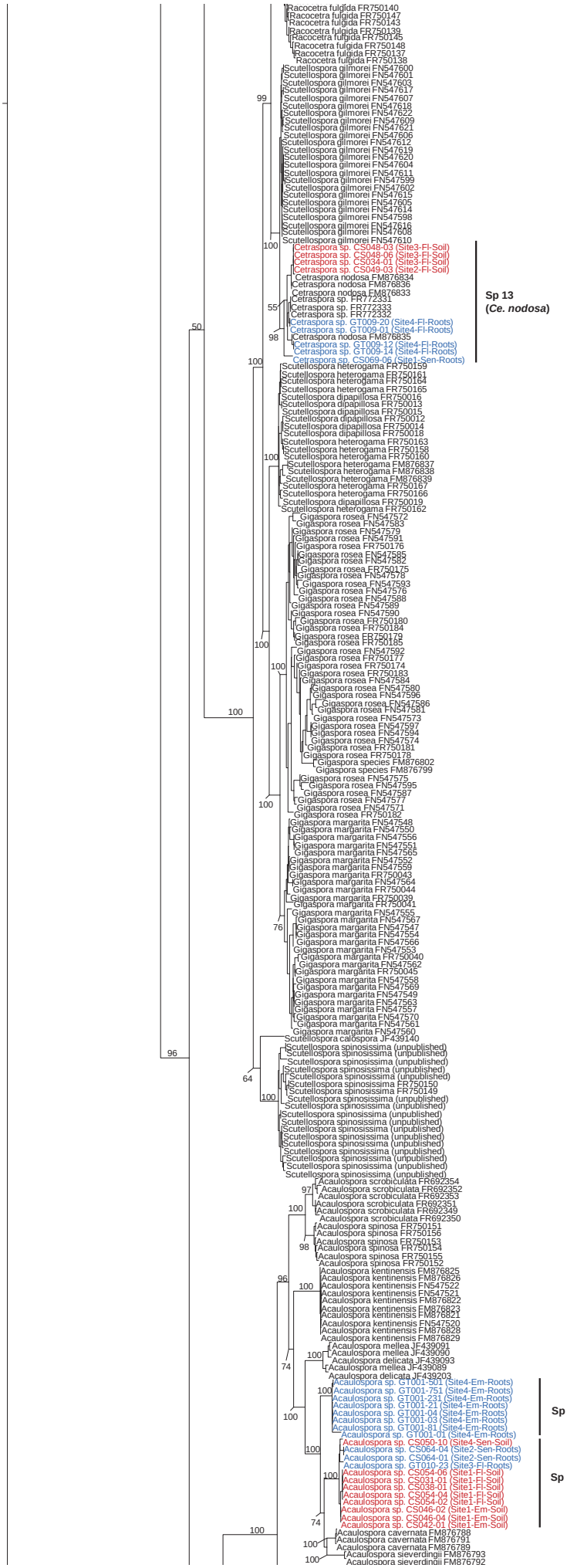
Rhizophagus sp. JF439101
Rhizophagus irregularis FR750196
Rhizophagus irregularis FR750193
Rhizophagus irregularis FR750085
Rhizophagus irregularis FR750079
Rhizophagus irregularis FR750091
Rhizophagus irregularis FR750080
Rhizophagus irregularis FR750081
Rhizophagus irregularis FM865613
Rhizophagus irregularis FR750078
Rhizophagus irregularis HE817881
Rhizophagus irregularis HE817884
Rhizophagus irregularis FR750097
Rhizophagus irregularis HE817883
Rhizophagus irregularis FR750100
CSN68 (Roots)
CSN61 (Roots)
CSS6 (Roots)
CSS17 (Roots)
CSN10 (Roots)
CSS5 (Roots)
Rhizophagus irregularis FM865595
Rhizophagus irregularis FM865591
Rhizophagus irregularis FM992380
Rhizophagus irregularis FM992383
Rhizophagus irregularis FM992377
Rhizophagus irregularis FM992387
Rhizophagus irregularis FM992378
Rhizophagus irregularis FM865594
Rhizophagus irregularis FM865592
Rhizophagus irregularis FM865593
Rhizophagus irregularis FM865596
Rhizophagus irregularis FM865589
Rhizophagus irregularis FM865588
Rhizophagus irregularis FR750105
Rhizophagus irregularis FR750104
Rhizophagus irregularis FR750102
CSN85 (Roots)
CSN62 (Roots)
CSN54 (Roots)
CSN94 (Roots)
CSN11 (Roots)
100
100
100
80
69
86
100
71
76
100
67
52
100
100


