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Phylogenetic relationships among arbuscular mycorrhizal fungal species in the deeply rooted archaeosporales (Glomeromycota)

Robert J. Bills

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**PHYLOGENETIC RELATIONSHIPS AMONG ARBUSCULAR MYCORRHIZAL
FUNGAL SPECIES IN THE DEEPLY ROOTED ARCHAEOSPORALES
(GLOMEROMYCOTA)**

Robert J. Bills

**Dissertation submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Agricultural Sciences**

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ABSTRACT

PHYLOGENETIC RELATIONSHIPS AMONG ARBUSCULAR MYCORRHIZAL FUNGAL SPECIES IN THE DEEPLY ROOTED ARCHAEOSPORALES (GLOMEROMYCOTA)

Robert J. Bills

Arbuscular mycorrhizal fungi (AMF) in the phylum *Glomeromycota* are globally distributed biotrophic fungi that form symbiotic associations with a majority of terrestrial plants. Their global beneficial role in plant and soil ecology warrants a better understanding of their evolutionary history and taxonomic relationships. This project focused on species relationships in two deeply rooted families *Ambisporaceae* and *Archaeosporaceae*. In one study, analysis of morphology and 28S ribosomal DNA (LSU) sequences resulted in two major changes that redefined *Ambispora* to include only species that were dimorphic for both acaulosporoid and glomoid spore types. First, species described as producing only glomoid spores (*Am. leptoticha*, *Am. fecundispora*, and *Am. callosa*), only acaulosporoid spores (*Am. jingerdemannii*), or both spore morphotypes (*Am. appendicula*) were synonymized with *Ambispora leptoticha*. LSU and more conserved 18S (SSU) sequences indicated little divergence between species. Second, *Ambispora fennica* was synonymized with *Ambispora gerdemannii* based on morphological and LSU sequence variation equivalent to that in the sister clade *Am. leptoticha*. With this analysis, *Ambispora* was reduced to three species: *Am. leptoticha*, *Am. gerdemannii*, and *Am. granatensis*. Morphological and molecular characters were given equal treatment in this study, as each data set informed and clarified grouping and ranking decisions. The two inner layers of the acaulosporoid spore wall were the only structural characters uniquely defining each of these three species; all other characters were shared. Phenotypes of glomoid spores were indistinguishable between species, and thus were informative only at the genus level. Evolution of sporogenous saccule was a convergent trait because it also evolved in other AMF families. In a second study, morphology and LSU sequence variation were compared in the only two *Archaeospora* species, *Ar. trappei* and *Ar. schenckii*. Both species form a precursor sporogenous saccule, but spores develop laterally (acaulosporoid) or within (entrophosporoid) the subtending hypha, respectively. In all other morphological characters, the two species are identical. One *Ar. schenckii* and ten *Ar. trappei* strains grouped all taxa into one highly supported monophyletic clade. However, LSU sequences of *Ar. trappei* strains segregated into two subclades, with *Ar. schenckii* grouping in one of the *Ar. trappei* clades. This evidence indicates the entrophosporoid mode does not represent a speciation event, but rather is a stable dimorphism within one or more populations of a single species. Therefore, *Ar. schenckii* and *Ar. trappei* were considered conspecific and synonymized as *Ar. trappei*. The combination of molecular and morphological evidence exposed the entrophosporoid mode as a polymorphism that likely was fixed in a founder asexual population and then was heritable and stable in the absence of recombination or negative selection pressures. Spore dimorphism is not a rare event, occurring also in the previous study. LSU sequence divergence between the two *Ar. trappei* clades was greater than that within the clade containing *Ar. trappei* and *Ar. schenckii* strains. This distinct subclade structure suggests fixation of discrete variants typical of clonal reproduction and possible retention of polymorphisms in rDNA repeats. The broader implication from this result is that clade topology alone may not provide an adequate criterion for ranking at the species level.

Dedication

Evidence for evolution, a natural phenomena about patterns of descent from common ancestry and relatedness of “kinds”, speaks for itself. For those who overlooked it in the Bible:

“Then God said, ‘Let the earth bring forth grass, the herb that yields seed, and the fruit tree that yields fruit according to its kind, whose seed is in itself, on the earth’; and it was so. And the earth brought forth grass, the herb that yields seed according to its kind, and the tree that yields fruit, whose seed is in itself according to its kind. And God saw that it was good.”

Genesis 1: 11–12 (NKJV).

The evolution of plants, ancestor descendant relationships, brought forth from this earth, “according to its kind.”

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Chapter 1

Literature Review

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi in the phylum *Glomeromycota* (Schüßler *et al.* 2001) are globally distributed, asexual fungi that form obligate symbioses with the roots of the majority of terrestrial plants (Smith & Read 2008) and one non mycorrhizal species that forms an endosymbiosis with cyanobacterium (Schüßler 2002). Fossil evidence suggests that AM fungi have been symbiotic with terrestrial vegetation for at least 400 million years (Remy *et al.* 1994). The origins of this ancient association may have been a critical factor for the emergence of plants on to land. Their subsequent intimate coevolution (Pirozynski & Malloch 1975; Morton 1990; Redecker *et al.* 2000a) and global distribution makes their symbiotic association one of the most widespread in nature (Fitter 2005; da Silva *et al.* 2006; Kivlin *et al.* 2011).

Arbuscular mycorrhizal fungi establish a symbiotic relationship colonizing roots of plants (intra radical hyphae) and the surrounding soil (extra radical hyphae) in a reciprocal exchange of carbohydrates moving from the host to the fungus and inorganic resources moving from the fungus to the plant (Allen 1991; Smith & Read 2008). Extra-radical hyphae growing out past the depletion zone of the host rhizosphere are extremely efficient at scavenging inorganic phosphorus, nitrogen, copper, and zinc (Cui & Nobel 1992; Smith & Read 2008). In addition to well documented improvement to host nutrition, AMF have also been shown to improve resistance to root pathogens (Newsham *et al.* 1995), tolerance to drought (Augé 2004), and increase plant fitness (Janos *et al.* 1980; Koide 1991). AMF diversity has also been shown to differentially influence plant diversity and productivity (van der Heijden *et al.* 1998; Vogelsang *et al.* 2006), influence plant tolerance to herbivory (Bennet & Bever, 2007), provide tolerance to heavy metals (Toler *et al.* 2005), and provide aluminum resistance in acidic soils (Kelly *et al.* 2005; Klugh & Cumming 2007).

Ecosystem factors that influence the diversity and distribution of AMF species have included investigations of edaphic characters and plant community composition (Lekberg *et al.* 2007; Antoninka *et al.* 2015; Krüger *et al.* 2015, Davison *et al.* 2015). These investigations included differences in soil type, soil pH, soil nutrient levels, land-use history, and the composition of the plant community at a variety of scales.

Species Concepts

One of the oldest problems in biology is the absence of a universally accepted definition of species, the basal category in the Linnaean taxonomic hierarchy (De Queiroz 1988). A species is a collection of populations delimited by diagnosable conserved traits that are stable across time and space (Mishler 1985; Morton 1990; Baum 1992; Davis & Nixon 1992; Davis 1996; Mishler & Theriot 2000). As units of evolution, members evolve together collectively because of shared selection pressure(s) or a common developmental constraint (Ereshefsky 1991), gene flow (Mayer 1942) or other biological process unique to the organisms being investigated (Mishler 1985). All species concepts explore a theory of a process of speciation and consist of two separate classification processes of grouping and ranking taxa (Mishler & Theriot 2000). Disagreements over how to conceptually define ranking and grouping criteria, both morphologically and molecularly, can lead to conflicting taxonomies, which can cause misinterpretation of phylogenetic patterns and processes (Ereshefsky 1991).

The number of species concepts developed by biologists is inexplicably large and varied (Coyne & Orr 2004). The species concept used for this study was based on the phylogenetic species concept. The phylogenetic species concept was first proposed by Joel Cracraft in 1983 to incorporate evolutionary processes into an explanation for the origin and maintenance of species (Cracraft 1983). The theoretical basis for this concept was that each species originates via an ancestor-descendant relationship regardless of a taxon's biology or ecology. Grouping criteria was based on monophyly, where taxa form clades based on relatedness of shared unique, homologous characters. Ranking criteria were based on congruence between all informative character sets that establish inclusiveness among members. The phylogenetic species concept is the most appropriate for delimiting asexual organisms, including AMF species, because evolution is a universal process. Phylogenetic methods are applicable to all characters, monophyly is applicable to all living organisms, and there is only one unique causal force for any given species, which serves to unite the taxon (Horvath 1997).

Testing for homology is different for morphological and molecular characters. Morphological characters are tested for homology by evaluating similarity in ontogeny, tree topography and composition and conjunction (co-occurring structures in the same organism) and congruence of phylogenetic characters (Patterson 1982; Patterson 1988; de Pinna 1991). None of these tests are available for molecular characters and so statistical algorithms have been

developed to assess if nucleotide and amino acid similarity is greater than might be anticipated by chance alone (Patterson 1988).

Analysis of molecular characters often starts with an alignment containing gene sequences from multiple taxa with an assumption that nucleotide position in one sequence is matched to homologous positions in the other. A distinction between orthologous sequences (latest common ancestry through gene divergence) and paralogous sequences (latest ancestry through gene duplication) must also be made (Patterson 1988; Olsen & Woese 1993; Baum & Smith 2013). Coyne & Orr (2004) state that the evolutionary history of each gene is unique. Although a gene phylogeny may provide a history of gene evolution that can be correlated to the evolution of a species, it may conflict with the phylogeny of a different gene. Caution is required to distinguish gene phylogenies (which represent evolution of a specific gene at one scale) with species phylogenies (evolution of all the characters of a species) (Pamilo & Nei 1988). The basal units of evolution are species, each of which is distinguished by discrete characters that identify unique common ancestry (Patterson 1982). All independent morphological and molecular character sets (gene phylogenies) that inform on the continuity of descent need to be in congruence to determine a true species phylogeny (Coyne & Orr 2004; Morton 2009).

Taxonomy and systematics of AMF

The taxonomy and systematics of AM fungi continue to be contentious as efforts to characterize taxa at a variety of levels have failed to achieve a biologically informative operational classification (versus a consensus classification) that has universal acceptance among researchers (Morton 2009; Oehl *et al.* 2011; Redecker *et al.* 2013). Redecker *et al.* (2013) recently published a revised monophyletic classification of the Glomeromycota based on the collaborative efforts of a group of systematists experienced in the biology and taxonomy of AM fungi (Fig 1). The reconstruction of evolutionary history and identification of ancestor/descendant relatedness among taxa helps organize a classification system of inclusive hierarchal groups from the least inclusive group (species) to the most inclusive (Phylum).

Mycorrhizal structures may appear to have similar structures and functions at all ranks in a classification and among species. During analysis, these must be identified as either homologous or analogous. Homology is a hypothesis of similarity due to descent from a common ancestor (Patterson 1988). Therefore, homologous characters are phylogenetically

informative because they define monophyletic groups (Patterson 1982; De Queiroz 1988). In contrast, analogous characters are similar due to independent evolution and are not considered to be derived from a recent common ancestor. Analogous characters have different evolutionary histories, so they create “noise” that can complicate phylogenetic analyses.

Efforts to characterize the fungal component of the mycorrhizal association functionally, taxonomically, ecologically, and by molecular analysis have been hampered by the absence of a universally accepted operational species concept (Røsendahl 2007; Redecker *et al.* 2014). Morton (1990) evaluated a variety of contemporary species concepts and their applicability to AM fungal evolutionary relationships. At that time he found that a combination of phenetic and evolutionary concepts provided a satisfactory method for defining the unique biological properties of these asexual fungi. Vegetative structures (arbuscules, vesicles) and biochemical features (staining reaction) in roots are highly variable and resolved only conflicts at the family level. Species level characters were important because they provided a more accurate assessment and explanation of the evolution and maintenance of the AM symbiosis (Morton 1990).

Morphology of AMF

Morphological characters that are stable and phylogenetically useful can be defined empirically from developmental patterns and processes for application to taxonomic problems. The identification of the initiation and termination of spore characters and the order and sequence of events of subcellular character differentiation and transformation can be hierarchically ordered and used for comparisons between individuals and other taxa. Comparative ontogeny has been described for species in *Scutellospora* (Franke & Morton 1994; Morton 1995), *Gigaspora* (Bentivenga & Morton 1995), *Glomus* (Stürmer & Morton 1997), and *Acaulospora* (Stürmer & Morton, 1999). Ontogenic interpretations (Franke and Morton 1994; Stürmer & Morton 1997, 1999) of the morphological features of AMF spores provide criteria to test for character homology and therefore help determine the phylogenetic significance of these characters (Morton 1988; Morton & Benny 1990). Mycorrhizal structure and organization, and mode of spore germination along with spore primary characters (i.e., spore wall, inner flexible walls, and germination shield) and secondary characters (layers that develop within each primary character) define genus and higher-level rankings. Mode of spore formation from a sporiferous saccule, which occurs early during spore ontogeny, has recently been shown to be a convergent trait that

discriminates at species level in some lineages (Kaonongbua *et al.* 2010) and as a population variant in others (Morton & Redecker 2001; Bills & Morton 2015). Tertiary characters, which contain all the variation that occurs within each layer (i.e., color, thickness, and ornamentation), define species (Franke & Morton 1994; Morton *et al.* 1995; Bills & Morton 2015).

AMF species

The use of spores and their subcellular structures to characterize species composition of AMF communities has some limitations (Morton *et al.* 1995). Some AMF species elude detection because they cannot be induced to sporulate in pot cultures. Often, spores collected from field-samples lack the complete range of phenotypic variation or are in poor condition due to degradation and senescence that diagnostic characters are missing or obscure (Morton 1993; Røsendahl 2007). Identification of spore diagnostic characters provides additional challenges. Adequate sampling and use of voucher specimens from many populations and closely related species is necessary to identify the range and stability of phenotypic variation. This helps reduce the misinterpretation of population level characters as discrete species level characters (Bills & Morton 2015). Due to the challenges and limitations of morphology-based identification, the advent of DNA analyses ushered in an era of new molecular tools that have been applied to classification of AMF.

