Mycorrhiza (2012) 22:501–513
DOI 10.1007/s00572-011-0425-8

ORIGINAL PAPER

Comparison of morphological and molecular genetic quantification of relative abundance of arbuscular mycorrhizal fungi within roots

P. Shi · L. K. Abbott · N. C. Banning · B. Zhao

Received: 4 September 2011 / Accepted: 8 December 2011 / Published online: 10 January 2012

Abstract Nested PCR amplicons of ribosomal RNA genes have been used to identify individuals within assemblages of arbuscular mycorrhizal (AM) fungi in roots and to estimate their relative abundance. Microscopy has also been used to identify their relative abundance in roots, but only at low resolution, usually the genus level. We evaluated the robustness of using nested PCR amplicons of ribosomal RNA genes to estimate the relative abundance of undefined AM fungi in uniformly aged roots in comparison to visual estimates. The relative abundance of AM fungi was assessed as per cent root length colonised by morphotypes and relative sequence type abundance in clone libraries. Plants were grown in coastal soil to obtain assemblages of unknown AM fungi at two times (spring and autumn). Relative abundance of dominant genera of AM fungi in roots (*Archaeospora* and *Glomus*) based on an analysis of ribosomal RNA genes did not consistently correspond with relative abundance of morphotypes. This microscopic vs. molecular genetic comparison supports previous conclusions that there can be limitations in using nested PCR amplicons for quantifying the relative abundance of AM fungi in roots, with a sampling bias likely to be of significance. Both molecular genetic and morphological methods are used to estimate relative abundance of AM fungi as a precursor to understanding mycorrhizal function in field soils, but they are rarely verified using alternative approaches although this may be necessary.

Keywords Glomeromycota · Clone library · Microscopy · *Acacia cochlearis* · *Olearia axillaris*

Introduction

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are ubiquitous soil organisms that are important to overall biodiversity, productivity and stability of terrestrial ecosystems (Rosendahl 2008). Investigations of the spatial distribution of AM fungi have included estimates of the abundance and species diversity of spores or hyphae found in soil. However, spore number and diversity are not well correlated with root colonisation (Wilde et al. 2009). Furthermore, seasonal dynamics occur within AM fungal communities in roots (Santos-Gonzalez et al. 2007), and the number of AM fungi co-colonising the same root system can range from a few to many species (Table 1). Both species diversity and dynamics of relative abundance of AM fungal communities in roots is fundamental to understanding their roles in plant–fungal interactions (Mandyam and Jumpponen 2008).

At the genus level, AM fungi have been distinguished inside roots based on characteristic colonisation patterns (Merryweather and Fitter 1998a). This approach uses either a standard bait plant (Abbott 1982) or roots from the field (Merryweather and Fitter 1998a, b). Quantitative morphological descriptions (morphotypes) of root colonisation by AM fungi (Merryweather and Fitter 1998a) at genus level

Electronic supplementary material The online version of this article (doi:10.1007/s00572-011-0425-8) contains supplementary material, which is available to authorized users.

P. Shi · B. Zhao (✉)
State Key Laboratory of Agricultural Microbiology,
Huazhong Agricultural University,
Wuhan 430070, People's Republic of China
e-mail: binzhao@mail.hzau.edu.cn

P. Shi · L. K. Abbott (✉) · N. C. Banning
Soil Biology Group, School of Earth and Environment, UWA
Institute of Agriculture, Faculty of Natural and Agricultural
Sciences, The University of Western Australia,
Crawley 6009 WA, Australia
e-mail: lynette.abbott@uwa.edu.au

Table 1 Examples of published studies assessing the relative abundance of arbuscular mycorrhizal (AM) fungi in roots using standard PCR-based molecular genetic methods followed by cloning, sequencing, RFLP, T-RFLP or pyrosequencing approaches