CSN17 (Roots)
CSS65 (Roots)
CSS66 (Roots)
CSS96 (Roots)
CSS-Bb34 (Soil)
CSS13 (Roots)
CSS25 (Roots)
CSN10 (Roots)
CSS99 (Roots)
CSN113 (Roots)
CSS91 (Roots)
CSN-Bb37 (Soil)
CSN19 (Roots)
Rhizophagus irregularis FR750088
Rhizophagus sp. CS063-01 (Site2-Sen-Roots)
CSN97 (Roots)
Rhizophagus irregularis FM992381
CSN101 (Roots)
Rhizophagus irregularis (unpublished)
Rhizophagus irregularis (unpublished)
CSS94 (Roots)
CSS68 (Roots)
CSS43 (Roots)
CSS64 (Roots)
Rhizophagus irregularis JF439196
Rhizophagus irregularis FM992382
Rhizophagus irregularis JF439197
Rhizophagus irregularis FR750086
Rhizophagus irregularis FR750087
Rhizophagus irregularis FM865608
Rhizophagus irregularis FR750070
Rhizophagus sp. CS062-05 (Site2-FI-Roots)
Rhizophagus irregularis FR750084
Rhizophagus irregularis HE817880
CSS5 (Roots)
Rhizophagus irregularis FR750082
Rhizophagus sp. CS070-06 (Site1-Em-Roots)
Rhizophagus irregularis FM865550
Rhizophagus irregularis FM865610
CSS10 (Roots)
Rhizophagus irregularis FM865555
Rhizophagus irregularis FM865557
Rhizophagus irregularis FM865553
Rhizophagus irregularis FM865609
Rhizophagus irregularis FM865617
Rhizophagus irregularis FM865611
Rhizophagus irregularis FM865556
Rhizophagus irregularis FM865554
Sp 9
(Rh. irregularis)
100
Glomus species FR750201
Glomus species FR750202
Glomus macrocarpum FR750526
100
Glomus macrocarpum FR750538
Glomus macrocarpum FR750541
Glomus macrocarpum FR750543
Glomus macrocarpum FR750371
Glomus macrocarpum FR750363
100
Glomus macrocarpum FR750365
Glomus macrocarpum FR750368
Glomus macrocarpum FR750370
Glomus macrocarpum FR750367
Glomus macrocarpum FR750528
Glomus macrocarpum FR750529
Glomus macrocarpum FR750532
Glomus macrocarpum FR750537
Glomus macrocarpum FR750535
Glomus macrocarpum FR750530
Glomus macrocarpum FR750366
Glomus macrocarpum FR750527
Glomus macrocarpum FR750364
Glomus macrocarpum FR750542
Glomus macrocarpum FR750533
Glomus macrocarpum FR750534
Glomus macrocarpum FR750531
Glomus macrocarpum FR750544
Glomus macrocarpum FR750540
CSS-Bb15 (Soil)
Septoglomus sp. JF439177
Septoglomus sp. JF439178
Septoglomus sp. JF439155
Septoglomus sp. JF439190
Septoglomus sp. JF439163
Septoglomus sp. JF439138
CSS-Bb31 (Soil)
CSS-Bb13 (Soil)
CSS-Bb21 (Soil)
CSN-Bb33 (Soil)
CSS-Bb9 (Soil)
100
Septoglomus viscosum HF548862
Septoglomus viscosum HF548859
Septoglomus viscosum HF548854
Septoglomus viscosum HF548853
Septoglomus viscosum HF548860
Septoglomus sp. JF439159
Septoglomus sp. JF439131
Septoglomus sp. JF439132
Septoglomus viscosum HF548858
Septoglomus viscosum HF548863
Septoglomus viscosum HF548855
Septoglomus viscosum HF548861
Septoglomus viscosum HF548856
Septoglomus constrictum JF439180
98
Septoglomus constrictum JF439176
Septoglomus sp. CS029-02 (Site3-Em-Soil)
Septoglomus sp. CS029-01 (Site3-Em-Soil)
Septoglomus sp. CS044-02 (Site3-Em-Soil)
Septoglomus sp. CS043-05 (Site4-Em-Soil)
Septoglomus sp. CS028-04 (Site4-Em-Soil)
Sp 10
(Se. constrictum ?)
CSS-Ba60 (Soil)
CSS-Bb9 (Soil)
Septoglomus constrictum JF439167
CSS-Bb43 (Soil)
Funneliformis sp. CS043-02 (Site4-Em-Soil)
CSS-Bb27 (Soil)
CSS-Bb5 (Soil)
CSS-Bb6 (Soil)
CSN-Ba1 (Soil)
CSN-Ba10 (Soil)
Funneliformis sp. GT025-23 (Site2-Em-Roots)
Funneliformis mosseae FR750024
Funneliformis mosseae FN547485
Funneliformis mosseae FR750026
Funneliformis mosseae FN547488
Funneliformis mosseae FN547484
Funneliformis mosseae FR750031
Funneliformis mosseae FN547474
Funneliformis mosseae epitype-FN547486
Funneliformis mosseae FN547487
Funneliformis mosseae FR750025
Funneliformis mosseae FR750032
Funneliformis mosseae FN547490
Funneliformis mosseae FR750033
Funneliformis mosseae FR750028
Funneliformis sp. CS045-05 (Site2-Em-Soil)
Funneliformis mosseae FN547475
Funneliformis mosseae FN547493
Funneliformis mosseae FN547483
Funneliformis mosseae FN547476
Funneliformis mosseae FN547491
Funneliformis mosseae FN547482
Funneliformis mosseae FN547492
Funneliformis sp. CS045-04 (Site2-Em-Soil)
Funneliformis sp. CS032-01 (Site2-Em-Soil)
Funneliformis sp. CS045-02 (Site2-Em-Soil)
Funneliformis sp. GT006-01 (Site2-Em-Roots)
Funneliformis sp. CS051-04 (Site3-Sen-Soil)
Funneliformis sp. CS047-02 (Site4-FI-Soil)
Funneliformis sp. CS033-04 (Site4-FI-Soil)
Funneliformis sp. GT004-22 (Site3-Em-Roots)
Funneliformis sp. CS033-02 (Site4-FI-Soil)
Funneliformis sp. CS033-03 (Site4-FI-Soil)
Funneliformis sp. CS047-05 (Site4-FI-Soil)
Funneliformis sp. CS024-11 (Site4-Sen-Soil)
Funneliformis sp. CS036-01 (Site3-Sen-Soil)
Funneliformis sp. CS036-05 (Site3-Sen-Soil)
Funneliformis sp. CS057-04 (Site3-Sen-Roots)
Funneliformis sp. CS057-03 (Site3-Sen-Roots)
Funneliformis sp. CS057-02 (Site3-Sen-Roots)
Funneliformis sp. CS051-02 (Site3-Sen-Soil)
100
Funneliformis mosseae JF439135
Funneliformis mosseae JF439130
Funneliformis sp. CS050-06 (Site4-Sen-Soil)
Funneliformis sp. CS035-04 (Site4-Sen-Soil)
Funneliformis sp. CS039-02 (Site4-Sen-Soil)
Funneliformis sp. CS050-08 (Site4-Sen-Soil)
Funneliformis mosseae JF439106
Funneliformis mosseae JF439112
Funneliformis sp. GT002-14 (Site4-Em-Roots)
Funneliformis sp. GT002-31 (Site4-Em-Roots)
Funneliformis sp. GT002-19 (Site4-Em-Roots)
Funneliformis sp. CS053-10 (Site1-Sen-Soil)
Sp 12
(Fu. mosseae)
Funneliformis coronatus FM876795
100
Funneliformis coronatus FM876797
Funneliformis coronatus FM876794
Funneliformis coronatus FM876798
Funneliformis coronatus FM876796
Funneliformis sp. FR750123
Funneliformis sp. FR750124
Funneliformis sp. FR750122
Funneliformis sp. FR750118
Funneliformis sp. FR750120
63
Funneliformis sp. CS032-05 (Site2-Em-Soil)
Funneliformis sp. GT001-11 (Site4-Em-Roots)
Funneliformis sp. CS047-07 (Site4-FI-Soil)
Funneliformis sp. GT001-51 (Site4-Em-Roots)
Funneliformis caledonius FN547495
Funneliformis caledonius FN547494
66
Funneliformis caledonius FN547496
Funneliformis caledonius FN547497
Funneliformis caledonius FN547499
90
Funneliformis caledonius FN547498
79
Funneliformis sp. CS035-02 (Site4-Sen-Soil)
Funneliformis sp. CS043-01 (Site4-Em-Soil)
Funneliformis sp. CS050-07 (Site4-Sen-Soil)
77
Funneliformis sp. CS039-01 (Site4-Sen-Soil)
Funneliformis sp. CS045-01 (Site2-Em-Soil)
Sp 11
(Fu. caledonius)
Funneliformis sp. FM876813
Funneliformis sp. FN547480
100
Funneliformis sp. FN547481
Funneliformis sp. FN547477
Funneliformis sp. CS028-01 (Site4-Em-Soil)
Sp 12
(Fu. mosseae)
100
Pacispora scintillans W4545 FM876832
Pacispora scintillans W4545 FM876831
100
Racocetra weresubiae FR750134
Racocetra weresubiae FR750135
82
Racocetra fulgida FR750136
Racocetra fulgida FR750141
Racocetra fulgida FR750144
100
Racocetra fulgida FR750146
Racocetra fulgida FR750142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Racocetra fulgida FR750148
Racocetra fulgida FR750137
Racocetra fulgida FR750138
Scutellospora gilmorei FN547600
Scutellospora gilmorei FN547601
Scutellospora gilmorei FN547603
Scutellospora gilmorei FN547617
Scutellospora gilmorei FN547607
Scutellospora gilmorei FN547618
Scutellospora gilmorei FN547622
Scutellospora gilmorei FN547609
Scutellospora gilmorei FN547621
Scutellospora gilmorei FN547606
Scutellospora gilmorei FN547612
Scutellospora gilmorei FN547619
Scutellospora gilmorei FN547620
Scutellospora gilmorei FN547604
Scutellospora gilmorei FN547611
Scutellospora gilmorei FN547599
Scutellospora gilmorei FN547602
Scutellospora gilmorei FN547615
Scutellospora gilmorei FN547605
Scutellospora gilmorei FN547614
Scutellospora gilmorei FN547598
Scutellospora gilmorei FN547616
Scutellospora gilmorei FN547608
Scutellospora gilmorei FN547610
Cetraspora sp. CS048-03 (Site3-Fl-Soil)
Cetraspora sp. CS048-06 (Site3-Fl-Soil)
Cetraspora sp. CS034-01 (Site3-Fl-Soil)
Cetraspora sp. CS049-03 (Site2-Fl-Soil)
Cetraspora nodosa FM876834
Cetraspora nodosa FM876836
Cetraspora nodosa FM876833
Cetraspora sp. FR772331
Cetraspora sp. FR772333
Cetraspora sp. FR772332
Cetraspora sp. GT009-20 (Site4-Fl-Roots)
Cetraspora sp. GT009-01 (Site4-Fl-Roots)
Cetraspora nodosa FM876835
Cetraspora sp. GT009-12 (Site4-Fl-Roots)
Cetraspora sp. GT009-14 (Site4-Fl-Roots)
Cetraspora sp. CS069-06 (Site1-Sen-Roots)
Sp 13
(Ce. nodosa)
Scutellospora heterogama FR750159
Scutellospora heterogama FR750161
Scutellospora heterogama FR750164
Scutellospora heterogama FR750165
Scutellospora dipapillosa FR750016
Scutellospora dipapillosa FR750013