The number of described AM fungal species continues to be a matter of debate due to the lack of a universally accepted species concept among researchers and a failure to identify suitable morphological and molecular criteria for grouping and ranking taxa (Redecker & Raab 2006; Morton 2009; Schüßler & Walker 2010; Redecker *et al.* 2013). Many recent taxonomic revisions by Oehl *et al.* (2011) have been criticized and rejected due evidence that analyses and interpretations were based on selective and poor data (Redecker *et al.* 2013). Historically, confusing and inconsistent morphological terminology used to describe spore wall characters (Walker 1983), the relatively low number of morphologically described and sequenced taxa, and the preferential use of rRNA genes for phylogenetic analyses all contribute to maintaining a confusing and contentious species concept (Morton 2009). Another issue is a necessity for accurate identification of homologous characters that can be used to group and rank AMF into clades as unique and discrete entities that are stable over time, regardless of environment (Morton 1993). The majority of biologists define species based on a lineage that maintains its

identity following a unique origin (Røsendahl 2007). Differences between AMF research groups on how to define the evolutionary significance of diagnostic spore wall characteristics confuses identification of novel characters that are needed to group and rank taxa. Contradictory phylogenies based solely on either morphological data or molecular analysis of SSU rDNA has also produced conflicting phylogenies that warrant further analyses (Walker & Schüßler 2004; Morton 2009; Schüßler & Walker 2010).

With the advent of tools for DNA analyses, additional controversies occurred when phenotypic patterns of relatedness were contradicted by molecular phylogenies (Morton & Benny 1990; Morton 2009). Morton & Benny (1990) erected two suborders, *Glomineae* and *Gigasporineae*, based on differences in the inner wall development, mycorrhizal structures, infectivity patterns of hyphal fragments, fatty acid chemistry and cell wall chemistry. Specifically, β (1-3) glucan were detected in Glomineae (species now identified from the genera *Rhizophagus*, *Funneliformis*, and *Acaulospora*) but not in *Gigasporineae* (*Gigaspora* and *Scutellospora*) (Gianinazzi-Pearson *et al.* 1994). Schüßler *et al.* (2001) SSU gene phylogeny showed a conflicting history reclassifying *Acaulospora* (*Acaulosporaceae*) as a sister clade to *Gigaspora* and *Scutellospora* (*Gigasporaceae*).

AMF protein gene phylogenies

AMF investigators pursued other genomic regions for single copy genes that could be phylogenetically informative. These studies included elongation factor 1-alpha (*EFL1*), actin (*act*), α -tubulin, β -tubulin, H⁺-ATPase, and two RNA polymerase II subunits (*RPB1*, *RPB2*) (Helgason *et al.* 2003; Corradi *et al.* 2004a, 2004b; James *et al.* 2006; Redecker & Raab 2006; Msiska & Morton 2009a, 2009b; Morton & Msiska 2010a, 2010b; Stockinger *et al.* 2014). All supported the rDNA phylogeny that the *Glomeromycota* are a monophyletic group. Most protein gene trees were also discordant with morphology based hypothesis and placed *Acaulosporaceae* and *Gigasporaceae* as sister clades. The presence of gene copy number polymorphisms and pseudo genes in some clades was also detected (Corradi *et al.* 2007; Msiska & Morton 2009a, 2009b). Other incongruent topologies at deeper nodes also revealed conflicting identity with a sister clade to *Glomeromycota* (Helgason *et al.* 2003; Corradi *et al.* 2004a, 2004b; James *et al.* 2006).

Ribosomal RNA genes

Ribosomal RNA genes are found in the DNA of all living organisms making them ideal candidates for testing hypotheses about species, evolution, and relatedness of life. The rDNA gene is the most utilized marker for phylogenetic reconstructions that included bacteria, archaea, plants, animals, and fungi including AMF (Sogin 1990; Olsen & Woese 1993; Buckler *et al.* 1997; Reddy *et al.* 2005; Sonnenberg *et al.* 2007; Røsendahl 2007). In eukaryotes, these genes are located in a nucleolus, a membraneless compartment within the nucleus. The cellular demand for ribosomes and their protein products required for cell function and growth require tandem arrays of multiple repeats of the rRNA gene (Olson & Dunder 2005; Eickbush & Eickbush 2007). These tandem arrays of repeats are located either on single or multiple chromosomes, which are collectively called the nucleolus organizer regions (NORs) and are the location of nucleolus formation (Fatica & Tollervey 2002; Dez & Tollervey 2004; Fromont-Racine *et al.* 2005; Raška *et al.* 2006; and references therein). The proximity rRNA gene repeats within the nucleolus during cell cycle may play a role in homogeneity observed in these genes.

AMF rDNA phylogenies

Simon *et al.* (1992) and Helgason *et al.* (1998) were among the earliest that developed PCR primers to amplify SSU rDNA from AM fungal colonized roots. However, these early primers failed to amplify sequences from all AMF groups or lacked AMF specificity leading to amplification of non-mycorrhizal organisms. Schüßler *et al.* (2001) overcome these challenges with near full-length SSU sequences. This provided evidence for monophyly of AMF and a new phylum, *Glomeromycota* (Schüßler *et al.* 2001). Current use of SSU gene phylogenies have been for identification of AMF community composition in roots in a variety of ecosystems (Öpik *et al.* 2009). The low level of DNA variation in SSU fragments, amplified with primers AM1-NS31, often lacked species level resolution (Stockinger *et al.* 2010). An inability to match all environmental sequences to well-defined AMF isolates led to phylogenetic reconstructions of sequence groups to be defined as virtual taxa (VT) (Öpik *et al.* 2009, 2013).

New primers targeting ITS region were developed (White *et al.* 1990; Gardes & Bruns 1993). The ITS sequences contained greater variability than the SSU, but was also highly polymorphic due to the high number of insertions and deletions both within spores, within isolates and among closely related species. This rendered ITS sequences difficult to interpret,

especially when comparing closely related species (Sanders *et al.*, 1995; Lloyd-Macgilp *et al.* 1996, Stockinger *et al.* 2010). In contrast, the LSU gene region has been shown to resolve species (van Tuinen *et al.* 1998a, 1998b; Gollette *et al.* 2004; Krüger *et al.* 2009).

Van Tuinen *et al.* (1998b) developed primers at the 5' end of the LSU for the identification of AMF colonizing roots. The amplification product obtained from single spore and root DNA was a fragment roughly 750 bp in length and spanned the D1 and D2 region (Fig 2; van Tuinen *et al.* 1998). These primers have been used successfully to genotype a variety of AMF species (Kjøller & Røsendahl 2000; Kjøller and Røsendahl 2001; Rodriguez *et al.* 2005; Stukenbrock and Røsendahl, 2005). The LSU gene amplified from single spores has also been used to clarify phylogenetic relatedness among closely related species (Kaonongbua *et al.* 2010; Morton and Msisk 2010a, 2010b; VanKuren *et al.* 2013; Bills & Morton 2015). The LSU gene phylogenies were congruent with morphological analyses making the LSU a suitable tool to resolve species. This was attributed to presence of conserved regions of the LSU that allowed discrimination among species and variable regions that allowed discrimination within species (Buckler *et al.* 1997; Eickbush & Eickbush 2007; Sonnenberg *et al.* 2007; Morton & Msiska 2010a, 2010b; Bills & Morton 2015). The LSU phylogenies have been shown not to be suitable for resolving deeper nodes in phylogenetic reconstructions (Stockinger *et al.* 2010).

Krüger *et al.* (2009, 2012) and Stockinger *et al.* (2010) developed AM fungal specific degenerate primers that amplify a 1.5 kb rDNA fragment that spanned the SSU-ITS-5.8S-LSU. Stockinger *et al.* (2010) compared sequence data from all rDNA gene regions to identify a suitable bar code, defined as a tool for sequence-based identification of species. Their analyses confirmed the highly polymorphic nature of rDNA genes within some species, but were unable to identify a universal level of interspecific variation suitable for identifying species. Their interpretation of their results also recommended the use of their primers for the best species level resolution. Although the benefit of the longer 1.5kb amplification product allowed greater resolution at deeper nodes in phylogenetic analyses, the inclusion of the highly variable ITS required use of LSU region to anchor the alignment (Krüger, personal communication).

rRNA gene homogeneity

Concerted evolution is a universal mechanism that was introduced to explain the pattern of gene evolution in which repetitive gene families evolve, in concert, as single homogenous units (Liao 1999). Selection would be expected to maintain the homogeneity of functional gene regions, but homogeneity in nonfunctional regions was unexpected because these regions are free to accumulate mutations by drift (Liao 1999; Nei & Rooney 2005; Ganley & Kobayashi 2007; Naidoo *et al.* 2013). This pertained to the intergenic or nonfunctional regions of repetitive gene families, which are more similar within a species than between two closely related species (Liao 1999; Eickbush & Eickbush 2007).

During interphase, NORs converge in the nucleolus for rRNA gene transcription. Shared nucleolar localization brings homologous and non-homologous chromosomes in close proximity that may allow mitotic recombination events leading to homogenization of rRNA gene repeats. These opportunities allow much more frequent interactions within chromosomes than interactions between chromosomes that may be spatially separated (Seperak *et al.* 1988). Although VanKuren *et al.* (2013) showed rRNA gene variants in a *Claroideoglossum* spp. that co-occurred near each other within the same nucleoli were spatially separated. This organization would impair gene homogenization.

The project documented in this dissertation consisted of two studies, both of which involved comparison of species in two deeply rooted clades in *Glomeromycota* using spore morphology, spore development and mode of formation, and sequence data from a 700-725 bp region near the 5' end of the LSU gene. The first study (Chapter 2) compared eight species in *Ambispora* (*Ambisporaceae*) to test the null hypothesis that sufficient morphological and LSU divergence separated eight species in *Ambispora* and spore type (acaulosporoid, glomoid, or both) resolved species level separation. The second study (Chapter 3) compared the only two species in *Archaeospora* (*Archaeosporaceae*) to test the null hypothesis that differences in mode of spore formation from a sporiferous saccule and LSU divergence support identity of two discrete species in *Archaeospora*.

Phylum: *Glomeromycota* Walker & Schüßler
 Class: *Glomeromycetes* Cavalier-Smith
 Order: *Diversisporales* Walker & Schüßler
 Family: *Diversisporaceae* Walker & Schüßler
 Genus: *Diversispora* Walker & Schüßler
 Genus: *Redeckera* Walker & Schüßler
 Family: *Acaulosporaceae* Morton & Benny
 Genus: *Acaulospora* (Gerdemann & Trappe) Berch
 Family: *Pacisporaceae* C. Walker, Blaszkowski
 Schüßler & Schwarzott
 Genus: *Pacispora* C. Oehl & Sieverd.
 Family: *Gigasporaceae* Morton & Benny
 Genus: *Scutellospora* Walker & Sanders
 Genus: *Gigaspora* (Gerdemann & Trappe) Walker & Sanders
 Genus: *Dentiscutata* Oehl, Souza & Sieverding
 Genus: *Cetraspora* Oehl, Souza & Sieverding
 Genus: *Racocetra* Oehl, Souza & Sieverding
 Order: *Glomerales* Morton & Benny
 Family: *Claroideoglomeraceae* Walker & Schüßler
 Genus: *Claroideoglomus* Walker & Schüßler
 Family: *Glomeraceae* Pirozynski & Dalpé
 Genus: *Glomus* Tulasne & Tulasne
 Genus: *Funneliformis* Walker & Schüßler
 Genus: *Septoglomus* Sieverding, Silva & Oehl
 Genus: *Rhizophagus* (Dangeard) Walker & Schüßler
 Genus: *Sclerocystis* Berkeley & Broome
 Order: *Archaeosporales* Walker & Schüßler
 Family: *Ambisporaceae* Walker, Vestberg & Schüßler
 Genus: *Ambispora* Walker, Vestberg & Schüßler
 Family: *Geosiphonaceae* (Engler & Gilg) Walker & Schüßler
 Genus: *Geosiphon* Wettstein
 Family: *Archaeosporaceae* Morton & Redecker
 Genus: *Archaeospora* Morton & Redecker
 Order: *Paraglomerales* Walker & Schüßler
 Family: *Paraglomeraceae* Morton & Benny
 Genus: *Paraglomus* Morton & Redecker

Fig 1 – Consensus classification of the phylum *Glomeromycota* down to genera based on rDNA phylogeny in Redecker *et al.* (2013). Taxa proposed by Goto *et al.* (2012) excluded.

Fig 2 – Schematic representation of a partial single nuclear ribosomal RNA (rRNA) gene. Primer arrows indicate the direction of amplification of each primer. Top red arrows indicate universal eukaryotic-species primers used in first round PCR amplification. Bottom blue arrows indicate fungus-specific primers used in second round PCR (White *et al.* 1998; van Tuinen *et al.* 1998b; Trouvelet *et al.*, 1999).

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CHAPTER 2

A COMBINATION OF MORPHOLOGY AND 28S RRNA GENE SEQUENCES PROVIDE GROUPING AND RANKING CRITERIA TO MERGE EIGHT INTO THREE *AMBISPORA* SPECIES (*AMBISPORACEAE*, *GLOMEROMYCOTA*)¹

Introduction

Classification of arbuscular mycorrhizal fungi in the phylum *Glomeromycota* has undergone numerous changes during the past decade as application of sequence data from ribosomal RNA and beta-tubulin genes together with some input from morphology inferred more natural phylogenetic relationships (Redecker and Raab 2006; Msiska and Morton 2009; Oehl et al. 2011; Krüger et al. 2012; Redecker et al. 2013). Some clades were well resolved and highly supported by both morphological and molecular data, so they evoked little controversy. Others were more problematic because of conflicts between traditional morphological characters and rDNA sequence data. Fungal species in the order *Archaeosporales* (Schüßler et al. 2001) exemplify some of these problems and the difficulties in resolving them. Dimorphic species, with spore phenotypes bridging two families, created nomenclatural and phylogenetic confusion. Complicating this story were misleading interpretations of degraded type specimens and treating any perceived difference as a species-defining character. Also, use of molecular data to provide independent verification of morphological interpretations was not possible for species lacking living material.

Archaeosporales presently consists of three monogeneric families. *Geosiphonaceae* contains one nonmycorrhizal species *Geosiphon pyriformis* (Schüßler et al. 2001; Schüßler 2002). *Archaeosporaceae* consists of *Archaeospora trappei* (Morton and Redecker 2001), described first as *Acaulospora trappei* (Ames and Linderman 1976) and *Archaeospora schenckii* (Krüger et al. 2012), described initially as *Entrophospora schenckii* (Sieverding and Toro 1987). *Ambisporaceae* contains eight species in the genus *Ambispora* (Walker et al. 2007a; Walker 2008) and this group is the focus of this study. The nomenclatural history of species in *Ambispora* is summarized in Fig. 1 to clarify events in a complex timeline.