Primer region	Method	Source of root samples	Relative abundance information used	Main findings	Reference
SSU	Cloning and sequencing	Roots from field	Yes	19 sequence types. Sequence Glo50a most widespread. Four most common sequence types were *Glomus* representing 65% sequences	Santos-Gonzalez et al. (2007)
ITS (nested)	Cloning and sequencing	Roots from field	Yes	7 families and species groups. *Glomus* group Ab dominant	Hempel et al. (2007)
LSU (hemi-nested)	Cloning and sequencing	Roots from field	Yes	8 sequence types, in *Glomus* groups A and B; >90% of sequences corresponded to *Glomus intraradices*	Cesaro et al. (2008)
SSU (nested)	Cloning and sequencing	Roots from field	Yes	113 different sequences, 4 plant species. Glo G2b was the most widespread. Glo G2c was distributed equally in three shrub species	Alguacil et al. (2011)
SSU (nested)	Cloning, RFLP and sequencing	Roots from field	Yes	9 phylotypes, 7 belonging to *Glomus* group A (GA). GA-1 phylotype dominant	Liu et al. (2011)
ITS (nested)	Cloning, RFLP and sequencing	Roots from field	Yes	9 AM fungal types; 1 type only found in the highly contaminated area. *Glomus* A predominant	Zarei et al. (2010)
LSU (nested)	T-RFLP, cloning and sequencing	Roots from field	Yes (also presence/absence analysis)	71 sequences types. *Glomus* Ab2 (*G. intraradices*) was the most frequently found	Verbruggen et al. (2010)
LSU (nested)	T-RFLP, cloning and sequencing	Roots from field	Yes	17 sequence types. Clades Glo1 containing two known *G. intraradices* sequences and dominant in all four plant species	Wu et al. (2007)
SSU	Pyro-sequencing	Roots from field	Yes (also presence/absence analysis)	48 Glomeromycota taxa. *Glomus* VT 113, most abundant taxon, representing 37.5% sequences	Opik et al. (2009)
SSU (semi-nested)	Pyro-sequencing	Roots from field	Yes	70 MOTUs of AM fungi. There were distinct AM fungal assemblages in each season	Dumbrell et al. (2011)

have been used to assess competitive interactions among AM fungi (Abbott and Robson 1984; Pearson et al. 1993), seasonal variation in colonisation by AM fungi (Scheltema et al. 1987) and success of field inoculation with AM fungi (Bell et al. 2003). The demonstration of seasonal dynamics of relative abundance of morphotypes of AM fungi inside roots in bluebells (*Hyacinthoides non-scripta*) from the field (Merryweather and Fitter 1998a, b) is a classical ecological study of AM fungi. Molecular analyses have confirmed the identity of the fungi at the same field site (Clapp et al. 1995; Helgason et al. 1999). In another study, Beck et al. (2007) compared the identity of AM fungi using DNA sequences and microscopic evaluation of morphotypes in the same roots and tentatively attributed two morphotypes to two rDNA sequence types of *Glomus* group A.

Species identification of AM fungi now relies primarily on phylogenetic sequence analysis (Gamper et al. 2009; Redecker et al. 2000). Several AM-specific polymerase chain reaction (PCR) primers have been designed using large subunit (LSU) and small subunit (SSU) rDNA sequences (Krüger et al. 2009). Most research has been based on a fragment of the SSU rDNA marker, amplified by the PCR primers NS31 and AM1 (Helgason et al. 1998) which amplify most, but not all, groups of AM fungi (Redecker et al. 2003). However, due to co-amplification of non-AM fungi and insufficient resolution, this methodology can be inadequate for species recognition in environmental samples (Stockinger et al. 2010). In contrast, the LSU rDNA region provides better resolution, allowing separation of closely related AM fungal taxa such as *Glomus caledonium* and *Glomus geosporum* (Rosendahl and Matzen 2008).

With respect to the accuracy of molecular genetic quantification of AM fungi, in contrast to their identification, there are unresolved issues (Robinson-Boyer et al. 2009). For example, Santos-Gonzalez et al. (2007) referred to the need to use clone libraries generated from mixtures of 18S rRNA cautiously because of potential sources of bias (such as PCR drift and selection), great variation in the AM fungal genome size and the number of copies of the rRNA gene among AM fungal species. For evaluations based on PCR and cloning, biases can be induced by template dosage and difference in binding energies, universal primer selectivity and phylogenic specificity, PCR amplicon size and preferential ligation of TA cloning systems (Huber et al. 2009; Junier et al. 2008; Lueders and Friedrich 2003; Palatinszky et al. 2011; Polz and Cavanaugh 1998). The study of Cesaro et al. (2008) illustrated the potential for cloning bias to occur during detection of AM fungi in soil. In this case, a fungus belonging to *Glomus* group A (*G. geosporum*) was not detected using the cloning approach. Furthermore, the size of DNA sample used for PCR amplication is commonly small (up to 30 cm of root fragments; Table 1) which could lead to a sampling bias.

Several studies have sought to verify molecular genetic quantification of AM fungi in roots based on PCR amplification of ribosomal RNA genes under experimental conditions (Alkan et al. 2004; Isayenkov et al. 2005). In the study by Alkan et al., two approaches for verifying the amount of *Glomus intraradices* DNA detected in roots were tested. First, host roots were spiked with fungal DNA, and second, colonised and non-colonised roots were mixed to establish a series of colonisation levels. There was a good correlation between estimates of *G. intraradices* in roots based on qRT-PCR quantification and classical morphological estimates. The authors stated that it was not intended to replace morphological with the qRT-PCR method of quantification, but rather to use the molecular approach to enable rapid analysis of root samples based on the relationship between qRT-PCR quantification and morphological assessment. Subsequently, it was shown that real-time PCR used for quantifying AM fungal nucleic acids may be poorly correlated with visual measurements of colonisation because spatial heterogeneity could lead to the sampling bias (Gamper et al. 2008). An investigation of the relationship between molecular genetic and morphological estimates of the extent of colonisation of roots by different AM fungi for unknown assemblages associated with naturally occurring plant communities could contribute to understanding the robustness of each approach.