Scutellospora dipapillosa FR750015
Scutellospora dipapillosa FR750012
Scutellospora dipapillosa FR750014
Scutellospora dipapillosa FR750018
Scutellospora heterogama FR750163
Scutellospora heterogama FR750158
Scutellospora heterogama FR750160
Scutellospora heterogama FM876837
Scutellospora heterogama FM876838
Scutellospora heterogama FM876839
Scutellospora heterogama FR750167
Scutellospora heterogama FR750166
Scutellospora dipapillosa FR750019
Scutellospora heterogama FR750162
Gigaspora rosea FN547572
Gigaspora rosea FN547583
Gigaspora rosea FN547579
Gigaspora rosea FN547591
Gigaspora rosea FR750176
Gigaspora rosea FN547585
Gigaspora rosea FN547582
Gigaspora rosea FR750175
Gigaspora rosea FN547578
Gigaspora rosea FN547593
Gigaspora rosea FN547576
Gigaspora rosea FN547588
Gigaspora rosea FN547589
Gigaspora rosea FN547590
Gigaspora rosea FR750180
Gigaspora rosea FR750184
Gigaspora rosea FR750179
Gigaspora rosea FR750185
Gigaspora rosea FN547592
Gigaspora rosea FR750177
Gigaspora rosea FR750174
Gigaspora rosea FR750183
Gigaspora rosea FN547584
Gigaspora rosea FN547580
Gigaspora rosea FN547596
Gigaspora rosea FN547586
Gigaspora rosea FN547581
Gigaspora rosea FN547573
Gigaspora rosea FN547597
Gigaspora rosea FN547594
Gigaspora rosea FN547574
Gigaspora rosea FR750181
Gigaspora rosea FR750178
Gigaspora species FM876802
Gigaspora species FM876799
Gigaspora rosea FN547575
Gigaspora rosea FN547595
Gigaspora rosea FN547587
Gigaspora rosea FN547577
Gigaspora rosea FN547571
Gigaspora rosea FR750182
Gigaspora margarita FN547548
Gigaspora margarita FN547550
Gigaspora margarita FN547556
Gigaspora margarita FN547551
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Gigaspora margarita FN547552
Gigaspora margarita FN547559
Gigaspora margarita FR750043
Gigaspora margarita FN547564
Gigaspora margarita FR750044
Gigaspora margarita FR750039
Gigaspora margarita FR750041
Gigaspora margarita FN547555
Gigaspora margarita FN547567
Gigaspora margarita FN547547
Gigaspora margarita FN547554
Gigaspora margarita FN547566
Gigaspora margarita FN547553
Gigaspora margarita FR750040
Gigaspora margarita FN547562
Gigaspora margarita FR750045
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Gigaspora margarita FN547557
Gigaspora margarita FN547570
Gigaspora margarita FN547561
Gigaspora margarita FN547560
Scutellospora calospora JF439140
Scutellospora spinosissima (unpublished)
Scutellospora spinosissima (unpublished)
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Scutellospora spinosissima FR750150
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Scutellospora spinosissima (unpublished)
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Scutellospora spinosissima (unpublished)
Scutellospora spinosissima (unpublished)
Acaulospora scrobiculata FR692354
Acaulospora scrobiculata FR692352
Acaulospora scrobiculata FR692353
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Acaulospora scrobiculata FR692349
Acaulospora scrobiculata FR692350
Acaulospora spinosa FR750151
Acaulospora spinosa FR750156
Acaulospora spinosa FR750153
Acaulospora spinosa FR750154
Acaulospora spinosa FR750155
Acaulospora spinosa FR750152
Acaulospora kentinensis FM876825
Acaulospora kentinensis FM876826
Acaulospora kentinensis FN547522
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Acaulospora kentinensis FN547520
Acaulospora kentinensis FM876828
Acaulospora kentinensis FM876829
Acaulospora mellea JF439091
Acaulospora mellea JF439090
Acaulospora delicata JF439093
Acaulospora mellea JF439089
Acaulospora delicata JF439203
Acaulospora sp. GT001-901 (Site4-Em-Roots)
Acaulospora sp. GT001-751 (Site4-Em-Roots)
Acaulospora sp. GT001-231 (Site4-Em-Roots)
Acaulospora sp. GT001-21 (Site4-Em-Roots)
Acaulospora sp. GT001-04 (Site4-Em-Roots)
Acaulospora sp. GT001-03 (Site4-Em-Roots)
Acaulospora sp. GT001-81 (Site4-Em-Roots)
Acaulospora sp. GT001-01 (Site4-Em-Roots)
Sp 15
Acaulospora sp. CS050-10 (Site4-Sen-Soil)
Acaulospora sp. CS064-04 (Site2-Sen-Roots)
Acaulospora sp. CS064-01 (Site2-Sen-Roots)
Acaulospora sp. GT010-23 (Site3-Fl-Roots)
Acaulospora sp. CS054-06 (Site1-Fl-Soil)
Acaulospora sp. CS031-01 (Site1-Fl-Soil)
Acaulospora sp. CS038-01 (Site1-Fl-Soil)
Acaulospora sp. CS054-04 (Site1-Fl-Soil)
Acaulospora sp. CS054-02 (Site1-Fl-Soil)
Acaulospora sp. CS046-02 (Site1-Em-Soil)
Acaulospora sp. CS046-04 (Site1-Em-Soil)
Acaulospora sp. CS042-01 (Site1-Em-Soil)
Sp 14
Acaulospora cavernata FM876788
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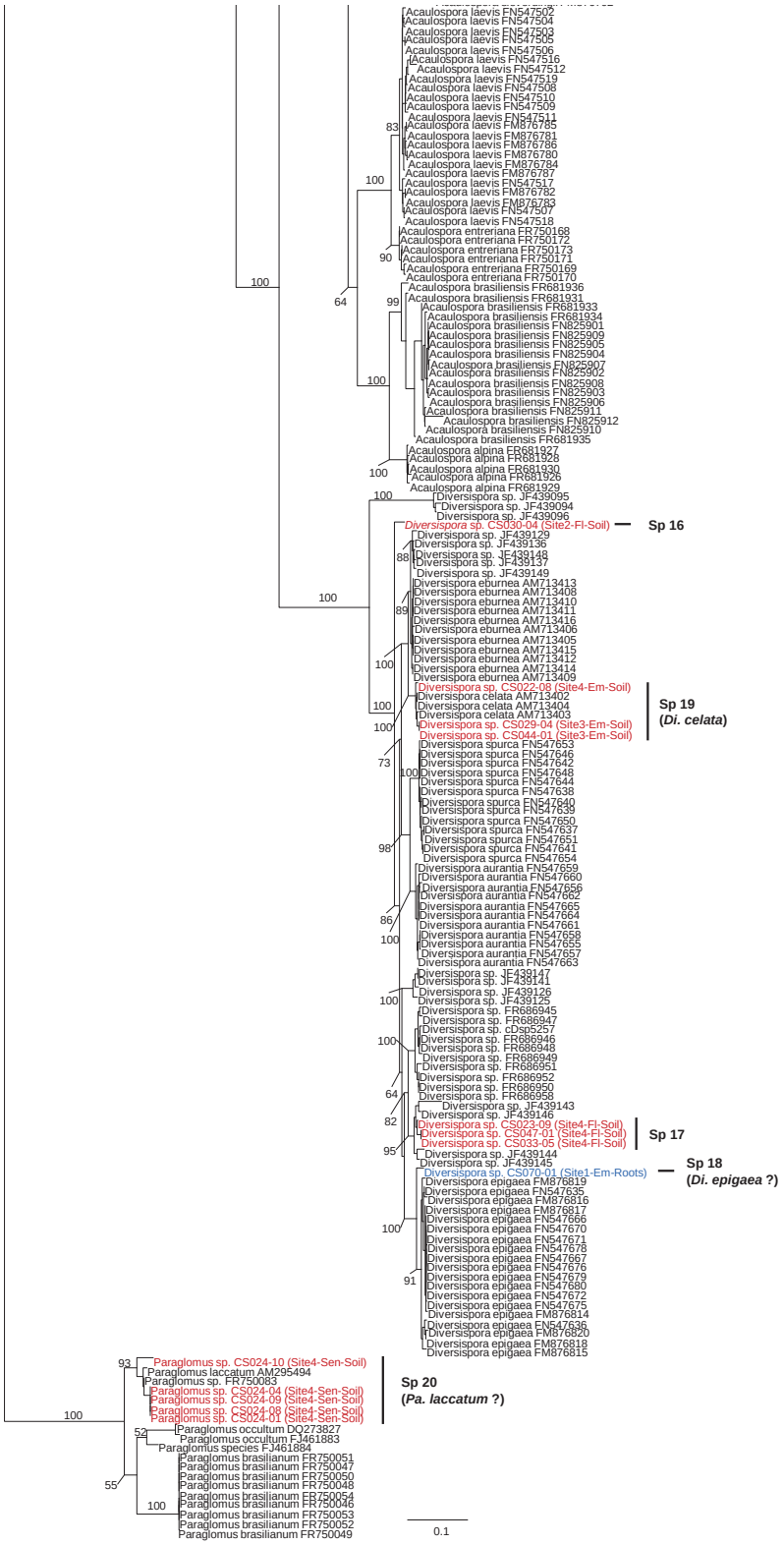
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Acaulospora entreriana FR750173
Acaulospora entreriana FR750171
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Acaulospora brasiliensis FR681936
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Acaulospora brasiliensis FN825910
Acaulospora brasiliensis FR681935
Acaulospora alpina FR681927
Acaulospora alpina FR681928
Acaulospora alpina FR681930
Acaulospora alpina FR681926
Acaulospora alpina FR681929
Diversispora sp. JF439095
Diversispora sp. JF439094
Diversispora sp. JF439096
Diversispora sp. CS030-04 (Site2-Fl-Soil)
Sp 16
Diversispora sp. JF439129
Diversispora sp. JF439136
Diversispora sp. JF439148
Diversispora sp. JF439137
Diversispora sp. JF439149
Diversispora eburnea AM713413
Diversispora eburnea AM713408
Diversispora eburnea AM713410
Diversispora eburnea AM713411
Diversispora eburnea AM713416
Diversispora eburnea AM713406
Diversispora eburnea AM713405
Diversispora eburnea AM713415
Diversispora eburnea AM713412
Diversispora eburnea AM713414
Diversispora eburnea AM713409
Diversispora sp. CS022-08 (Site4-Em-Soil)
Diversispora celata AM713402
Diversispora celata AM713404
Diversispora celata AM713403
Diversispora sp. CS029-04 (Site3-Em-Soil)
Diversispora sp. CS044-01 (Site3-Em-Soil)
Sp 19
(Di. celata)
Diversispora spurca FN547653
Diversispora spurca FN547646
Diversispora spurca FN547642
Diversispora spurca FN547648
Diversispora spurca FN547644
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Diversispora aurantia FN547659
Diversispora aurantia FN547660
Diversispora aurantia FN547656
Diversispora aurantia FN547662
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Diversispora aurantia FN547661
Diversispora aurantia FN547658
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Diversispora aurantia FN547657
Diversispora aurantia FN547663
Diversispora sp. JF439147
Diversispora sp. JF439141
Diversispora sp. JF439126
Diversispora sp. JF439125
Diversispora sp. FR686945
Diversispora sp. FR686947
Diversispora sp. cDsp5257
Diversispora sp. FR686946
Diversispora sp. FR686948
Diversispora sp. FR686949
Diversispora sp. FR686951
Diversispora sp. FR686952
Diversispora sp. FR686950
Diversispora sp. FR686958
Diversispora sp. JF439143
Diversispora sp. JF439146
Diversispora sp. CS023-09 (Site4-Fl-Soil)
Diversispora sp. CS047-01 (Site4-Fl-Soil)
Diversispora sp. CS033-05 (Site4-Fl-Soil)
Sp 17
Diversispora sp. JF439144
Diversispora sp. JF439145
Diversispora sp. CS070-01 (Site1-Em-Roots)
Sp 18
(Di. epigaea ?)
Diversispora epigaea FM876819
Diversispora epigaea FN547635
Diversispora epigaea FM876816
Diversispora epigaea FM876817
Diversispora epigaea FN547666
Diversispora epigaea FN547670
Diversispora epigaea FN547671
Diversispora epigaea FN547678
Diversispora epigaea FN547667
Diversispora epigaea FN547676
Diversispora epigaea FN547679
Diversispora epigaea FN547680
Diversispora epigaea FN547672
Diversispora epigaea FN547675
Diversispora epigaea FM876814
Diversispora epigaea FN547636
Diversispora epigaea FM876820
Diversispora epigaea FM876818
Diversispora epigaea FM876815
Paraglomus sp. CS024-10 (Site4-Sen-Soil)
Paraglomus laccatum AM295494
Paraglomus sp. FR750083
Paraglomus sp. CS024-04 (Site4-Sen-Soil)
Paraglomus sp. CS024-09 (Site4-Sen-Soil)
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Paraglomus sp. CS024-01 (Site4-Sen-Soil)
Sp 20
(Pa. laccatum ?)
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Paraglomus species FJ461884
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Paraglomus brasilianum FR750047
Paraglomus brasilianum FR750050
Paraglomus brasilianum FR750048
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Paraglomus brasilianum FR750052
Paraglomus brasilianum FR750049
0.1