¹ Published as Bills RB, Morton JB. 2015. *Mycorrhiza* **25**: 485–498. doi: 10.1007/s00572-015-0626-7.

Species in *Ambispora* were classified initially by mode of spore formation. Species forming glomoid spores with a subtending hypha were placed in *Glomus* and species with acaulosporoid spores attached to a terminal saccule were placed in *Acaulospora* (Gerdemann and Trappe 1974). Protologue descriptions of *Acaulospora gerdemannii* (Nicolson and Schenck 1979), *Glomus leptotichum* (Schenck and Smith 1982) and *Glomus fecundisporum* (Schenck and Smith 1982) were based on only one spore morphotype. *Acaulospora appendicula* (Schenck et al. 1984) also was described as monomorphic even though glomoid spores, described as being chlamydospore-like, were mentioned in the protologue. In strains deposited in the International Culture Collection of Vesicular Mycorrhizal Fungi (INVAM) by N.C. Schenck, glomoid and acaulosporoid spores of *Acaulospora appendicula* FL130A were indistinguishable from those in a dimorphic culture of *G. leptotichum* FL184B propagated from a single spore of each spore type (Morton et al. 1997). Examination of Oregon State University Herbarium (OSC) holotype and University of Florida (FLAS) isotype specimens revealed a similar result. Morton et al. (1997) then synonymized both *Acaulospora* and both *Glomus* species into a dimorphic species classified as *Acaulospora gerdemannii* to satisfy the criterion of nomenclatural priority. 18S rRNA gene (SSU) sequences verified dimorphism and also showed this species was basal to most other glomeromycotan clades (Sawaki et al. 1998; Redecker et al. 2000).

Rose et al. (1979) described *Glomus gerdemannii* from field-collected spores that were interpreted as possessing glomoid features. Similar novel ornamentations of spore wall layers and subtending hypha were observed on acaulosporoid spores of a dimorphic culture of INVAM accession AU215. This fungus also formed smaller glomoid spores that were phenotypically similar to those of *G. leptotichum* and type specimens of *G. fecundisporum* (Morton and Redecker 2001). A small sampling of SSU sequences grouped this species with *Ac. gerdemannii* (Redecker et al. 2000).

Analysis of SSU sequences also showed that *Ac. trappei* was more closely related to the two dimorphic species than to other *Acaulospora* species and so Morton and Redecker (2001) placed all three species in a new genus *Archaeospora* and in a new family *Archaeosporaceae*. Since *G. gerdemannii* and *Ac. gerdemannii* shared a common specific epithet, the former was renamed *Archaeospora gerdemannii* and the latter *Archaeospora leptoticha*.

Schüßler et al. (2001) used a broader sampling of SSU sequences that included the nonmycorrhizal species *Geosiphon pyriformis* to resolve three distinct monophyletic clades.

Geosiphon was placed in its own family, *Geosiphonaceae* in a new order *Archaeosporales*. The dimorphic species (*Ar. leptoticha* and *Ar. gerdemannii*) grouped into one clade and *Ar. trappei* into another. Spain et al. (2006) focused on the dimorphic clade and placed them in a new genus, *Appendicispora*. At the same time, these workers reversed all four synonymizations by Morton et al. (1997) based on their interpretations of morphological evidence. The dimorphic *Ar. leptoticha* was synonymized as the monomorphic *Glomus leptotichum*, and *Ac. appendicula* was resurrected as the dimorphic species *Appendicispora appendicula*. *Acaulospora gerdemannii* was restored as a monomorphic acaulosporoid species and renamed *Appendicispora jimgerdemannii*. *Glomus fecundisporum* was resurrected based on perceived differences in type specimens (spore wall outer layer). The dimorphic species *Ar. gerdemannii* was renamed *Appendicispora gerdemannii*.

Independently, Walker et al. (2007a) erected a new genus *Ambispora* typified by *Ambispora fennica*, a dimorphic fungus with SSU and ITS sequences that diverged from those of the other dimorphic species. The acaulosporoid spore was of similar phenotype to that of *Ar. gerdemannii*, but a single, short SSU and an ITS sequence of *Ar. gerdemannii* published by Redecker et al. (2000) did not cluster with *Am. fennica* sequences. These data, then, were used to discriminate the latter as a distinct species. They also transferred *Glomus callosum* (Sieverding 1988) to *Ambispora callosa*, relying on two glomoid strains from Japan (OK1, MAFF520057 and V1, MAFF520058) annotated as *G. leptotichum* in the National Institute of Agricultural Sciences (NIAS) Genbank databases (Murakoshi et al. 1998, Saito 2001, Kojima et al. 2004). ITS sequence divergence was used as the basis for separating *Am. callosa* from *Am. leptoticha*.

Two publications with classifications involving the same taxa (Spain et al. 2006; Walker et al. 2007a) created a confusing nomenclature. Walker et al. (2007b) resolved this problem by assigning priority to *Appendicispora*, erecting a new family *Appendicisporaceae*, and transferring all *Ambispora* species to this genus. *Appendicispora* was determined later to be a homonym, so *Ambispora* was resurrected and *Appendicisporaceae* was synonymized with a new family *Ambisporaceae* (Walker 2008). Palenzuela et al. (2011) then described a new dimorphic species, *Ambispora granatensis*, based on a combination of morphological and SSU and ITS sequence data. In this study, both comparative morphology and sequences of a 700—750 bp region of the 5' end of the 28S rRNA (LSU) gene were used to reexamine the eight species currently circumscribed in *Ambispora* (Fig. 1). *Ambispora reticulata* has been added more

recently (Oehl et al. 2012), but its taxonomic status will be discussed in a separate analysis because there is no evidence it is even a mycorrhizal fungus.

Materials and methods

Specimens

Type materials of *Ambispora* species consisted of *Ac. appendicula* (OSC 41495, FLAS F53673); *Ac. gerdemannii* (OSC 37514, FLAS F51804); *Am. fennica* (EPITYPE Att550-30 from M. Vestberg); *Gl. fecundisporum* (OSC 40250, FLAS F52579); *Gl. gerdemannii* (OSC 39476); and *Gl. leptotichum* (OSC 40249, FLAS F52577). Spores of *G. callosum* (OSC 147148) preserved in 5% formalin were used for morphological analyses. Only slide vouchers were available for analysis of *Am. granatensis* (OSC 134712, Z+ZT 1626).

Ambispora leptoticha strains consisted of INVAM accessions used by Morton et al. (1997) and Morton and Redecker (2001) for comparison with type specimens of *Ac. appendicula*, *Ac. gerdemannii*, *Gl. fecundisporum* and *Gl. leptotichum*. Living cultures included FL130A, FL184B, MX982A, NC169, NC171, NC176, WV109C and VZ856B. Accessions FL184B and FL130A were identified as reference strains for *G. leptotichum* and *Ac. appendicula*, respectively, because both were deposited by N.C. Schenck, an author of both protologues. They were not designated here as ex-types because provenance with the type pot cultures could not be established unequivocally. Cultures of *Ambispora gerdemannii* strains consisted of INVAM accessions MT106 and ON205A. The reference strain for *Am. gerdemannii* AU215A used by Redecker et al. (2000) and Morton and Redecker (2001) died before this study began, so only slide vouchers were available for morphological reevaluation.

The plant host used to culture all fungi was *Sorghum sudanense* (Staph.) Piper, according to protocols described by Morton et al. (1993). Voucher specimens consisted of spores from each culture mounted permanently on glass slides in polyvinyl alcohol-lactic acid-glycerol (PVLG, Koske and Tessier 1983) and PVLG mixed with Melzer's reagent (1:1 v/v). Specimens were examined with a Nikon Eclipse E600 microscope and photographed with a Nikon DS-Ri1 digital camera. All slides are stored in the INVAM voucher library.

DNA extraction.

DNA was extracted from single spores of *Am. leptoticha* and *Am. gerdemannii* crushed in a 0.2 mL microcentrifuge tube containing 14 μ L 10X Taq polymerase buffer (New England Biolabs, Ipswich, MA) with a UV sterilized micro pestle. Tube contents were transferred to 94 °C water bath for 4 min, snap-chilled and stored on ice until amplification.

Two rounds of PCR amplification were performed to generate enough product of the LSU gene. For the first round of amplification, primer pair ITS1 and NDL22 was used, followed by a nested amplification using primers LR1 and FLR2 (White et al. 1990, van Tuinen et al. 1998, Trouvelet et al. 1999). PCR was performed in 50 μ L volumes containing 4 μ L spore DNA, 3 μ L 10x PCR buffer (New England BioLabs, Ipswich, MA), 0.2 mmol of each dNTP, 1.5 mmol $MgCl_2$, 5 pmol of each primer, and 0.1 μ L of Taq DNA polymerase (New England BioLabs, Ipswich, MA). The second round of PCR was similar to the first but used 1.0 μ L of first amplification product as template with 7 μ L 10x PCR buffer. Cycling parameters for both rounds of amplification entailed initial denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products (8 μ L) were stained with ethidium bromide and electrophoresed on a 1.5% agarose gel and visualized by UV illumination. Amplification products were purified with QIAquick PCR Purification kit (Qiagen USA, Valencia, CA) and cloned with pCR®4-TOPO plasmid vector and transformed using One Shot® TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Positive transformants were verified with colony PCR using LR1/FLR2 primers. Colony PCR reactions were performed in 12.5 μ L containing 1/4 volume of PCR reagents, transformants sampled by pipette tip in 5.93 μ L nuclease free water, and 0.1 μ L of Taq DNA polymerase. Cycling parameters were identical to regular PCR. Plasmid DNA containing the insert was purified using QIAprep Miniprep kit and DNA sequenced at Davis Sequencing (Davis, CA, USA) using M13 primers. New LSU sequences were deposited in NCBI as accession numbers KC166251–KC166283. Sequence alignment is available at INVAM (<http://invam.wvu.edu/>). Published LSU sequences of *Am. appendicula* and *Am. fennica* were obtained from the National Center for Biotechnology Information (NCBI) database.

Phylogenetic analyses

Sequence chromatographs were inspected in Biological Sequence Alignment Editor (BioEdit, www.mbio.ncsu.edu/BioEdit/bioedit.html) and subjected to a search on the NCBI nucleotide Basic Local Alignment Search Tool (nBLAST) to verify homology with other glomeromycotan fungal sequences. Nucleotide sequences were aligned using MUSCLE (Edgar 2004).

Phylogenetic trees were inferred from Bayesian analysis in MrBayes 3.2.2 (Ronquist et al. 2011, <http://mrbayes.net>) and maximum likelihood (ML) criteria implemented in MEGA6 (Tamura et al. 2013). Model test for 24 different nucleotide substitution models was performed in MEGA6 (Tamura et al. 2013). Kimura 2-parameter model plus gamma (K2P+G) had the lowest Bayesian Inference Criterion (BIC) score and was selected for analyses (Nei & Kumar 2000). MrBayes settings were 4 x 4 nucleotide model, general time-reversible (GTR) converted to K2P by fixing stationary state frequencies to equal and ploidy set to haploid. The analysis included two runs of 10,000,000 generations with burn-in of 2,500,000 generations. The Bayesian tree was visualized in FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree>). ML analysis was performed in MEGA6 with K2P + G model, with four discrete gamma categories and 1000 replications to assess bootstrap support (Tamura et al. 2013).

Results

Molecular analysis

Bayesian and ML analyses of LSU sequences analyses grouped acaulosporoid and glomoid spores of *Am. leptoticha* and *Am. appendicula* strains in a highly supported monophyletic clade, thus providing evidence of conspecificity (Fig. 2). In the absence of living source material, LSU sequences could not be obtained from *G. fecundisporum*, *Am. callosa*, or *Am. granatensis*.

LSU sequences from the one representative strain of *Am. fennica* grouped with those of two North American strains of *Am. gerdemannii* in a highly supported clade (Fig. 2), but they also formed a distinct subclade. When equivalence in branching pattern and sequence variation within sister clades was used as the criterion to determine rank at the molecular level (Redecker et al. 2013), then *Am. fennica* and *Am. gerdemannii* were conspecific. A third-party LSU sequence of *Gl. gerdemannii* AU215 (GenBank accession AJ510233) grouped with *Am. leptoticha*, verifying that this sequence was annotated incorrectly when it was submitted to NCBI (Kaonongbua et al. 2010).

Morphological analyses

All of the *Ambispora* species described in this paper are potentially dimorphic, and each spore type exhibits unique patterns of morphological divergence and hence taxonomic significance. Holotype and isotype specimens of many species were parasitized or degraded in a lactophenol storage medium, thus altering color and dimensions of spore wall layers of many specimens. However, important diagnostic features still were identifiable from some specimens for comparative purposes. Range of spore diameters has been reviewed elsewhere (Walker et al. 2007a and references therein).

Glomoid spores

Glomoid type specimens (Fig. 3a-b) and reference culture *G. leptotichum* FL184B (Fig. 3c-d) were indistinguishable from those of *G. fecundisporum* (Figs. 3e), *Ac. gerdemannii* (Fig. 3f-g), *Ac. appendicula* (Fig. 3h), *Am. gerdemannii* (Fig. 3i-j) and *Am. granatensis* (Fig. 3k-l). Glomoid spores of all *Ambispora* species described to date, whether examined as type or fresh specimens, possessed a similar bilayered spore wall continuous with a bilayered subtending hyphal wall (Figs. 3-4). The outer layer (L1) often thins with age and then appears granular, flaky, or is absent after sloughing. An “alveolate reticulum” of shallow ridges rarely was observed on the surface of the spore wall outer layer of *G. leptotichum* (Fig. 3a-b), a trait used by Schenck and Smith (1982) and Spain et al. (2006) to distinguish this species from *G. fecundisporum* (Fig. 3e). The spore wall inner layer (L2) is permanent and is semi-pliable even in preserved specimens because it changes shape with applied pressure in all species and sometimes has a wrinkled inner surface, all of which likely contribute to the observed phenotypic variability. Spores also varied in degree of browning, (Fig. 3e, f-g) which is likely an artifact because spores were hyaline to pale yellow in all healthy living cultures. Glomoid spores from *Am. gerdemannii* MT106 and those from isotype specimens of *Am. granatensis* were indistinguishable from glomoid spores of other *Ambispora* species except they were smaller and thus had thinner spore wall layers (Fig. 3i-l). The wide range in spore size observed in a culture of *G. leptotichum* FL184B (50-280 μm) also was found in vials of glomoid type specimens of *G. leptotichum*, *G. fecundisporum*, *Ac. gerdemannii*, and *Ac. appendicula*. Glomoid spores of an *Am. granatensis* in pot culture

(Paluena et al. 2011) have a smaller size range (40-70 μm) than that of *Am. gerdemannii* MT106/ON205A (40-190 μm), which overlaps the size range of *Am. leptoticha* spores.

Glomus callosum was reclassified as *Ambispora callosa* because of SSU relatedness and ITS clade structure (Walker et al. 2007a). Glomoid spores of holotype *G. callosum* (Fig. 4a-c) did not possess any traits that distinguished this species from fresh spores of *G. leptotichum* FL184B (Fig. 4d-f), even though type spores had been preserved in 5% formalin for 15 years. The range in spore size overlapped between species and all were within the range reported by Walker et al. (2007a). As with other *Ambispora* species (Fig. 3), the hyaline to pale yellow spore wall of both species was bilayered, with a somewhat friable outer layer and a thicker semi-pliable inner layer (Fig. 4b-c, e-f). In particular, the continuity of bilayered organization in spore and attached hyphal walls was a consistent phenotype shared among glomoid spores of all species in *Ambispora*. The absence of distinctive morphological traits renders this spore synanamorph taxonomically uninformative below the rank of genus.

Acaulosporoid spores

All *Ambispora* species form a glomoid-like subtending hypha (pedicel) that branches from the neck of a sporiferous saccule, hence the term “acaulosporoid” (Fig. 5a-b). The spore wall in all taxa consists of three discrete layers and a developmentally separate semi-pliable hyaline inner wall (Fig. 5a-c). Only the two inner layers of the spore wall (L2-L3) diverge as discrete and stable phenotypes, and so they are the taxonomically significant morphological traits at the species level. The L2 and L3 layers of the spore wall form hemispherical protrusions and depressions, respectively, in *Am. leptoticha* (Fig. 5a). Both layers are smooth and rigid, but L3 is brittle and breaks into shards that are birefringent in polarized light when crushed in *Am. gerdemannii* (Fig. 5b). Both L2 and L3 are rigid, but L2 is quite thin ($< 2 \mu\text{m}$) with a faintly wrinkled surface in *Am. granatensis* (Fig. 5c). All other characters vary somewhat, but much of that variation is encompassed within and between strains of any given species. The semi-flexible hyaline inner wall is indistinguishable between species (Fig. 5a-c).

Some overlapping traits have been used erroneously to separate species. Spain et al. (2006) distinguished *Ac. gerdemannii* (reclassified as *Am. jimgerdemannii*) from *Ac. appendicula* (reclassified as *Am. appendicula*) based solely on two phenotypes of the outer spore wall layer that tend to degrade and slough readily (Fig. 6a): (i) “cerebriform” folds consisting of prominent

ridges that resembled convolutions of a mammalian brain (e.g. Fig. 6b) and (ii) a “crazed” surface consisting of fine friable cracks and fissures that easily crumbled (e.g. Fig. 6c). The cerebriform phenotype documented in the protologue and type specimens of *Am. jingerdemannii* (Fig. 6b) also was found on spores from *Ac. appendicula* FL130A (Fig. 6d) and *G. leptotichum* FL184B. From a population of spores in active culture FL130A (Fig. 6f), phenotypes from crazed to cerebriform phenotypes were present in a continuum (Fig. d-f). The crazed phenotype used to distinguish *Ac. appendicula* also predominated in fresh spores extracted from a culture of *Am. leptoticha* WV109. Moreover, the same continuum of phenotypes chronicled above for FL130A also was present in this pot culture. Clearly, the character states of the outer layer of the spore wall can co-occur as a range of phenotypes because of friability and susceptibility to decomposition. Even absence of the outer two layers of the acaulosporoid spore wall (Fig. 6a) is not unusual because it has been observed in all 19 INVAM accessions of *Am. leptoticha*. Since these character states are neither stable nor consistent, they are taxonomically uninformative at any level.

Spore morphology is indistinguishable between *Am. fennica*, *Am. gerdemannii*, and the holotype of *G. gerdemannii* when population level variation, age, degradation and preservation artifacts are taken into account (Fig. 7). The acaulosporoid spore of all three of these species consists of a three-layered spore wall and a semi-pliable inner wall of separate origin during development (Morton and Redecker 2001). The outer two layers of the spore wall in specimens of both *Am. fennica* (Fig. 7a-c) and *Am. gerdemannii* MT106 (Fig. 7d-f) are adherent and friable, breaking apart readily when crushed, and produce a dark dextrinoid staining reaction in Melzer’s reagent. These two layers vary greatly in appearance depending on degree of degradation or sloughing and can be partly to mostly sloughed or absent in some spores. The inner layer of the spore wall (L3) is novel, with a fracture pattern that can produce sharp-edged shards that are birefringent in polarized light. The thick semi-pliable inner hyaline wall may appear as only one layer, but sometimes separates into a number of very thin layers on either or both proximal and distal surfaces (Fig. 7c, f).

Analysis of type specimens and phylogenetic analysis by Palenzuela et al. (2011) support *Am. granatensis* as a discrete species in the *Ambispora*. No LSU sequences were available for analysis of *Ambispora granatensis* in this study, but SSU phylogeny positions this species as a monophyletic clade in the genus (Palenzuela et al. 2011; Krüger et al. 2012). Phylogenetic tree

branch lengths reported in their analysis suggests greater sequence variation than has been measured in other *Ambispora* species. Type material verified that *Am. granatensis* is a dimorphic species with both glomoid spore types (Fig. 3k, l) of similar morphology to those produced by *Am. leptoticha* and *Am. gerdemannii* and is unique in phenotypes of the L2 and L3 layers of the acaulosporoid spore wall (Fig. 5c).

Revised classification

Ambisporaceae C. Walker, Vestberg & Schüßler emend. R.J. Bills & J.B. Morton

Synonym: *Appendicisporaceae* C. Walker, Vestberg & Schüßler, Mycol. Res. 111:254 (2007); nom. illegit. (Arts 53.1, 53.3)

Typus: *Ambispora* (C. Walker, Vestberg & Schüßler) R.J. Bills & J.B. Morton 2013

Arbuscular mycorrhizal fungi producing dimorphic propagules of both glomoid and acaulosporoid spores, although fungal genotypes and environment impact on expression of either or both spore types. Spores formed singly or in clusters, from the terminal tip of a sporiferous hypha (glomoid synanamorph) or subtending an acaulosporoid spore (acaulosporoid synanamorph). Glomoid spores are hyaline with bilayered spore and hyphal walls lacking any discrete species-level characters. Acaulosporoid spores formed from a pedicel branching from the neck of a hyaline sporiferous saccule. Acaulosporoid spores possess a three-layered spore wall and a separate semi-pliable hyaline inner wall with layers of variable number and thickness. Spore wall outer layer tends to degrade and slough with age and the middle and inner layers of the spore wall express unique phenotypes that differentiate species. Distinguished from other families in the *Archaeosporales* by potential dimorphism, unique acaulosporoid spore morphology, and monophyly derived from rDNA sequence variation.

Ambispora C. Walker, Vestberg & Schüßler emend. R. Bills & J.B. Morton

Type species: *Ambispora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) R. Bills & J.B. Morton, comb. nov.

Dimorphic arbuscular mycorrhizal fungi capable of producing either or both glomoid and acaulosporoid spores. Glomoid spores hyaline with bilayered spore and subtending hyphal walls lacking discrete species-level characters; formed singly or in clusters, from the terminal tip of a sporiferous hypha or hyphae subtending an acaulosporoid spore. Acaulosporoid spores formed from a pedicel branching from the neck of a hyaline sporiferous saccule, with a spore wall consisting of three layers: a degradable and sloughing outer layer, a middle layer developing from a bilayered pedicel, and a rigid inner layer. The inner two layers of the acaulosporoid spore wall are unique in each species. An inner semi-pliable multi-layered hyaline wall forms independent of the spore wall and is indistinguishable among species.

Ambispora leptoticha (N.C. Schenck & G.S. Sm.) R.J. Bills & J.B. Morton comb. nov. (Figs. 2, 3a-h, 4, 5a, 6).

The species is described in Morton & Redecker (2001). It is distinct from other species in the genus *Ambispora* by its rDNA characteristics (Fig. 2) and acaulosporoid spore wall morphology (Figs. 5a, 6).

Basionym: *Acaulospora gerdemannii* N.C. Schenck & T.H. Nicolson, *Mycologia* 71:193 (1979).

Acaulospora appendicula Spain, Sieverd. & N.C. Schenck, *Mycologia* 76:686 (1984).

Glomus leptotichum N.C. Schenck & G.S. Sm., *Mycologia* 74:82–83 (1982).

Glomus fecundisporum N.C. Schenck & G.S. Sm., *Mycologia* 74:81 (1982).

Synonyms: *Archaeospora leptoticha* (N.C. Schenck & G.S. Sm.) J.B. Morton & D. Redecker, *Mycologia* 93:184 (2001).

Appendicispora leptoticha (N.C. Schenck & G.S. Sm.) C. Walker, Vestberg & Schüßler, *Mycol. Res.* 111:255 (2007).

Pseudoglomus leptotichum (N.C. Schenck & G.S. Sm.) S.P. Gautam & U.S. Patel, *The Mycorrhizae, Diversity, Ecology and Applications* (Delhi):5 (2007).

Ambispora leptoticha (N.C. Schenck & G.S. Sm.) C. Walker, *Mycol. Res.* 112:297 (2008).

Appendicispora jimgerdemannii (N.C. Schenck & T.H. Nicolson) Spain, Oehl & Sieverd., *Mycotaxon* 97:176 (2006).

Ambispora jimgerdemannii (Spain, Oehl & Sieverd.) C. Walker, *Mycol. Res.* 112:298 (2008).

Appendicispora fecundispora (N.C. Schenck & G.S. Sm.) C. Walker, Vestberg & Schüßler, Mycol. Res. 111:254 (2007).

Ambispora fecundispora (N.C. Schenck & G.S. Sm.) C. Walker, Mycol. Res. 112:298 (2008).

Glomus callosum Sieverd., Angew. Botanik 62:374 (1988).

Appendicispora callosa (Sieverd.) C. Walker, Vestberg & Schüßler, Mycol. Res. 111:254 (2007).

Ambispora callosa (Sieverd.) C. Walker Mycol. Res. 112:298 (2008).

Appendicispora appendicula (Spain, Sieverd. & N.C. Schenck) Spain, Oehl & Sieverd., Mycotaxon 97:170 (2006).

Paracaulospora appendicula (Spain, Sieverd. & N.C. Schenck) S.P. Gautam & U.S. Patel, The Mycorrhizae, Diversity, Ecology and Applications (Delhi):5 (2007).

Ambispora appendicula (Spain, Sieverd. & N.C. Schenck) C. Walker Mycol. Res. 112:298 (2008).

Ambispora gerdemannii (S.L. Rose, B.A. Daniels & Trappe) R.J. Bills & J.B. Morton comb. nov. (Figs. 2, 3i-j, 5b, 7).

LSU sequence data (Fig. 2) and acaulosporoid spore wall morphological (Figs. 5b, 7) together provide strong support that *Am. fennica* and *Am. gerdemannii* are conspecific and distinct from other species in *Ambispora*. Both *Am. fennica* and *Am. gerdemannii* clearly are dimorphic in living cultures (Morton and Redecker 2001; Spain et al. 2006; Walker et al. 2007a).

Basionym: *Glomus gerdemannii* S.L. Rose, B.A. Daniels & Trappe, Mycotaxon 8:297 (1979).

Synonyms: *Archaeospora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) J.B. Morton & D. Redecker, Mycologia 93:186 (2001).

Appendicispora gerdemannii (S.L. Rose, B.A. Daniels & Trappe) Spain, Oehl & Sieverd., Mycotaxon 97:174 (2006).

Ambispora gerdemannii (S.L. Rose, B.A. Daniels & Trappe) C. Walker, Vestberg & Schüßler, Mycol. Res. 111:148 (2007).

Ambispora gerdemannii (S.L. Rose, B.A. Daniels & Trappe) C. Walker, Mycol. Res. 112:298 (2008).

Ambispora fennica C. Walker, Vestberg & Schüßler, Mycol. Res. 111:148 (2007).

Appendicispora fennica (C. Walker, Vestberg & Schüßler) C. Walker, Vestberg & Schüßler, Mycol. Res. 111:254 (2007).

Ambispora fennica (C. Walker, Vestberg & Schüßler) C. Walker, Mycol. Res. 112:298 (2008).

Epitype: *Ambispora gerdemannii* INVAM accession MT106 submitted by C. Rosier, University of Montana, Missoula, MT, March 26, 2003.

Discussion

A species is a hypothesis that sometimes requires revision as new information becomes available. The proliferation of species based on weak or selective evidence jeopardizes the information content of a classification and how that information may be utilized in comparative studies. The taxonomic history of taxa that now comprise *Ambispora*, the only genus in the family *Ambisporaceae*, exemplifies this issue. As an outcome of this study, eight species classified in *Ambispora* have been reduced to three species based on a congruent combination of morphological and rDNA evidence.

The redefinition of *Ambispora leptoticha* to include *Am. fecundispora*, *Am. appendicula*, and *Am. jimgerdemannii* has come full circle and represents a return to the original species hypothesis of Morton and Redecker (2001). In addition, a reinterpretation of type material and published SSU/ITS sequences of *Am. callosa* indicates conspecificity with *Am. leptoticha*. The four species resurrected from *Archaeospora leptoticha* by Spain et al. (2006) relied on their interpretations of morphology, which were based on three faulty premises. The first premise involved distinguishing acaulosporoid spores of putatively monomorphic *Am. jimgerdemannii* from dimorphic *Am. appendicula* based on the presumption that “cerebriform” and “crazed” phenotypes in type material were novel, stable, and discrete enough to differentiate species. Comparative analysis of these spores in 19 INVAM accessions of *Am. leptoticha* propagated over a 20 year period indicated that both phenotypes are extremes in a continuum of variation. These characters, therefore, are not informative either taxonomically or phylogenetically. Instead, they represent population-level variation associated with age, senescence or environmental degradation.