10.3 Supplementary data – Chapter 6

The following data are supplementary material for the publication **A conserved AM fungal core-species community colonizes potato roots in the Andes.**

Sample name	Country	Province	Coordinates	Average temperature (°C) - high/low	Altitude (mamsl)	Cultivar	Field size (m^2)	pH
B1	Bolivia	Cochabamba	17°28' 48.1''S 65°38' 36.5''W	24.5/7.8	3560	Waycha	X	X
B2	Bolivia	Cochabamba	17°9' 51.7''S 66°5' 3.8''W	24.5/7.8	3700	Waycha	X	X
B3	Bolivia	Cochabamba	17°26' 57.7''S 65°36' 56.7''W	24.5/7.8	4007	Waycha	X	X
B4	Bolivia	La Paz	17° 18' 47.3"S 62° 07' 31.3"W	13/1	4070	Waycha	X	X
E1	Ecuador	Loja	3°32'03.7''S 79°13'17.2''W	22.9/6.8	2759	Fripapa	X	4.45
E2	Ecuador	Loja	3°32'21.8''S 79°13'32.6''W	22.9/6.8	2676	Guata	X	4.40
E3	Ecuador	Azuay	3°20'15.9''S 79°09'25.4''W	20.3/9.2	3000	Superchola	X	4.49
E4	Ecuador	Cañar	2°37' 20.4''S 78°56' 4.7''W	20.3/9.2	3561	Superchola	X	4.07
P1	Peru	Tayacaja	12°14' 40.6''S 75°03' 03.9''W	20/3	4075	Yungay	1500	4.36
P2	Peru	Huancayo	11°53' 14.4''S 75°25' 05.1''W	17/0	3751	Yungay	1400	7.36
P3	Peru	Huancayo	12°01' 42.9''S 75°16' 02.7''W	17/0	3245	Yungay	1800	5.71
P4	Peru	Tarma	11°16' 02.2''S 75°06' 56.8''W	17/0	2658	Unica	1300	6.23