Spain et al. (2006) used the same premise to separate *Am. fecundispora* (as *Gl. fecundisporum*) from *Am. leptoticha* (as *Gl. leptotichum*), whereby the surface appearance of the spore wall outer layer (of two layers) was “reticulate” (former species) or not (latter species). This layer is somewhat friable and undergoes some degradation, so a range of variation is expressed that include both phenotypes. The synonymization of each pair of species remains strictly morphological in the study here, because ex-type materials of *Am. jimgerdemannii* and *Am. fecundispora* have never been available to test hypotheses of gene phylogeny. Records indicate that no cultures were established in INVAM (as *Ac. gerdemannii*, *G. fecundisporum*) when the collection was curated by N.C. Schenck, one of the authors of both protologues.

The second premise of Spain et al. (2006) was that consistent sporulation of only one spore morphotype constituted evidence of a strict monomorphic habit. The absence of the acaulosporoid morphotypes in cultures of *G. fecundisporum* and *G. leptotichum* is not proof that this behavior does not exist because expression of dimorphism is both unpredictable and highly variable. For example, *Acaulospora appendicula* FL130A has produced varying frequencies of both morphotypes over 17 propagation cycles (Morton et al. 1997; Redecker et al. 2000; Morton and Redecker 2001). Another strain, *Am. leptoticha* VZ856, sporulates only as the acaulosporoid morph (Morton et al. 1997; Morton and Redecker 2001). In contrast, *G. leptotichum* FL184B has sporulated predominantly, or often exclusively, as the glomoid morph through 16 propagation cycles over 22 years (Morton et al. 1997; Morton and Redecker 2001). In their analysis, Spain et al. (2006) also failed to consider the presence of several acaulosporoid spores in the type specimen of *G. leptotichum* that supported dimorphism.

The taxonomy of *Ambispora callosa* has a confusing history. When Sieverding (1988) described *Gl. callosum*, dimorphism within a species had not been discovered yet. The presence of any acaulosporoid spores would likely have been interpreted as a different species. Subsequent cultures (Kojima et al. 2004; Walker et al. 2007a) appear to be exclusively monomorphic. Murakoshi et al. (1998) earlier had reported a dimorphic strain (F3b) from a single spore culture with an acaulosporoid morph similar to *Ac. gerdemannii* (= *Am. leptoticha*) and glomoid spores identified as *G. leptotichum*. Walker et al. (2007a) relied on the dimorphic strain F3b and two glomoid strains from Japan (OK1, MAFF520057 and V1, MAFF520058) annotated as *G. leptotichum* in the National Institute of Agricultural Sciences (NIAS) Genebank databases (Murakoshi et al. 1998, Saito 2001, Kojima et al. 2004) to undertake a comparative

molecular approach. Those data were not congruent, however, with an ML SSU tree grouping the Japanese isolates with *Am. leptoticha* and an unrooted maximum parsimony (MP) tree of ITS sequences positioned them into separate clades. Despite these conflicting trees, the ITS data was used to define the monomorphic isolates as *Am. callosa* and to separate this species from *Am. leptoticha*. An ML phylogeny reconstructed from SSU-ITS-LSU rDNA sequences (Krüger et al. 2012) supports the SSU tree and ranks *Am. callosa* isolates with *Am. leptoticha* and *Am. appendicula* in a clade of equivalent topology with other *Ambispora* species. The inclusion of isolate F3b as an equivalent subclade, with its *Am. leptoticha* acaulosporoid morphotype, provides morphological evidence for synonymy.

The third premise of Spain et al. (2006) was that morphological differences between species, regardless of how small, are important for delimiting and ranking of species. However, some phenotypic differences (novelties), no matter how stable or discrete they might be, may not reflect speciation events at all but instead indicate fixation of character variants that evolved as disjunct clonal populations (Morton and Msiska 2010a, 2010b; vanKuren et al. 2013). The intercalary spore formation considered by Spain et al. (2006) to be unique to *G. fecundisporum* is an example of a minor character of no diagnostic merit because it occurs so rarely that it isn't even represented in type material. The same conclusion applies to phenotypes generated by artifacts of storage medium, age or parasitism.

Analysis of *G. fecundisporum* posed somewhat of a problem. Even though the species was described from cultures by Schenck and Smith (1982), no representative living cultures exist. Records indicate this species was never deposited as a coded accession in Florida-INVAM or in any other lab. Hence, comparative morphological evidence rested only with the type material, which was in poor condition. Phenotypic variation in glomoid spores still were not distinguishable from that in spores of other merged species when state of the material was considered.

The revision of *Ambispora gerdemannii* in this study corrects the interpretation of *Ambispora fennica* as a distinct species. Walker et al. (2007a) described *Am. fennica* as being “very close morphologically” to *Am. gerdemannii* and the comparative evidence provided in this study goes further and concludes they are indistinguishable. Dimorphism is well established, and the glomoid morph shares all of the traits of the glomoid spores of *Am. leptoticha* except that the size

range is smaller. Morphology thus provides additional robust criteria to rank *Am. gerdemannii* within the *Am. leptoticha* clade.

The separation of *Am. fennica* from *Am. gerdemannii* by Walker et al. (2007a, 2007b) and Walker (2008) was based solely on SSU sequence data. This decision warrants discussion because it highlights two common problems associated with molecular data: (i) misannotations in public databases and (ii) taxon sample size. These workers relied on a single divergent SSU sequence annotated erroneously in NCBI as *Am. gerdemannii*. INVAM accession AU215 produced spores that matched the phenotype of *Am. gerdemannii*, but the representative sequences submitted to NCBI were actually those of *Am. leptoticha*. This pattern also was true for an AU215 ITS sequence (AJ012111). The addition of LSU sequences from two living cultures of *Am. gerdemannii* did not cluster with isolate AU215, providing conclusive evidence that AU215 sequences were not from spores of *Am. gerdemannii*.

Second, sample size greatly influences tree topology and thus ranking decisions (Pollock et al. 2002; Heath et al. 2008). rDNA sequences from *Am. fennica* were limited to a spore from one strain (Walker et al. 2007a; Walker 2008; Krüger et al. 2012). When additional rDNA sequences from *Am. gerdemannii* INVAM MT106 and Chinese strain n8_9 (JF439210) were added in an analysis by Krüger et al. (2012), these strains grouped with *Am. fennica*. In this study, *Am. gerdemannii* LSU sequences and those from one population of *Am. fennica* placed both taxa in a monophyletic clade equivalent in topology to the *Am. leptoticha* sister clade. The topology of *Am. fennica* as a distinct subclade likely is a reflection of limited sample size (both in number of isolates representing the species and number of samples within a population). Only 13 *Am. fennica* rDNA sequences published by Krüger et al (2012, Fig. 3) contained homologous LSU sequences for comparison in our study. All of these transformant clones were of a single isolate (Att200-23) and 12 were sequences from a single spore (clones MK096-1—MK096-12). Therefore, these data may not represent the scope of genetic variation in the source population, and by extension, the range of populations comprising the putative species. A similar distinct topology could be generated within *Am. leptoticha* when only selected strains were analyzed (result not shown). The genetic distance observed in the *Am. gerdemannii* clade is low, being much less than that of strains representing species clades in other genera such as *Paraglomus occultum* (this study), *Acaulospora paulinae* (Kaonongbua et al. 2010), *Claroideoglomus etunicatum* (van Kuren et al. 2013) and *Dentiscutata heterogama* (Morton and Msiska 2010b).

Although no LSU sequence analysis was performed on *Am. granatensis*, enough molecular and morphological evidence exists to support the retention of *Am. granatensis* as a phylogenetically discrete species in *Ambispora*. An SSU gene tree grouped *Am. granatensis* in a monophyletic clade with greater sequence variation than clades that grouped sequences from *Am. fennica* and the *Am. callosa*/*Am. appendicula* clade (Palenzuela et al. 2011). At the organismal level, *Am. granatensis* is dimorphic in pot cultures (Palenzuela et al. 2011). Glomoid spores possess the same bilayered spore wall and subtending hyphae phenotype shared by other species in *Ambispora*. Acaulosporoid spores shared the same three-layered spore wall and hyaline multilayered semi-pliable inner wall, and uniqueness was expressed in divergent inner layers of the spore wall.

Phylogenetic analysis of morphology relies on shared derived characters (synapomorphies) (Hillis 1987; Davis and Nixon 1992) of discrete and stable spore phenotypes (Morton 1990; Morton and Msiska, 2010a). Results of this study indicate that novel synapomorphies separating species described to date reside in divergent phenotypes of the inner two layers of the acaulosporoid spore wall. This pattern follows a more general one in *Glomeromycota*, where essential species-level traits of AMF, regardless of clade, are found mostly in spore wall characters (Morton 1995; Stürmer and Morton 1997; Stürmer and Morton 1999). Unlike other glomeromycotan clades, however, *Ambispora* species have a capacity for dimorphism and the glomoid morphotype appears to lack any capacity for divergence as discrete and stable traits. Possibly, genes involved specifically in glomoid spore formation are historically constrained and there is little pressure to select for any emergent variants or alternatively, the phenotypic space is too narrow for expression of new and distinctive variation.

This study reveals some of the inherent difficulties in systematic interpretations of evidence at all levels. Comparative morphology relies on a broad enough sampling of specimens to distinguish variation between populations versus species so that the former are not mistaken for the latter (Morton and Msiska 2010b; Redecker et al. 2013). That was not done by Spain et al. (2006) in resurrecting four species from *Ar. leptoticha*. Equally important, however, are analyses that identify those morphological characters which provide unique and consistent markers of speciation. Other phenotypic differences that are the product of mutation events fixed readily because of clonal reproduction in populations must be excluded, and nowadays gene sequence analysis contribute to exposing these traits (Kaonongbua et al. 2010; Morton and Msiska 2010b).

Similar considerations apply to comparative molecular data, either for rDNA gene repeats where concerted evolution is a critical process in homogenizing variants or where duplicate copies of protein-encoding genes exist. Linked rRNA gene polymorphisms are present in *C. etunicatum* strains that form a distinct clade, but they do not disrupt monophyly of the species (VanKuren et al. 2013). The polymorphisms appear to be maintained in disjunction nucleoli, with concerted evolution occurring within each localized rRNA gene array. Phylogeny of the beta-tubulin gene also reveals distinct subclade structure in *Dentiscutata heterogama* (Msiska and Morton 2009), but there is no evidence of divergent paralogs. Rather, exposure of this genetic diversity is attributed to extensive sampling of transformant clones among a range of fungal strains. As was done in this study, resolution of such conflicts resides in consideration of available evidence at all scales.

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Protologue species (see text for authors)	Morton & Redecker (2001) (synonymizations)	Spain et al. (2006) (species separations)	Walker et al. (2007b) (nomenclature changes)	Walker (2008) (nomenclature changes)	This study (synonymizations)
<i>Acaulospora gerdemannii</i>	→ <i>Archaeospora leptoticha</i>	<i>Appendicispora jimgerdemannii</i>	<i>Appendicispora jimgerdemannii</i>	<i>Ambispora jimgerdemannii</i>	→ <i>Ambispora leptoticha</i>
<i>Glomus leptotichum</i>		<i>Glomus leptotichum</i>	<i>Appendicispora leptoticha</i>	<i>Ambispora leptoticha</i>	
<i>Glomus fecundisporum</i>		<i>Glomus fecundisporum</i>	<i>Appendicispora fecundispora</i>	<i>Ambispora fecundispora</i>	
<i>Acaulospora appendicula</i>		<i>Appendicispora appendicula</i>	<i>Appendicispora appendicula</i>	<i>Ambispora appendicula</i>	
<i>Glomus callosum</i>			<i>Appendicispora callosa</i>	<i>Ambispora callosa</i>	
<i>Glomus gerdemannii</i>	→ <i>Archaeospora gerdemannii</i>	<i>Appendicispora gerdemannii</i>	<i>Appendicispora gerdemannii</i>	<i>Ambispora gerdemannii</i>	→ <i>Ambispora gerdemannii</i>
<i>Ambispora fennica</i>			<i>Appendicispora fennica</i>	<i>Ambispora fennica</i>	
<i>Ambispora granatensis</i>					→ <i>Ambispora granatensis</i>

Fig. 1 Timeline summarizing major taxonomic revisions in *Ambispora* (*Ambisporaceae*).

Fig. 2 Phylogenetic tree from partial 28S rDNA (LSU) sequences reconstructed using the Kimura-2 parameter and the maximum likelihood criterion in MEGA6. Bayesian posterior probabilities greater than 0.97 represented by thickened branches. Branch labels specify: spore morphotype (ac = acaulosporoid, gl = glomoid) and source material (spore1, spore2, spore3) for each transformant clone, INVAM accession, and NCBI accession code. Only bootstrap values above 70% are designated. **A third party sequence submitted to GenBank and erroneously annotated as *Glomus gerdemannii*.

Fig. 3 Comparative morphology of glomoid spores produced by *Ambispora* species. All form a bilayered spore wall consisting of a somewhat friable hyaline to pale brown outer layer (L1) and a hyaline semi-pliable inner layer (L2) that is continuous with a bilayered (L1-L2) subtending hyphal wall. **a** Small specimen of *Glomus leptotichum* holotype OSC40249. **b** Typical specimen from *G. leptotichum* OSC40249. **c** Spore of *Ambispora leptoticha* from active culture FL184B. **d** Spore and subtending hypha of *Am. leptoticha* FL184B. **e** Spore and subtending hypha of *Glomus fecundisporum* OSC40250. **f** Spores and attached hyphae from *Acaulospora gerdemannii* holotype OSC37514. **g** Small spore from *Ac. gerdemannii* OSC37514. **h** Transition between spore and subtending hyphal wall of *Acaulospora appendicula* isotype FLAS F53673. **i** Spore of *Ambispora gerdemannii* MT106 with transition between bilayered spore wall and subtending hyphal wall. **j** Spore of *Am. gerdemannii* ON205. **k-l** Spores from *Ambispora granatensis* isotype Z+ZT isotype 55-5503. Bar = 10 µm.

Fig. 4 Comparative morphology of whole and broken spores of *Glomus callosum* holotype OSC147148 (**a-c**) and glomoid spores of *Ambispora leptoticha* FL184B after nine months in refrigerated dry storage (**d-f**). **a** Whole spores in 5% formalin (size range 60–310 μm). **b** Typical spore with a friable outer layer (L1) and thicker semi-pliable inner layer (L2). **c** Thinner-walled spore. **d** Whole spores in water (size range 72–290 μm). **e** Typical spore showing semi-pliable phenotype of inner spore wall layer (L2). **f** Thinner-walled spore. Bar for a, d = 250 μm, all others = 10 μm.

Fig. 5 Comparative morphology of acaulosporoid spores produced by known *Ambispora* species. All form a friable and sloughing outer spore wall layer (L1), with divergence between species expressed in phenotypes of the second (L2) and inner (L3) layers of the spore wall. The middle spore wall layer (L2) is continuous with the wall of the pedicel (P) which branches from the neck of a sporiferous saccule. **a** *Am. leptoticha* FL130A, **b** *Am. gerdemannii* ON205A **c** *Am. granatensis* Z+ZT isotype 55-5504. A thick hyaline semi-pliable inner wall that forms separately from the spore wall (iw) varies in number and thickness of separable layers, but this phenotypic variation is shared by all three species. Bar = 5 μ m.

Fig. 6 Comparative morphology of the acaulosporoid spores of *Ambispora* species. **a** Spores of *Am. leptoticha* FL130A in water showing various stages of degradation/sloughing of the outer spore wall layer (OL) exposing the bright white ornamented inner spore wall layer (L3). **b** Cerebriform phenotype of spore wall outer layer from *Acaulospora gerdemannii* OSC37514. **c** Crazed phenotype of spore wall outer layer from *Ac. appendicula* OSC41495. **d-f** Spores sampled at the same time from an active culture of *Am. leptoticha* FL130A showing variation in phenotypes of the outer spore wall layers. **d** Crazed phenotype. **e** Crazed and cerebriform regions of the outer layer on the same spore. **f** Cerebriform phenotype. Bar for a = 200 μ m, for all others = 25 μ m.

Fig. 7 Equivalence in comparative morphology of acaulosporoid spores of *Ambispora fennica* and *Ambispora gerdemannii*. Spores of *Am. fennica* spores mounted on slides labeled Att550-30 from J. Blaszkowski (**a-c**). **a** In Melzer's reagent, showing the dextrinoid reaction of a friable outer spore wall layer (L1), a more permanent second layer (L2), a fracturing rigid inner layer (L3), and a semi-flexible inner wall (iw). **b** In PVLG, with the outer layer of the spore wall (L1) mostly sloughed. **c** Spore with all layers of the spore wall (L1-L3) and inner wall (iw) present. Spores of *Am. gerdemannii* strain MT106 (**d-f**). **d** In Melzer's reagent. **e** In PVLG with only remnants of the outer spore wall layer (L1) present. **f** All layers of the spore wall (L1-L3) and inner wall (iw) present. Bar = 10 μ m.

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CHAPTER 3

MORPHOLOGY AND AN LSU PHYLOGENY REVEAL *ARCHAEOSPORA TRAPPEI* AND *AR. SCHENCKII* ARE SYNONYMOUS AND MODE OF SPORE FORMATION RESOLVES ONLY STABLE POPULATION-LEVEL VARIATION²

Introduction

Classification of arbuscular mycorrhizal fungi (AMF) into species requires a broad sampling of specimens for comparative morphology and molecular analysis in order distinguish population-level from species-level variation (Morton & Msiska 2010b; Bills & Morton 2015). The asexual mode of reproduction in AMF can complicate ranking decisions because it may lead to fixation of heritable mutations at any level (Kaonongbua *et al.* 2010; Msiska & Morton 2010b; Bills & Morton 2015). *Archaeospora trappei* and *Ar. schenckii* are two species in the family *Archaeosporaceae* (*Archaeosporales*), which exemplify some of the challenges in defining species boundaries (Schüßler & Walker 2010; Krüger *et al.* 2012).

Both *Ar. trappei* and *Ar. schenckii* produce tiny hyaline spores that originate from the hypha of a precursor sporiferous saccule and they share identity in spore morphology (Morton & Redecker 2001; Sieverding & Oehl 2006; Schüßler & Walker 2010; Oehl *et al.* 2011). The two species differ in only one trait, and that is position of the spore as it originates from the hypha of a precursor sporiferous saccule. Spores of *Ar. trappei* form laterally from the saccule hypha. This similarity in spore ontogeny shared by species in *Acaulospora* led to classification of this species first as *Acaulospora trappei* (Ames & Linderman 1976). Spores of *Ar. schenckii*, in contrast, form within the saccule hypha (Sieverding *et al.* 1987). Similarity in mode of spore formation shared by species was used for higher taxonomic rankings. Between then and now, phylogenies reconstructed from rRNA genes led to several revisions in classification within the AMF. Morton and Redecker (2001) identified *Ac. trappei* as one of several deeply rooted species of AM fungi, and reclassified the species as a member of the new genus *Archaeospora* in a new family *Archaeosporaceae*. Sieverding & Oehl (2006) recognized *E. schenckii* and *Ar. trappei* as being closely related based on identical spore subcellular structure and similar weakly staining mycorrhizae roots. However, these workers still gave mode of spore formation primacy and

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transferred *E. schenckii* to the monospecific genus *Intraspora* in *Archaeosporaceae*. Evidence began to accumulate indicating the entrophosporoid mode was a widely convergent trait in *Glomeromycota*. In *Acaulosporaceae*, species develop either acaulosporoid or entrophosporoid spores (Kaonongbua *et al.* 2010; Palenzuela *et al.* 2010; Oehl *et al.* 2011; Krüger *et al.* 2012). In *Diversisporaceae*, the glomoid mode of spore development dominates, but taxa that develop entrophosporoid spores also are present (Palenzuela *et al.* 2010; Oehl *et al.* 2011). Recognition of this pattern led to synonymization of *Intraspora* and *Archaeospora* as congeneric (Schüßler & Walker 2010), thus grouping *Ar. schenckii* and *Ar. trappei* as sister taxa. Sequences of a 1.5 kb rDNA fragment from transformants of single *Ar. trappei* and *Ar. schenckii* strains (Krüger *et al.* 2012) further supported this move.

The goal of this study was to determine whether the respective acaulosporoid and entrophosporoid modes of spore formation by the otherwise morphologically identical *Ar. trappei* and *Ar. schenckii* resolved species-level differences or indicated the rare occurrence of dimorphism among conspecific populations. Morphology was reexamined to assess the degree of variability amongst a broad range of strains and a 700-725 bp sequence near the 5' end of the LSU gene was used to assess genetic relatedness.

Materials and methods

Fungal materials

Archaeospora schenckii was represented by exotype OSC46030 of *E. schenckii*, a living culture of INVAM accession CL401, and four cultures started from single spores (CL401-ss1-4).

Acaulospora trappei was represented by holotype OSC35917 and 10 strains from seven countries (see Fig 1). Phylogenetic analysis also included living cultures of two species in *Paraglomus*, another deeply rooted clade (Fig 1).

All fungal strains were propagated on *Sorghum sudanense* (Staph.) according to protocols described by Morton *et al.* (1993). Voucher specimens consisted of spores permanently mounted on glass slides in polyvinyl-lactic acid-glycerol (PVLG, Koske & Tessier 1983) and PVLG mixed with Melzer's reagent (1:1 v/v). Specimens were examined with a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, New York) equipped with differential interference contrast (DIC) optics and photographed with a Nikon DS-Ri1 digital camera. Images were

captured with Nikon NIS-Elements imaging software (ver. 2.34). Spore morphological characters use the terminology of Morton & Redecker (2001).

DNA extraction, amplification and sequencing of rDNA

Identity of all *Ar. schenckii* and *Ar. trappei* spores used for DNA extraction was verified by presence of an attached sporiferous saccule and then handpicked with a single-use micropipette. Only single sonicated spores of *Ar. schenckii* and *Ar. trappei* were selected for each amplification. A spore was crushed in a 0.2 mL microcentrifuge tube containing 14 µL 10X *Taq* polymerase buffer (New England Biolabs, Ipswich, MA) with a UV sterilized micropestle (Kontes Pellet Pestle). Tube contents were transferred immediately to 94 °C water bath for 4 min, snap chilled and stored on ice until amplification.

Two rounds of 50 µl PCR amplification were performed to generate enough product of a 700-750bp LSU fragment for cloning (vanTuinen *et al.* 1998). Primer pair ITS1 and NDL22 were used for the first round of amplification, followed by a nested amplification using primers LR1 and FLR2 (White *et al.* 1990; van Tuinen *et al.* 1998; Trouvelet *et al.* 1999). The first PCR amplification contained 4 µl spore DNA, 3 µl 10x PCR buffer (New England BioLabs, Ipswich, MA), 0.2 mM of each dNTP, 1.5 mM MgCl₂, 5 pM of each primer, and 0.1 µl of *Taq* DNA polymerase (New England BioLabs, Ipswich, MA). The second round of PCR was similar but used 1 µl of first amplification product as template in 7 µl 10x PCR buffer. Cycling parameters for both PCR reactions entailed initial denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplicons (8 µl) were stained with ethidium bromide and electrophoresed on a 1.5% agarose gel and visualized by UV illumination, purified with QIAquick PCR Purification kit (Qiagen USA, Valencia, CA), cloned with pCR®4–TOPO plasmid vector and transformed using One Shot® TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). Positive transformants were verified with colony PCR using LR1/FLR2 primers. Colony PCR reactions were performed in 12.5 µL containing 1/4 volume of PCR reagents, transformants sampled by pipette tip in 5.93 µL nuclease free water, and 0.1 µL of *Taq* DNA polymerase. Cycling parameters were identical to regular PCR. Plasmid DNA containing the insert was purified using QIAprep Miniprep kit and sequenced at Davis Sequencing (Davis, CA, USA) using M13

primers. LSU sequences were deposited in NCBI KT250767-KT250842. The sequence alignment is available on the INVAM website (<http://invam.wvu.edu/>).

Phylogenetic analyses

Sequence chromatographs were inspected in Biological Sequence Alignment Editor (BioEdit, www.mbio.ncsu.edu/BioEdit/bioedit.html) and subjected to a search on the NCBI nucleotide Basic Local Alignment Search Tool (BLASTn) to verify homology with other glomeromycotan fungal sequences. Nucleotide sequences were aligned using MUSCLE (Edgar 2004), inspected and edited in MEGA6 (Tamura *et al.* 2013). Phylogenetic trees were reconstructed using both Bayesian and maximum likelihood (ML) methods. MEGA6 was used to determine the best evolutionary model and nucleotide substitution pattern. The Kimura 2 parameter model plus gamma (K2P + G) had the lowest Bayesian inference criteria (BIC) score and was selected for analyses (Nei & Kumar 2000) with four discrete gamma categories. Bayesian phylogeny reconstruction was carried out in MrBayes 3.2.2 (Ronquist *et al.* 2012; <http://mrbayes.net>). Settings included the 4 by 4 general-type DNA model, general time reversible (GTR) substitution model converted to K2P by fixing stationary state frequencies to equal (<http://mrbayes.sourceforge.net/wiki>), and ploidy set to haploid. The analysis included two runs of 10,000,000 generations with a burn-in of 2,500,000 generations. MrBayes tree with posterior probabilities was visualized in FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtrees>). The ML method was implemented in MEGA6 with 1000 replications to assess bootstrap support (Tamura *et al.* 2013).

Results

Phylogenetic analyses

LSU sequences grouped *Ar. trappei* and *Ar. schenckii* strains in a highly supported monophyletic clade, thus providing molecular evidence of conspecificity (Fig 1). MrBayes and ML phylogenetic trees were identical in topology. Sequences of some *Ar. trappei* strains segregated into a highly supported subclade, but others grouped closely with isolates of *Ar. schenckii*. The divergent subclades, designated here as *Ar. trappei* subclade-1 and *Ar. trappei* subclade-2, did not compromise monophyly of *Ar. trappei*, based on equivalence in tree topology. Although clones and NCBI accessions of *Ar. schenckii* clustered within *Ar. trappei* subclade-2 with high

support, short branch lengths signified very low nucleotide divergence. In fact, sequences from some *Ar. trappei* and *Ar. schenckii* spores were identical. Some strains of *Ar. trappei* (FL327C, NC104B, SF119B) yielded identical sequences, as did *Ar. trappei* accessions from NCBI (FR750036-FR750038, clones of pCK082). Similarly, some sequences within and between spores of *Ar. schenckii*, were identical. A similar pattern of sequence variation was measured in *Paraglomus occultum*, a member of another deeply rooted clade.

Analysis of evolutionary divergence between groups of sequences in this study for both *Ar. trappei* subclades (without *Ar. schenckii* sequences), *Ar. schenckii*, *Ambispora gerdemannii*, *Am. leptoticha*, *Paraglomus brasilianum* and *P. occultum* were estimated in MEGA 6. The average distance was calculated from the arithmetic mean of all pairwise distances between groups in the inter-group comparisons (Table 1). Divergence between *Ar. trappei* subclade-1 and *Ar. trappei* subclade-2 was 0.168 (SE 0.016) while that between *Ar. trappei* subclade-2 and *Ar. schenckii* was 0.030 (SE 0.004). Divergence between the two *Ar. trappei* subclades was greater than divergence in *Ambispora leptoticha* and *Am. gerdemannii* (0.069 SE 0.010) and *Paraglomus brasilianum* and *P. occultum* (0.111 SE 0.012).

The sequence alignment was visually inspected for patterns of mutation and insertion/deletions and possible sources of divergence between *Ar. trappei* subclades. There were 110 point mutations (64 transitions, 38 transversions and 8 indels) distinguishing *Ar. trappei* subclade-1 from *Ar. trappei* subclade-2 (without *Ar. schenckii* sequences). *Archeospora schenckii* and *Ar. trappei* subclade-2 sequences contained the same nucleotide substitutions that distinguished *Ar. trappei* subclade-2 from *Ar. trappei* subclade-1. Within *Ar. trappei* subclade-2, sequence variation between *Ar. trappei* and *Ar. schenckii* consisted of only eleven discrete mutations (six transitions, four transversions and one indel).

Morphological analysis of specimens

Spore morphology of both type specimens and living reference cultures of *Ar. trappei* and *Ar. schenckii* were indistinguishable except for mode of spore formation (Fig 2A-I). Only rarely were both spore phenotypes found in the same culture of CL401, but detection was possible only from immature spores with attached saccules mounted on glass slides (Fig 2B, F). Spore size also overlapped among strains (Table 2).