For fields marked with an X, information was not available

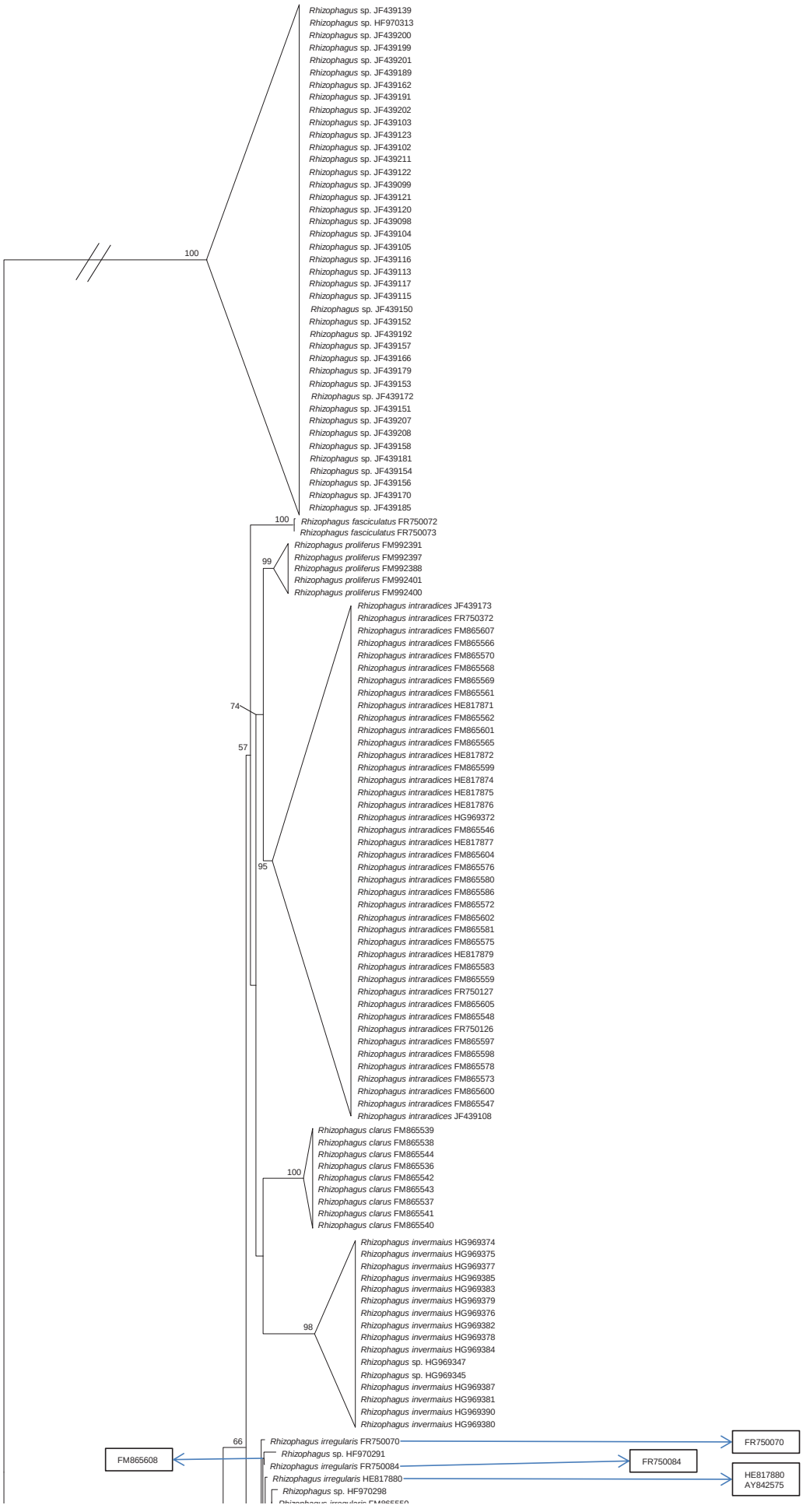
Online Resource 1: Description of edapho-climatic conditions of the study sites.

Soil properties												
EC dS/m	$CaCO_3$ (%)	Soil organic matter content (%)	N (%)	P (ppm)	K (ppm)	Texture	Sand	Silt	Clay	Ca^{+2}	Mg^{+2}	Na^{+}
X	X	X	X	X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X
X	X	X	X	X	X	X	X	X	X	X	X	X
0.47	0	9.01	X	7.2	275	X	56	26	18	7.07	1.72	0.27
0.35	0	5.19	X	11.9	242	X	50	32	18	6.22	1.2	0.46
0.20	0	13.40	X	4.0	275	Organic soil	X	X	X	5.17	1.09	0.42
0.25	0	31.70	X	7.0	95	Organic soil	X	X	X	0.96	0.18	0.37
0.62	0.00	7.00	0.41	64.5	418	Loam	46	46	8	X	X	X
0.82	19.40	2.28	0.14	50.5	236	Silt loam	32	54	14	X	X	X
1.13	0.00	2.98	0.28	56.0	268	Loam	40	40	20	X	X	X
1.82	0.10	2.34	0.18	61.7	253	Silt loam	40	52	8	X	X	X

Altitude category		
Category 4	> 4001 m	B3, B4, P1
Category 3	3561-4000 m	E4, B2, P2
Category 2	3001-3560 m	E3, P3, B1
Category 1	< 3000 m	E2, E1, P4

Online Resource 2: Description of the altitude groups and the sites that belong to each group.