The spore wall consisted of two discrete layers and an inner wall. The outer layer (*L1*) originated from the wall of the sporiferous saccule's subtending hypha and was hyaline, thin (1.0-1.2 μm), and somewhat flexible with applied pressure under a slide coverslip (Fig 2C-D, G-H). The inner layer (*L2*) also was thin (0.5-1.0 μm), hyaline and semi-flexible. The separate hyaline inner wall (*iw*) was thicker (1.3-2.5 μm) but also exhibited some flexibility. The spore wall outer layer, together with the sporiferous saccule, degraded with age and then become detached, leaving behind either one (acaulosporoid) or two (entrophosporoid) scars on the spore wall. The inner wall (*iw*) developed independently to form an endospore that enveloped spore contents (Fig 2C-E, H-I).

Revised classification

The absence of LSU sequence divergence (Fig 1) and identity in spore subcellular structure (Fig 2), collectively provide evidence indicating that *Ar. trappei* and *Ar. schenckii* are conspecific and that the entrophosporoid mode of spore development was an isolated mutational event that, once fixed, led to stable divergent populations. Therefore *Ar. schenckii* is combined with *Ar. trappei* into one species, with different populations of *Ar. trappei* producing either acaulosporoid spores or entrophosporoid spores or more rarely, both morphotypes.

Archaeospora trappei (J.B. Morton & Redecker) R.J. Bills & J.B. Morton, *combo. nov.* (Figs 1 and 2, Table 2).

Basionym: *Acaulospora trappei* R.N. Ames & Linderman, *Mycotaxon* **3**: 566. 1976.

Synonyms: *Entrophospora schenckii* Sieverd. & S. Toro, *Mycotaxon* **28**: 210 (1987)

Intraspora schenckii (Sieverd. & Toro) Oehl & Sieverd., *J. Appl. Bot. Food Quality (Angew. Botan.)* **80**:77 (2006)

Archaeospora schenckii (Sieverd. & S.Toro) C. Walker & A. Schüßler, *The Glomeromycota: a species list with new families and new genera*. The Royal Botanic Garden Edinburgh, The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University. Printed copy available under ISBN-13: 978-1466388048, ISBN-10: 1466388048 (2010).

Arbuscular mycorrhizal forming fungi producing either acaulosporoid or entrophosporoid spores formed singly or occasionally in pairs from the hypha of a sporiferous saccule. Acaulosporoid

spores form laterally and entrophosporoid spores form within the hypha. Spore subcellular structure, regardless of mode of formation, consists of a two-layered spore wall and a separate semi-flexible inner wall, all of which are hyaline (Morton & Redecker 2001). More variation exists in LSU sequences among acaulosporoid strains than between acaulosporoid and entrophosporoid strains.

Discussion

Testing species hypotheses requires appropriate sampling, both within and among geographically distant populations in order to identify the range of variation within a species (morphological and molecular) and then establishing the appropriate grouping and ranking criteria for both data sets (Redecker *et al.* 2013). The combined morphological and molecular analyses in this study indicate *Ar. trappei* and *Ar. schenckii* are not sufficiently distinct for species separation to be supported. The acaulosporoid and entrophosporoid modes of spore formation from a precursor sporiferous saccule are all convergent traits and divergent LSU gene polymorphisms are stable population-level variants that evolved in some *Glomeromycota* including *Ar. trappei*.

Morphological traits associated with formation of a sporogenous saccule, mode of spore formation, and spores that develop from them have three levels of resolution for classifying AMF. First, the sporogenous saccule that forms prior to spore development has few distinct character states, varying mostly in size. However, this saccule variation usually correlates with spore size and is not unique above the species level (Morton 1988). Therefore, it has no phylogenetic value. The independent evolution of a sporogenous saccule in five families (*Acaulosporaceae*, *Ambisporaceae*, *Archaeosporaceae*, *Diversisporaceae*, *Glomeraceae*) clearly establishes that it is a convergent character (Morton & Benny 1990; Morton & Redecker 2001; Walker *et al.* 2007; Palenzuela *et al.* 2010; Oehl *et al.* 2011; Bills & Morton 2015). Beyond the repeated evolution of this structure in genetically distant clades, little is known about the causal basis for emergence of this trait or its functional attributes beyond a precursor structure for spore development.

Second, the acaulosporoid and entrophosporoid modes of spore formation are also convergent traits within the *Glomeromycota*, but can resolve taxa within clades. The acaulosporoid mode evolved in two divergent families *Acaulosporaceae* and *Archaeosporaceae* (Gerdemannii & Trappe 1974; Morton *et al.* 1997; Redecker *et al.* 2000; Morton & Redecker

2001; Walker *et al.* 2007; Kaonongbua *et al.* 2010; Walker & Schüßler 2010; Bills & Morton 2015). In *Acaulosporaceae*, the acaulosporoid mode of spore formation is a heritable trait shared among species in *Acaulospora* and is informative as a grouping criterion. The acaulosporoid mode of spore formation as a grouping criterion in *Archaeosporaceae* remains enigmatic, as only one species is currently described in monogeneric *Archaeospora*.

Mode of spore formation in *Ambispora* is unique, as the clade contains three dimorphic species that form a bilayered glomoid phenotype and a more complex glomoid spore that forms from a bilayered “pedicel” that branches from hypha subtending a precursor saccule (Bills & Morton 2015).

The entrophosporoid mode of spore formation also originated multiple times in four different glomeromycotan families, but as it failed to define lineages above species level is only suitable for grouping populations. rDNA phylogenies have placed the phenotype in *Acaulosporaceae* (*Acaulospora colliculosa*, *Acaulospora columbiana*, *Acaulospora kentinensis*) *Archaeosporaceae* (*Ar. trappei*), *Claroideoglomeraceae* (*Entrophospora infrequens*) and *Diversisporaceae* (*Entrophospora baltica*, *Entrophospora nevadensis*) (Sieverding & Oehl 2006; Kaonongbua *et al.* 2010; Walker & Schüßler 2010; Oehl *et al.* 2011; Redecker *et al.* 2013). While the entrophosporoid phenotype discriminates at the species level *Ac. colombiana* from in lineages *Ac. denticulata*, *Ac. mellea*, and *Ac. morrowiae* (Msiska & Morton 2009a; Kaonongbua *et al.* 2010; Krüger *et al.* 2012; Krüger *et al.* 2013). In *Ar. trappei*, acaulosporoid and entrophosporoid modes of spore formation are rarely detected together in some isolates, rendering each phenotype unsuitable to reliably group populations that diverge into two discrete subclades using the LSU marker gene.

The independent evolution of the entrophosporoid mode of spore formation was extremely rare. Within the monogeneric *Acaulosporaceae*, the entrophosporoid phenotype arose in only three of the 37 described species in the clade (Kaonongbua *et al.* 2010). Regardless of spore position, later stages of spore ontogeny were similar in all taxa within the clade. Additional evidence from *Acaulospora* rDNA and β -tubulin gene phylogenies did not group the three entrophosporoid taxon, *Ac. colombiana*, *Ac. kentinensis*, and *Ac. colliculosa*, together into a single monophyletic clade but indicated that each shared more recent ancestry with a different acaulosporoid sister taxa (Morton & Msiska 2009a, Kaonongbua *et al.* 2010; Krüger *et al.* 2011, 2013).

Historically, detection of *Ar. schenckii* also was rare compared to globally distributed strains of *Ar. trappei*. Sieverding & Oehl (2006) reported the detection of *Ar. schenckii* in only five countries. Other diversity studies that identified *Ar. trappei* or *Ar. schenckii* reported the former 67 times and the latter only nine times (Stürmer & Morton in prep.). Both taxa were reported in the same study only twice and of these only once in same ecosystem (Castillo *et al.* 2006; Bashan *et al.* 2007). Additional evidence from the INVAM culture collection verifies the rarity of *Ar. schenckii*. Of the 27 *Ar. trappei* cultures in INVAM, only a single *Ar. schenckii* strain (CL401) has been submitted to the collection. Rarity of both phenotypes in the same isolate was also limited to intact immature spores from culture of CL401 (this study). The presence of both phenotypes can easily be missed in field-collected samples or overlooked if expressed at a low frequency in cultures due to degradation of outer layer, which sloughs and separates spore from the sporiferous saccule.

Taxonomically informative characters significant across all *Glomeromycota* for ranking genera and species were found in the spore subcellular structures in the former, and in the individual layers within the spore wall in the latter (Morton 1990). In *Acaulospora*, all species regardless of mode of spore formation have a bi- or tri-layered spore wall, two bilayered flexible inner germinal walls, and a germination orb that develops on the inner most germinal walls (Morton 1990; Stürmer & Morton 1999; Kaonongbua *et al.* 2010). In *Ambispora*, spores have a tri-layered spore wall and a thick semi-pliable inner wall with a variable number of sublayers (Morton *et al.* 1997; Morton & Redecker 2001; Bills & Morton 2015). In *Archaeospora*, both spore subcellular phenotypes of *Ar. trappei* are indistinguishable containing a simple, hyaline bilayered spore wall and a thicker flexible inner wall.

Phylogenetic reconstruction of *Ar. trappei* LSU sequences revealed population-level variants that diverged into two deeply lineages, with greater genetic distance between subclades than distances between species clades. The magnitude of the branch lengths suggests enough divergence exists to reclassify either *Ar. trappei* subclade as a new species. Although, other AMF have been shown to harbor highly polymorphic rDNA sequences. For example, VanKuren *et al.* (2013) showed clear evidence of divergent LSU gene evolution within single spores from globally distributed isolates of three *Claroideoglomus* species. Two distinct rRNA gene types, a long and a short type, were detected together within individual spores. Phylogenetic reconstructions showed the long and short type of sequences clustered together in two distinct

clades represented by sequences from all three species. Copies of both variants were found to exist in high copy number within individual nuclei and FISH analysis revealed both variants were close together but spatially separated within the same nucleoli.

One explanation for retention of polymorphic rRNA loci in *Claroideoglomus* spores physical separation of sequence types in the nucleoli inhibited homogenizing mechanisms of concerted evolution (vanKuren *et al.* 2013). A mechanism that explains the retention of divergent *Ar. trappei* sequences is not known. Our data did not detect both sequence types together within single spores, but both subclades were equally represented among geographically distinct isolates. A broader sampling of 13 clones from a single spore from South Africa (isolate SF119B) revealed identical sequences with North American isolates from North Carolina (NC105B) and Florida (FL327) indicating rRNA gene variants are highly conserved. A similar pattern also was observed within geographically differentiated isolates of *Paraglomus occultum* (This study; Colombian isolate CL700 and Florida isolate FL703), and within sequence variants of *Claroideoglomus claroideum*/*Claroideogloum luteum* and *Claroideoglomus etunicatum* (vanKuren *et al.* 2013). Identical polymorphisms in *Ar. trappei* shared across globally distinct isolates suggests fixation of these variants early in an ancestral population before descendant populations were separated due to a vicariance event such as continental movements (Baum & Smith 2013). The presence of minor intra-individual and intra-population LSU polymorphisms within each subclade suggest low rates of mutation occurred faster than localized homogenization of neighboring gene copies (VanKuren *et al.* 2013).

Our phylogenetic analysis indicates that evolutionary patterns in the ancestral clade *Archaeospora* in the *Glomeromycota* is much more complex than previously thought. The combined analysis of LSU gene sequence data in conjunction with analysis of the simple, spore wall subcellular characters indistinguishable between the two morphotypes has provided a powerful approach for testing hypotheses of evolutionary relationships and species boundaries in the *Glomeromycota* (Morton & Redecker 2001; Walker *et al.* 2007; Morton 2009; Kaonongbua *et al.* 2010; Morton & Msiska 2010a, 2010b; Krüger *et al.* 2011; Oehl *et al.* 2011; Schüßler *et al.* 2011; Redecker *et al.* 2013; Bills & Morton 2015). Effective use of comparative morphology and molecular data for classifying taxa relies on a broad sampling of specimens, in order to identify shared derived characters (synapomorphies), and prevents misinterpretation of population-level variation as species variation (Hillis 1987; Davis & Nixon 1992; Morton 2009; Msiska & Morton

2009a; Morton & Msiska 2010a; Redecker *et al.* 2013; Bills & Morton 2015). Phenotypic differences that are the product of mutation events fixed readily because of clonal reproduction in populations must be excluded, and current gene sequence analysis contributes to exposing these traits (Kaonongbua *et al.* 2010; Morton & Msiska 2010a; Kruger *et al.* 2011; Bills & Morton 2015).

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Table 1 – Estimates of evolutionary divergence (standard error) between groups of partial LSU rDNA sequences from deeply rooted taxa in *Glomeromycota*. Groups compared included both *Archaeospora trappei* subclades, *Ar. schenckii*, *Ambispora gerdemannii*, *Am. leptoticha*, *Paraglomus brasilianum*, and *P. occultum*. The average distance was calculated from the arithmetic mean of all pairwise distances between groups in the inter-group comparisons. Analysis conducted in MEGA6 using the Kimura 2-parameter model + gamma (gamma distribution = 4).

Clade group	<i>Ar. trappei</i> subclade-1	<i>Ar. trappei</i> subclade-2	<i>Ar. schenckii</i>	<i>Am. leptoticha</i>	<i>Am. gerdemannii</i>	<i>P. occultum</i>	<i>P. brasilianum</i>
^a <i>Ar. trappei</i> sub-1	XX	0.168 (0.016)	0.168 (0.017)	0.270 (0.025)	0.268 (0.025)	0.317 (0.025)	0.318 (0.026)
^b <i>Ar. trappei</i> sub-2		XX	0.030 (0.004)	0.254 (0.023)	0.267 (0.025)	0.357 (0.027)	0.339 (0.027)
^c <i>Ar. schenckii</i>			XX	0.259 (0.024)	0.270 (0.026)	0.366 (0.026)	0.340 (0.028)
^d <i>Am. leptoticha</i>				XX	0.069 (0.010)	0.289 (0.025)	0.292 (0.025)
^d <i>Am. gerdemannii</i>					XX	0.284 (0.023)	0.287 (0.024)
^e <i>P. occultum</i>						XX	0.111 (0.012)

^a*Ar. trappei* subclade-1 = *Archaeospora trappei* INVAM isolates FL327C, KE120, NC104B, SF119B; ^b*Ar. trappei* subclade-2 = *Archaeospora trappei* INVAM isolates AU219, AZ119; IL203B, ON201D, PE102, SF113 and NCBI accessions FR750034-FR750038; ^c*Ar. schenckii* = *Archaeospora schenckii* single spore isolates from INVAM strain CL401 and NCBI accessions FR750020-FR750023; ^d*Am* = *Ambispora*; ^e*P.* = *Paraglomus* isolates. Evolutionary analyses conducted in MEGA6 (Tamura *et al.* 2013).