Online Resource 3: Maximum-likelihood phylogenetic tree showing the EPA approach by using reference *Rhizophagus* sequences. Individual *R. irregularis* query sequences (approx. 760 bp) were placed in the tree branches by using EPA (shown by arrows). Query sequences that were placed in terminal nodes are marked with arrows starting at the name of the reference sequence. Reference numbers of the *R. irregularis* query sequences are shown in a box. *Paraglomus* sequences were used as outgroup.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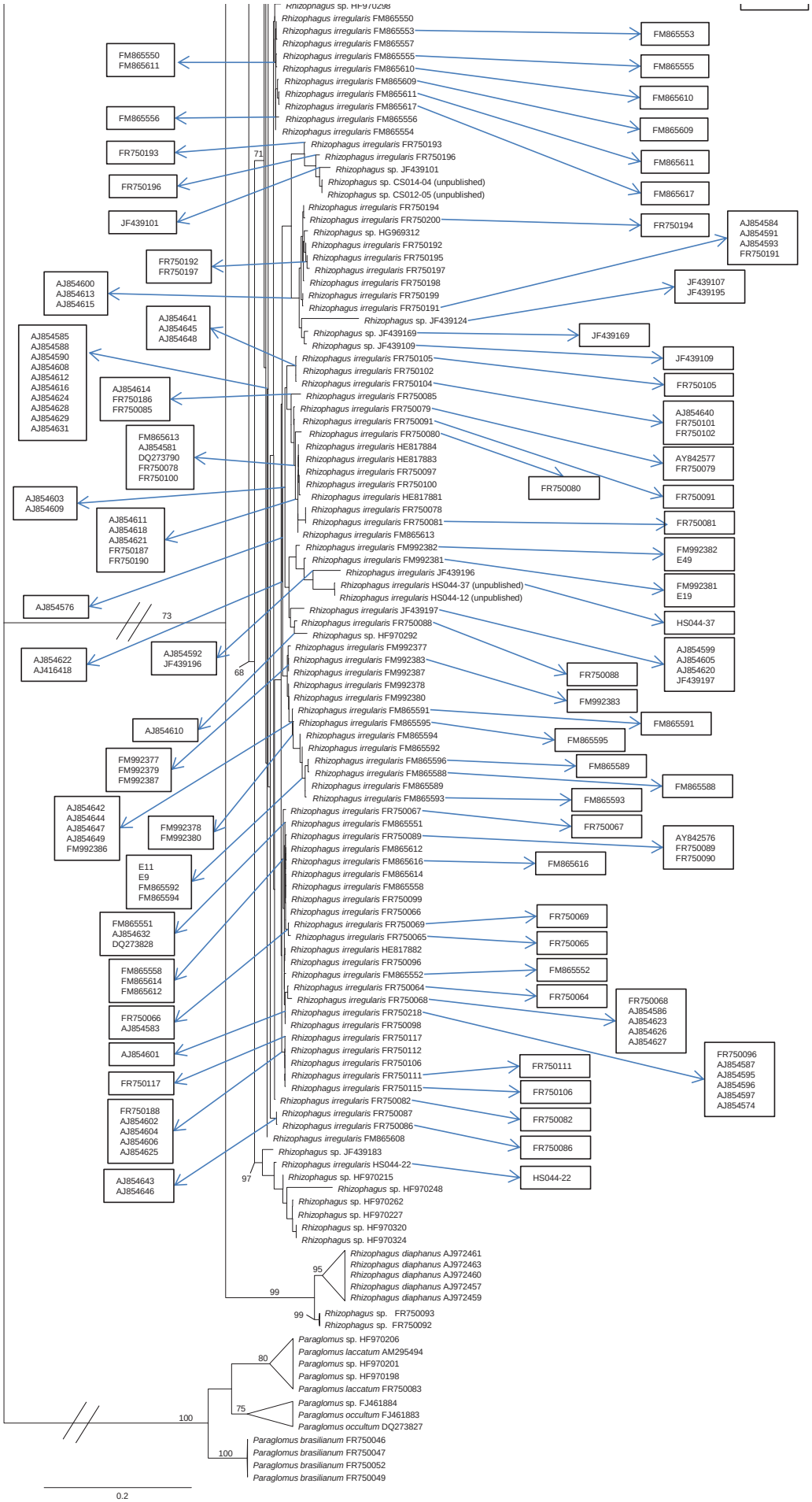
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71
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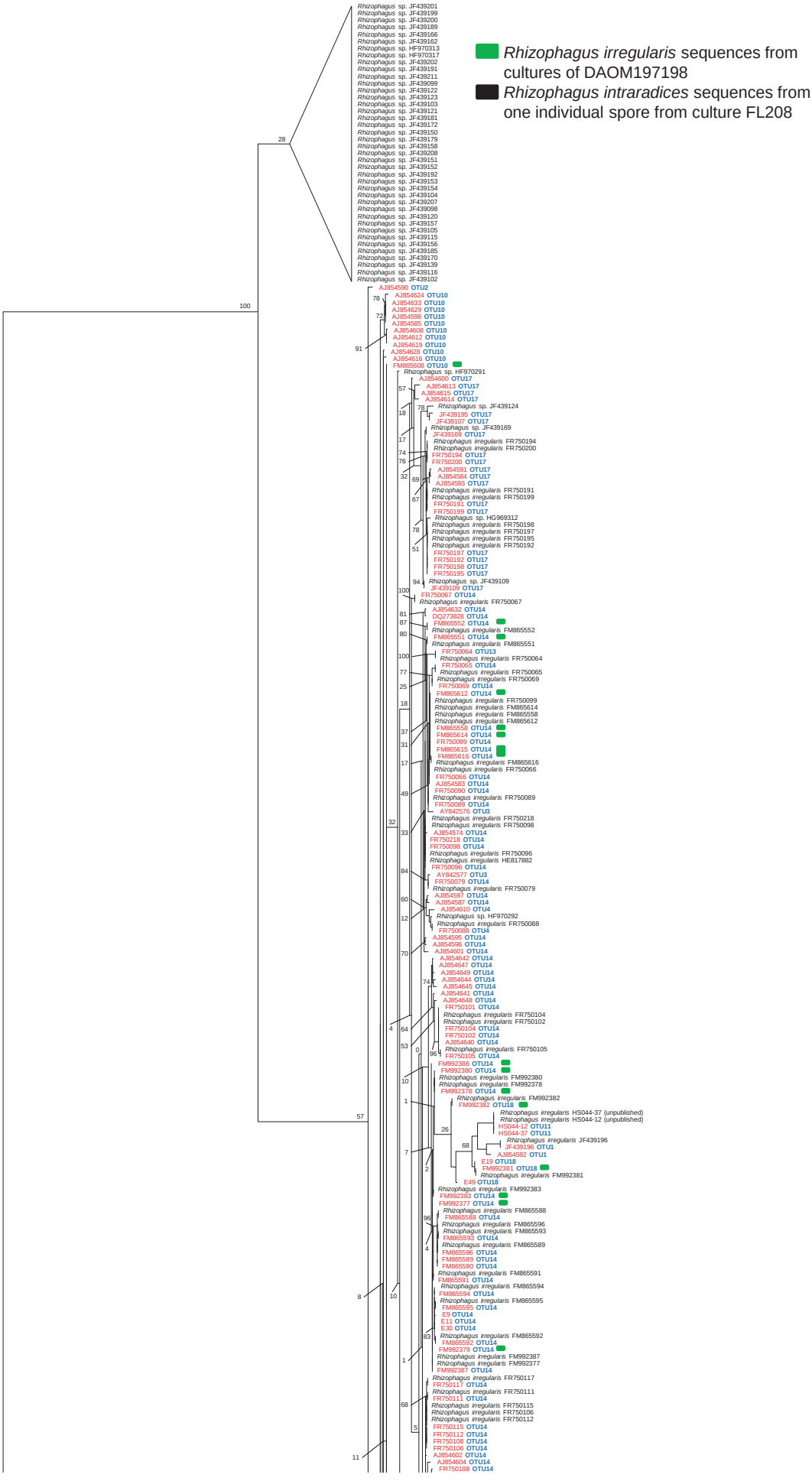
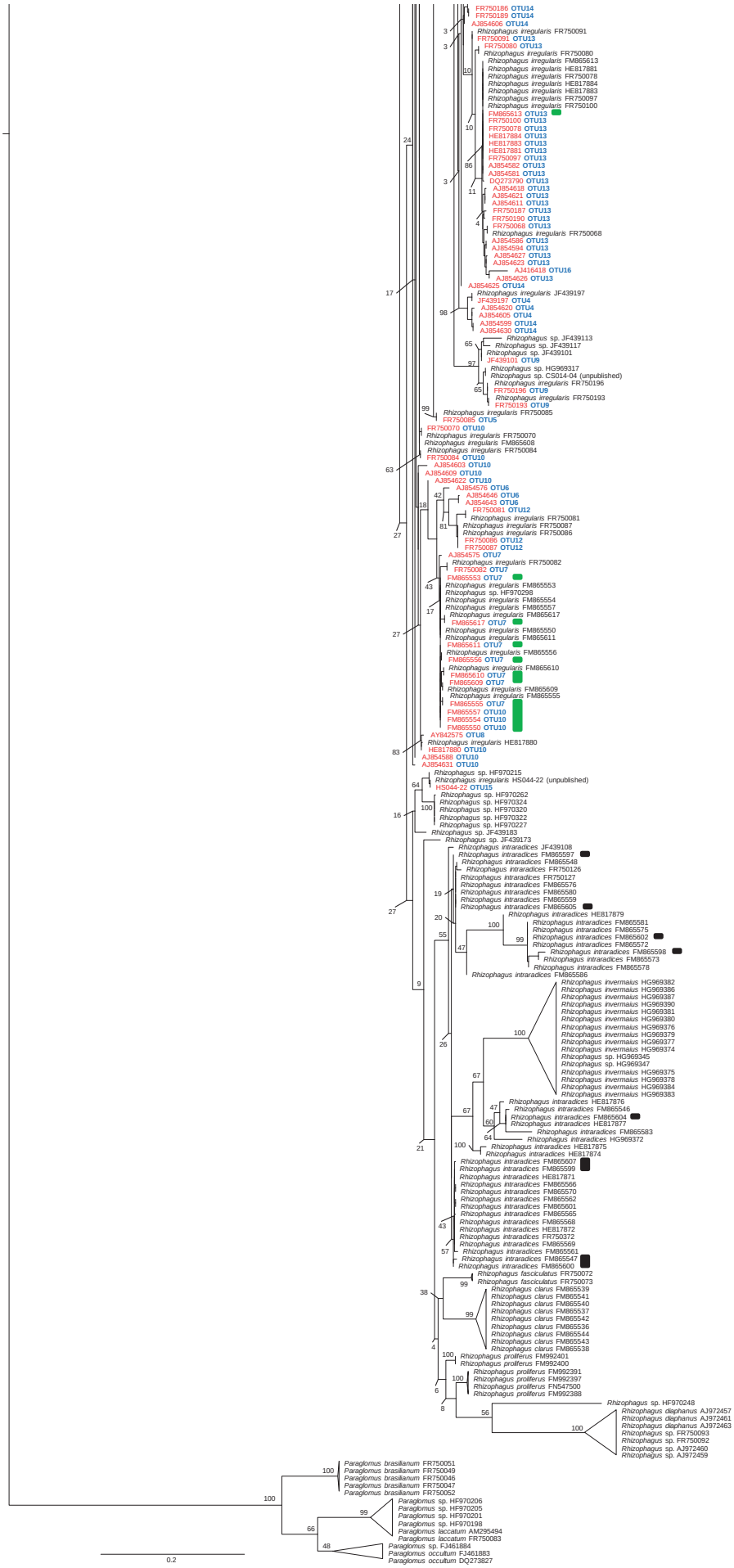
0.2

Online Resource 4: Maximum-likelihood phylogenetic tree showing results of the 97%-OTUs and the monophyletic clade approach. *Rhizophagus* together with the query sequences were used to compute the tree. A sequence similarity threshold of 97% was used to cluster the query sequences into 18 OTUs, marked in blue. Query sequences and their reference number are marked in red. Sequence variants belonging to isolates of *R. irregularis* DAOM 197198 or a single spore of *R. intraradices* FL208 are marked by colored squares. *Paraglomus* sequences were used as outgroup.