Table 2 – A summary of spore size and subcellular diagnostic characters of *Archaeospora schenckii* and *Archaeospora trappei* reported in previous studies.

Authors taxa described	Spore diameter (µm)	Spore shape	Saccule diameter (µm)	Spore wall characteristics	Outer structure(µm)	Inner structure & thickness (µm)
Ames & Linderman 1976 <i>Acaulospora trappei</i>	42-99 x 42-72	globose, ellipsoid, obovoid	50-82x42-72	1 wall	1 layer 1.2-2.3	No inner wall
Sieverding & Toro 1987 <i>Entrophospora schenckii</i>	(37-) 50-60 (-77) or 48-97 x 25-60	globose, subglobose or ellipsoid, ovoid	(37-)50-60(-77)	2 components	1 component, 0.5-1.0	2 components 0.4-1.0, 1.5-2.0
Morton & Redecker 2001 <i>Archaeospora trappei</i>	40-80	globose, subglobose, irregular	40-48x50-72	3 layers	layer 1 1.0-1.2	2 layers <0.5-0.7, 1.3-2.5
Fracchia <i>et al.</i> 2003 <i>Entrophospora schenckii</i>	(49-) 50-60 (-72)	globose, subglobose	50-60	3 layers	1 layers 0.5-1.0	2 layers 0.4-1.0, 1.5-2.0
Spain 2003 <i>Archaeospora trappei</i>	n.d.	n.d.	n.d.	2 walls	2 layers 0.5, 1.0	4 layers <0.5, 1.5-2.0, <0.5, 0.5
Hafeel 2004 <i>Archaeospora trappei</i>	82 x 95	Mostly globose	68-101	2 components	1 layer 1.0-2.0	2 layers 0.5-1.0, 1.5-3.0
Sieverding & Oehl 2006 <i>Intraspora schenckii</i>	45-75	Globose, subglobose, oblong	n.d.	2 walls, multiple layers	2 layers n.d.	3 “spore wall layers” n.d.
Walker <i>et al.</i> 2007 <i>Archaeospora trappei</i>	40-48 x 50-72	n.d.	n.d.	2 walls	1 component	bilayered component
<i>Intraspora schenckii</i>	40-48 x 50-72	n.d.	n.d.	2 walls	n.d.	n.d.

n.d.= no data reported.

Fig 1 – MrBayes phylogenetic tree reconstructed from partial 28S (LSU) gene sequences using the Kimura 2 parameter plus gamma (K2P + G) model. Maximum likelihood (ML) tree had identical topology. Bayesian posterior probabilities greater than 0.97 are depicted by thickened branches, and ML bootstrap values greater than 70 % are designated above the branches. Branch labels sequentially specify: source material, INVAM accession and (NCBI accession number). All new sequences *Ar. schenckii* CL401 sequences were generated from four single spore strains (ss1, ss2, ss3 or ss4). The tree was rooted by *Geosiphon pyriformis*.

Fig 2 – Equivalence in comparative spore morphology of *Archaeospora trappei* that develop from the hypha of a precursor sporiferous saccule, either laterally (acaulosporoid) or internally (entrophosporoid). Spores are hyaline, with a thin semi-flexible outer spore wall layer (*L1*), a more permanent, semiflexible second layer (*L2*) and a thicker flexible, finely laminate inner wall (*iw*) that form wrinkles and folds in crushed spores. The outer spore wall layer (*L1*) is continuous with the wall of the sporiferous saccule and subtending hyphal wall. Cicatrices, circular ridges or scars, may be detected at points of contact with the hypha subtending the saccule. Whole spores from *Ar. trappei* NB112 in water showing variation in acaulosporoid phenotype (A). Juvenile acaulosporoid spore (SP) forming laterally from the hypha subtending (SH) a sporiferous saccule (SS) from CL401 (B). Intact acaulosporoid spore with outer spore wall layer (*L1*) continuous with hyphal wall subtending a sporiferous saccule with fully differentiated second layer (*L2*) and inner wall (*iw*) from JA101C (C). Variation in acaulosporoid spore outer layer (*L1*) and hypha subtending a sporiferous saccule from AZ119A (D). Differentiated acaulosporoid spore with partially degraded spore wall outer layer (*L1*) and missing diagnostic hypha subtending a sporiferous saccule from MX116 (E). Juvenile entrophosporoid spore (SP) forming within the hypha subtending (SH) a sporiferous saccule (SS) from CL401 (F). Intact entrophosporoid spore within the hypha (*L1*) subtending a sporiferous saccule with second layer of the spore wall (*L2*) and inner wall (*iw*) from CL401 (G). Variation in differentiated entrophosporoid spore with partially degraded outer spore wall layer (*L1*) from CL401 (H). Differentiated entrophosporoid spore with partially degraded outer spore wall layer (*L1*) and missing diagnostic subtending hypha and saccule from CL401 (I). Bar A represents 100 μm Bars B–I represents 5 μm .

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CONCLUSIONS

A combination of morphological analysis of type specimens and live cultures and phylogenetic analysis of 28S rDNA (LSU) sequences was used to provide grouping and ranking criteria to merge species in *Ambispora* (*Ambisporaceae*) and *Archaeospora* (*Archaeosporaceae*).

Independent morphological and molecular data sets provided important criteria for distinguishing homologous characters that informed on common ancestry and exposed convergent characters that were not phylogenetically informative. Based on these combined data, four species (*Am. appendicula*, *Am. callosa*, *Am. fecundispora*, and *Am. jimgerdemannii*) were merged into *Am. leptoticha*; *Am. fennica* was merged into *Am. gerdemannii*; and *Ar. schenckii* was merged into *Ar. trappei*. The one other species in *Ambispora*, *Am. granatensis*, had a unique morphology and molecular signature but also shared the same phylogenetic patterns as three species in *Claroideoglossum*. Combined morphological and LSU data sets clarified species grouping and ranking criteria when given equal treatment. Glomoid spores in *Ambispora* provided resolution only at the genus level, whereas spore wall inner layers of acaulosporoid and entrophosporoid spores resolved species. Evolution of a sporogenous saccule was a convergent trait that evolved separately in *Ambispora*, *Archaeospora*, and in other AMF families.

LSU gene phylogeny from single spores of living cultures affirmed that spores produced by *Ambispora* and *Archaeospora* have the potential to be dimorphic. LSU sequences also produced divergent subclades within monophyletic groups that could be ranked definitively by the combination of morphological and molecular characters. Results from both studies show that rRNA genes in *Glomeromycota* are polymorphic, sometimes with sequence divergence within species equivalent to that between species of some AMF genera. The fixation of morphological and molecular characters in some populations may be explained by mutation events fixed rapidly due to genetic isolation and asexual mode of reproduction in haploid clonal organisms. The processes affecting the dynamics of polymorphic rRNA lineages within and among asexual species is poorly understood but encourages the search for other independent gene lineages that will clarify the biology and evolutionary relationships in *Glomeromycota*.

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RESEARCH EXPERIENCE

2007–2015 **Graduate Research Assistant**, *West Virginia University, Morgantown, WV.*

- Experience with molecular and morphological characterization of undescribed and described species of arbuscular mycorrhizal fungi (AMF) stored in the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM). Isolated, cultured and contributed more than 20 AM fungal species to the INVAM collection.
- Experience with maintenance of live AM fungal cultures and growing host plants in greenhouse and growth room environments. Hands-on experience using standard aseptic techniques working with soil micro-organisms.
- Hands-on experience developing methods for AMF spore extraction from cultures, DNA extraction, PCR, cloning, plasmid DNA preparation for sequencing, rDNA sequence alignments, and phylogenetics. Contributed more than 200 partial LSU rDNA gene sequences to the INVAM database. Experience using standard molecular aseptic techniques working with DNA.
- Hands-on experience in use of Nikon Eclipse E600 microscope equipped with DIC optics, Nikon DS-Ri1 digital camera and NIS-Elements imaging software.
- Experience training multicultural staff, international visitors, graduate and undergraduate students in microscope, molecular and fungal propagation methods.

2006–2007 **Research Technician**, *USDA Systematic Botany and Mycology Laboratory, Beltsville, MD.*

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2003–2006 **Graduate Research Assistant**, *Arizona State University*, Tempe, AZ

- Experience collecting, culture and identification of arbuscular mycorrhizal fungi.
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TEACHING EXPERIENCE

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- Experience teaching biology majors (160 students) the scientific method for hypothesis testing, experimental design, data collection, analysis, interpretation of data, and scientific writing.
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MEMBERSHIPS

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PUBLICATIONS

Bills RJ, Morton JB, *in prep.* Morphology and an LSU phylogeny reveal *Archaeospora trappei* and *Ar. schenckii* are synonymous and mode of spore formation resolves only stable population-level variation.

Bills RJ, Morton JB, 2015. Morphology and 28S rRNA gene sequences provide grouping and ranking criteria to merge eight into three *Ambispora* species (*Ambisporaceae*, *Glomeromycota*). *Mycorrhiza* 25(6): 485–498. doi:10.1007/s00572-015-0626-7.

Bills RJ, Stutz JC, 2007. AMF associated with indigenous and non indigenous plants at urban and desert sites in Arizona. Pages 207–220 In: *Mycorrhizas - Functional Processes and Ecological Impact*. (eds) C. Azcón-Aguilar, J.M. Barea, S. Gianinazzi, and V. Gianinazzi-Pearson. Springer-Verlag. Heidelberg.

PRESENTATIONS

Bills RJ, Stutz JC. Building a research experience: From morphotyping species of arbuscular mycorrhizae in and around Phoenix, Arizona to the molecular analysis of rust fungi from around the world. Systematic Botany and Mycology Laboratory, USDA Beltsville, MD. May 30, 2007

Bills RJ, Stutz JC. Arbuscular mycorrhizal fungal community associated with indigenous and non-indigenous plants in and around a desert city. Mid-Atlantic States Mycological Congress Beltsville, MD April 21, 2007

Bills RJ, Stutz JC. Arbuscular mycorrhizal fungal community associated with indigenous and non-indigenous plants in and around Phoenix, Arizona USA. 5th International Conference on Mycorrhiza, Granada, Spain. July 23–27, 2006.

Bills RJ. Mycorrhizal diversity and effects on brittlebush at a Sonoran Desert urban ecosystem. Thesis defense, Arizona State University, Tempe, Arizona. May 15, 2006.

Bills RJ. Effects of arbuscular mycorrhizal suppression on the productivity of *Encelia farinosa* (brittlebush) at an urban and a desert site. Global Institute for Sustainability, Arizona State University, Tempe, Arizona. November 14, 2005.

Bills RJ. Arbuscular mycorrhizal fungi: a fungal-plant mutualism in the upper Sonoran Desert and Phoenix, Arizona. Ecology Explorers K-12 Teacher Training Workshop, International Institute for Sustainability, Arizona State University, Tempe, AZ. June 28, 2005.

Bills RJ. Arbuscular mycorrhizal fungi in Phoenix: the fungal infection that doesn't itch. School of Life Sciences Graduate Student Brown Bag Seminar, Arizona State University, Tempe, AZ. April 1, 2005

Bills RJ. Arbuscular mycorrhizal fungi and the changing biodiversity between the Sonoran Desert and the Phoenix metropolitan area. 6th Annual Graduates in Earth, Life, and Social Sciences (GELSS) Research Symposium, Arizona State University, Tempe, AZ. February 11, 2005.

Bills RJ. AM fungal communities in the Phoenix metropolitan area and the adjacent Sonoran desert: a comparison. 4th Annual California-Oregon Mycorrhizal Meeting. Desert Studies Center, Zyzyx, CA. January 13–15, 2005.

Bills RJ. Arbuscular Mycorrhiza: The Rodney Dangerfield of the ecosystem. Ecology Explorers K-12 Teacher Training workshop, International Institute for Sustainability, Arizona State University, Tempe, AZ. June 21, 2004

POSTER PRESENTATIONS

Bills RJ, Morton JB. Morphology and an LSU phylogeny reveal *Archaeospora trappei* and *Ar. schenckii* are population level variants of one species. 8th International Conference on Mycorrhiza, Flagstaff, AZ. August 3–7, 2015.

Bills RJ, Morton JB. West Virginia University Graduate Student Research Competition, Morgantown, WV. April, 2013

Bills RJ, Morton JB. Combined morphological and molecular analyses to assess the community composition of glomeromycotan fungi in North American grasslands. International Conference of Mycorrhiza at Belo Horizonte, Brazil, August 9–14, 2009

Bills RJ, Stutz JC. Arbuscular mycorrhizal suppression and effects on the productivity of *Encelia farinosa* (brittlebush) at an urban and a desert site. 5th Annual Western Mycorrhizal Gathering, University of California Bodega Marine Laboratory, Bodega Bay, CA. February 10–12, 2006

Bills RJ, Stutz JC. Effects of arbuscular mycorrhizal fungal suppression on the productivity of *Encelia farinosa* (brittlebush) at an urban and a desert site. Central Arizona Phoenix-Long Term Ecological Research 8th Annual Poster Symposium. Tempe, AZ. January 19, 2006.

Bills RJ, Stutz JC. Arbuscular mycorrhizal fungal species richness is lower in the Phoenix metropolitan area in comparison to the surrounding desert. 7th Annual Central Arizona Phoenix-Long Term Ecological Research Symposium. Tempe, AZ. January 19, 2005.

Bills RJ, Whitcomb S, Cousins JR, Stutz JC. Comparisons between arbuscular mycorrhizal fungal communities in the Phoenix metropolitan area and surrounding desert. 89th Annual Meeting for the Ecological Society of America. Portland OR. August 1–6, 2004.

Bills RJ, Whitcomb S, Cousins JR, Stutz JC. Differences in arbuscular mycorrhizal fungal community structure at residential and desert land use types. Arizona State University East Graduate Student Poster Symposium, Mesa, AZ. April 16, 2004.

Bills RJ, Whitcomb S, Cousins JR, Stutz JC. Differences in arbuscular mycorrhizal fungal community structure at residential and desert land use types within the CAP LTER. 6th Annual Central Arizona Phoenix Long-Term Ecological Research Symposium. Tempe, AZ. February 23, 2004.

SERVICE

2014–present **Co-leader** 4th Grade Children’s Ministry, Chestnut Ridge Church, Morgantown, WV
2010–2013 **Coach**, Upward Youth Basketball, Morgantown, WV
2000–2003 **Literacy Tutor**, Truckee Meadows Community College, Department of Adult Basic Education, Reno, NV