Rhizophagus irregularis sequences from cultures of DAOM197198
Rhizophagus intraradices sequences from one individual spore from culture FL208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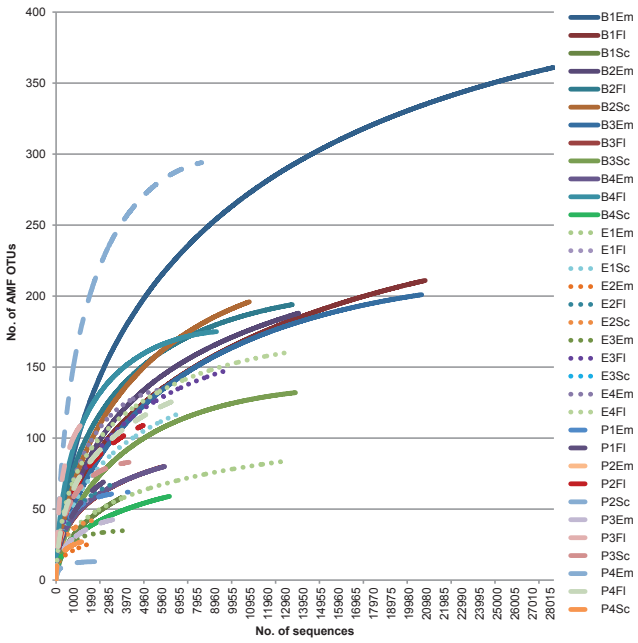
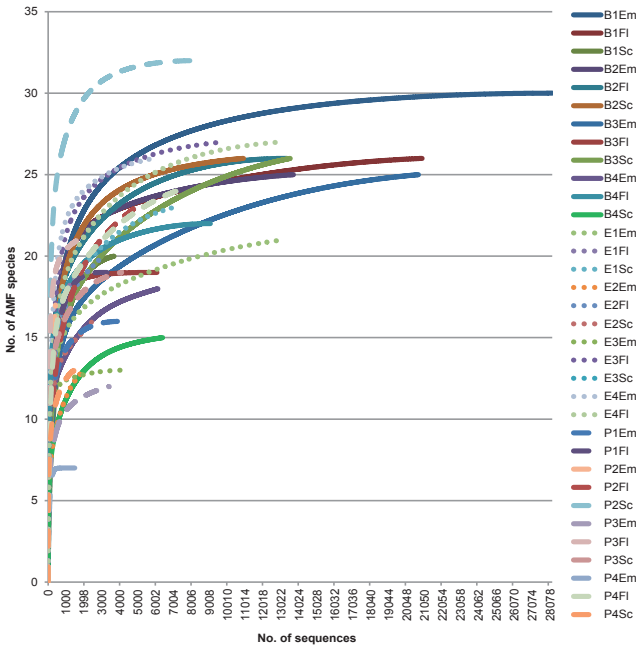
0.2

a)



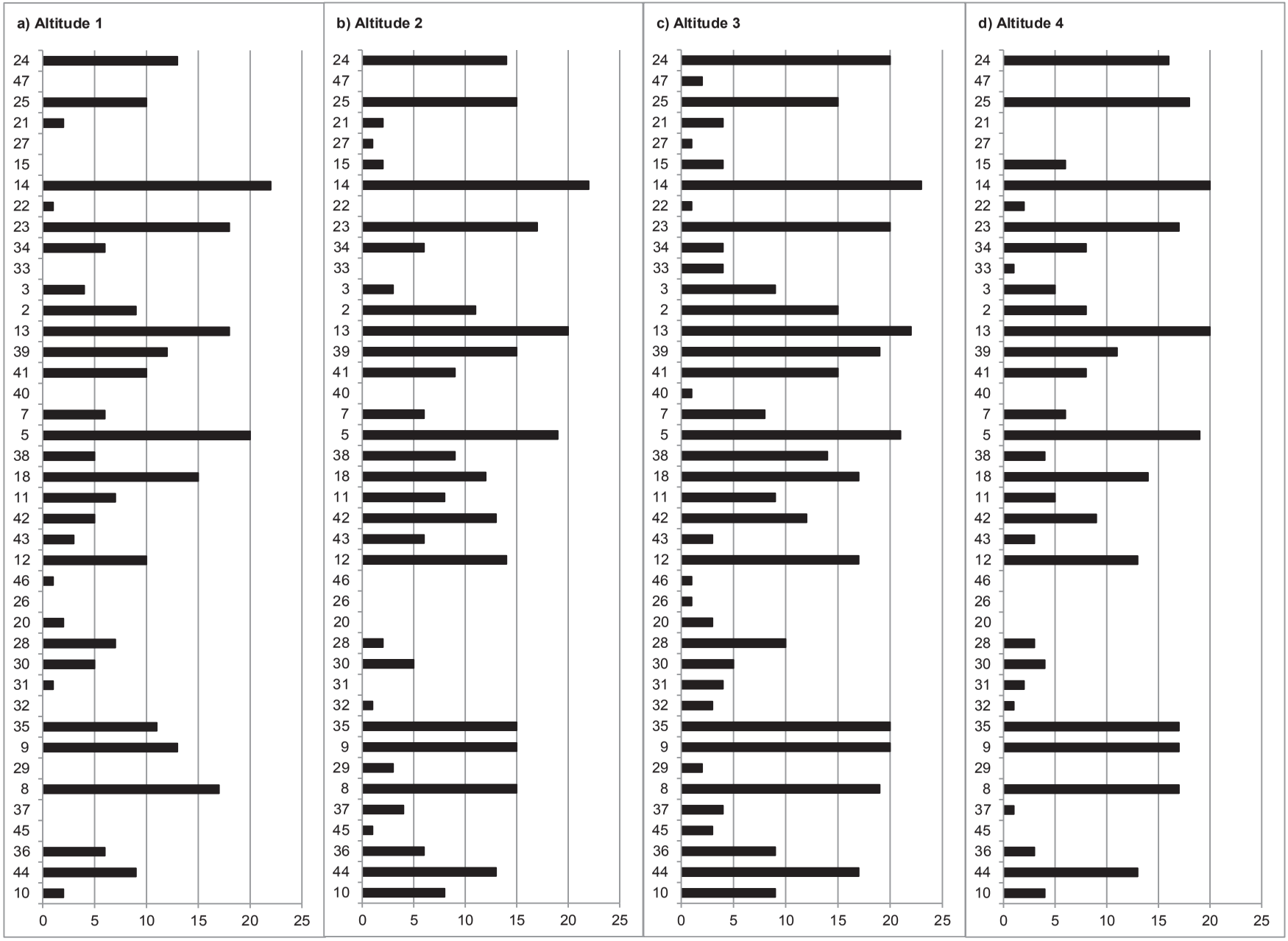
b)



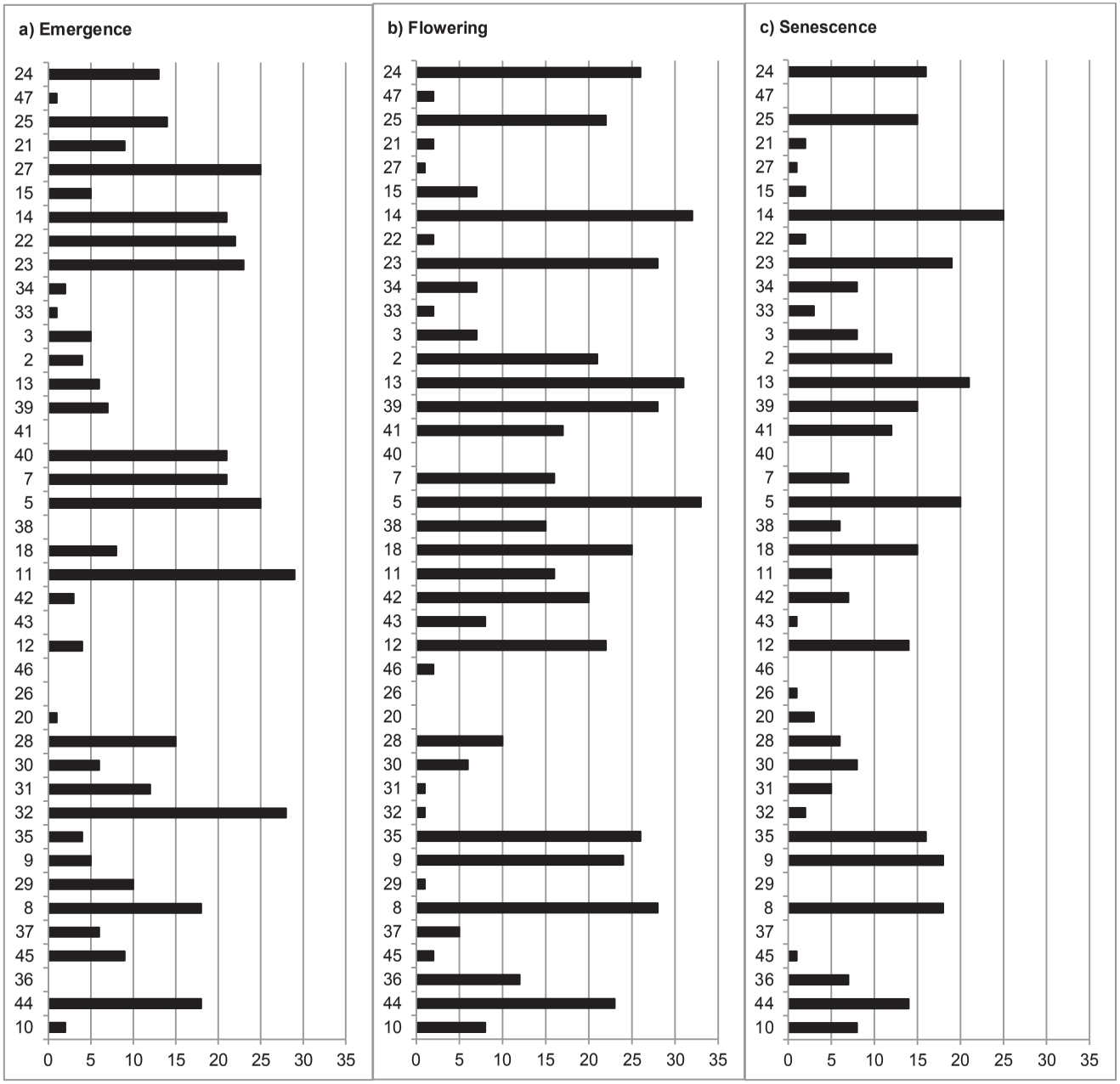
Online Resource 5: Rarefaction curves of the amount of AMF OTUs (a) and species (b).

Online Resource 7: Number of individual root samples in which 41 annotated AMF species were found at different altitudes categories and plant stages.

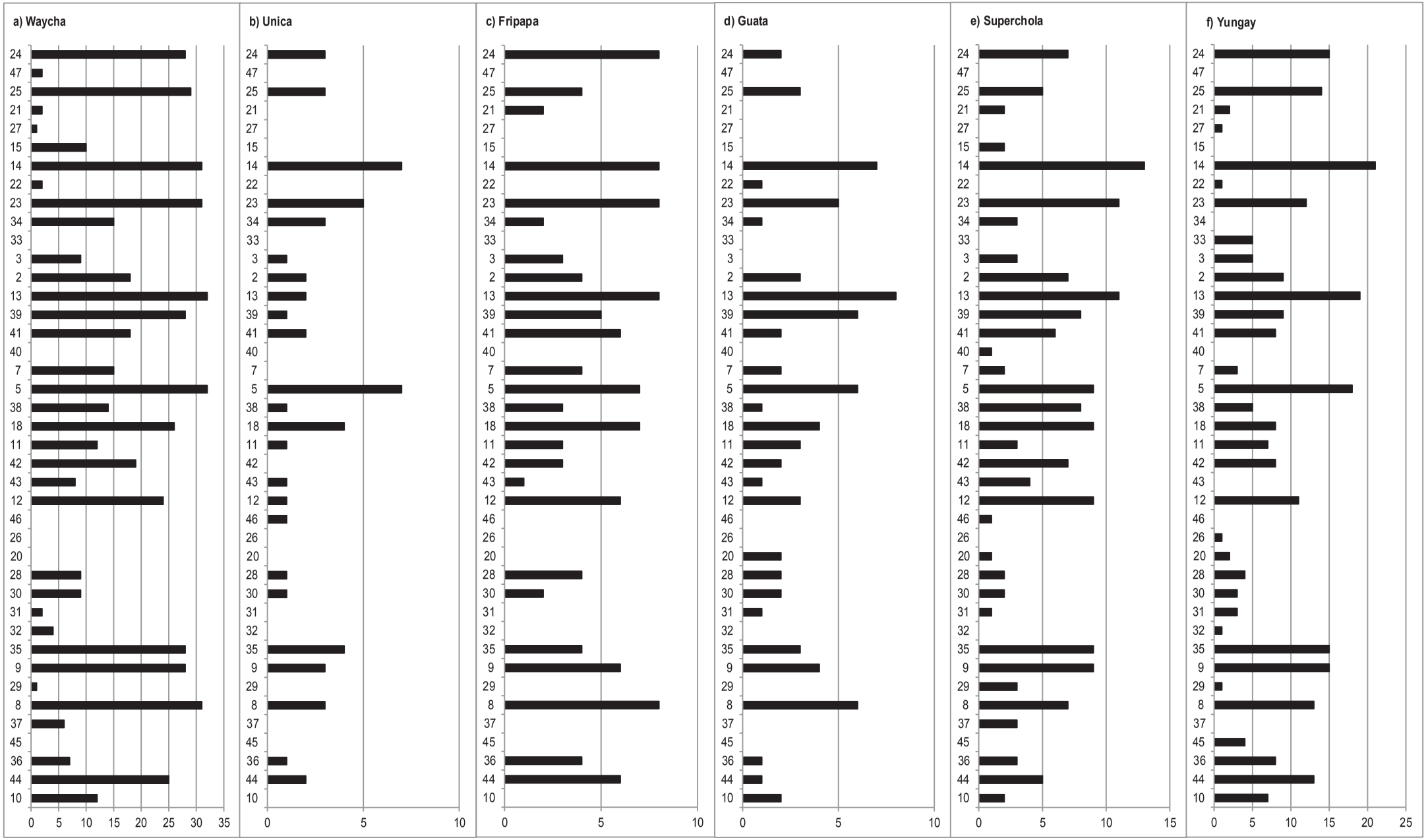
a)


a) Altitude 1
b) Altitude 2
c) Altitude 3
d) Altitude 4
24
47
25
21
27
15
14
22
23
34
33
3
2
13
39
41
40
7
5
38
18
11
42
43
12
46
26
20
28
30
31
32
35
9
29
8
37
45
36
44
10
0
5
10
15
20
25

b)


a) Emergence
b) Flowering
c) Senescence
24
47
25
21
27
15
14
22
23
34
33
3
2
13
39
41
40
7
5
38
18
11
42
43
12
46
26
20
28
30
31
32
35
9
29
8
37
45
36
44
10
0
5
10
15
20
25
30
35

c)


a) Waycha
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10 15 20 25 30 35
b) Unica
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10
c) Fripapa
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10
d) Guata
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10
e) Superchola
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10 15
f) Yungay
24 47 25 21 27 15 14 22 23 34 33 3 2 13 39 41 40 7 5 38 18 11 42 43 12 46 26 20 28 30 31 32 35 9 29 8 37 45 36 44 10
0 5 10 15 20 25

	Degrees of freedom	*F*-value	R^2	*P*-value
Altitude	3	1.5887	0.1322	**0.043**
Stage	2	1.6421	0.0911	0.061
Residuals	28		0.7767	
Total	33		1.000	

Online Resource 8: Influence of altitude and plant stage on the beta-diversity of the AMF communities annotated as species. P-values are based on 999 permutations.

11. Acknowledgements

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Quiero dar las gracias a mi familia y amigos por su apoyo a pesar de la distancia.
Mi tesis está dedicada a Juan, Adriana y Rafaela.

12. Curriculum vitae

Carolina Senés Guerrero

Date of birth:	11.01.1984
Place of birth:	Mexico

Education

01/2003-07/2007	Bachelor in Chemistry, Bacteriology and Parasitology. Autonomous University of Nuevo Leon (UANL, Mexico). Thesis: Determination of the prevalence of infection with hepatitis C virus in patients infected with HIV-1 and analysis of polymorphisms associated to the co-infection. Degree: Bachelor degree.
01/2008-12/2009	Master in Science in Biotechnology. Monterrey Institute of Technology (ITESM, Mexico). Thesis: Acclimatization of a microalgae environmental sample to high concentrations of CO_2 and description of its associated bacteria populations. Degree: Master in Science in Biotechnology.
09/2010-08/2014	Doctoral studies. Ludwig-Maximilians University (LMU, Munich) Thesis: DNA-based characterization of arbuscular mycorrhizal fungi associated to potato roots from the Andean region.