The relative abundance of AM fungi in roots varies with time (e.g. Pearson et al. 1993) and this has functional significance (Jansa et al. 2008). There are many examples of estimates of the relative abundance of AM fungi in roots which have been made using PCR-based molecular genetic methods followed by cloning, sequencing, restriction fragment length polymorphism (RFLP), terminal fragment length polymorphism (T-RFLP) or pyrosequencing approaches (see Table 1). Nested PCR has commonly been conducted to enlarge the amount of AM fungal DNA for downstream analysis in these studies using different primers (LSU or SSU rDNA). PCR amplicons were then used for cloning and RFLP, T-RFLP analysis or sequencing directly (such as pyrosequencing). In these studies, the relative abundance of AM fungi was estimated in terms of the proportion of clones or sequences representing different genotypes.

In contrast to the high resolution of molecular genetic methods for identification of AM fungi (e.g. Alkan et al. 2006), discrimination among of morphotypes of AM fungi colonising roots from a soil community is generally limited to genus level (Alkan et al. 2006; Pearson et al. 1993). Mycorrhizal colonisation is usually recorded as % root length colonised (Smith and Read 2008). Estimates of the length of root colonised (Tawaraya et al. 1999) is sometimes also reported, but the density of fungus within roots (Gazey et al. 1992; Plenchette and Morel 1996) is seldom assessed. Therefore, estimates of root colonised by AM fungi based

on morphological estimates do not usually quantify the actual amount of fungus present.

The function of AM fungal assemblages is of considerable significance in terrestrial ecology (Gamper et al. 2010) but it is not well quantified. Quantification of AM fungi within roots rarely includes fungal activity or its variability over time (Dumbrell et al. 2011; Jansa et al. 2008; Zhao et al. 1997). Since the activity of AM fungi in roots is important, the reliability of estimates of the relative abundance of AM fungi is also important because of their functional diversity. Therefore, we investigated the robustness of commonly used microscopic and molecular genetic approaches for estimating the relative abundance of AM fungi in roots by using unknown assemblages associated with coastal dune vegetation.

We tested the hypothesis that estimates of relative abundance of genera of AM fungi inside roots based on an analysis of ribosomal RNA genes correspond well with morphological (microscopic) estimates. We tested this hypothesis using the most common method for quantifying the abundance of AM fungi in roots (proportion of roots colonised (see Smith and Read 2008)) with discrimination at genus level (Abbott 1982), and a commonly used molecular genetic method for detecting and quantifying the presence of AM fungi in roots (nested PCR amplicons of ribosomal RNA genes; see Table 1). The mycorrhizal roots used to test this hypothesis were from uniformly aged root systems of subterranean clover bait plants grown in coastal sand dune soil. There had been no previous study of mycorrhizal diversity at this site. The evaluation was repeated using roots from bioassays conducted 5 months apart. As a cultivation bias can influence the community structure of AM fungi in roots (Sýkorová et al. 2007), the comparisons made here represent only those AM fungi in the field soil that were infective (and colonised subterranean clover roots) at the time the soil was sampled.

Materials and methods

Study site and experimental design

The study was conducted using plant and AM fungal communities at Yalgorup National Park (32°41′ S; 115°38′ E). This park forms part of the Quindalup Dune coastal ecosystem in south-west Western Australia with two dominant native understorey plant species, *Acacia cochlearis* (Labill.) and *Olearia axillaris* (DC) Benth.

A bait plant, subterranean clover (*Trifolium subterraneum* L.), was grown in undisturbed soil cores (11 cm in diameter and 11 cm in height) collected from the field. The first of the bioassays (Djuuna et al. 2009) used soil cores collected under *A. cochlearis*, and the second bioassay used soil cores from under *A. cochlearis* and *O. axillaris* collected 5 months later. Roots from the two bioassays contained unknown assemblages of AM fungi for quantification using either nested PCR amplicons of ribosomal RNA genes or microscopy.

Sampling and bioassay

In order to compare methodologies for estimating the relative abundance of AM fungi in root systems of similar age, subterranean clover was planted in 12 undisturbed soil cores collected under three replicate *A. cochlearis* plants (four cores per plant) in November (late spring) 2008. In the following autumn, this was repeated for 18 cores collected from under another three replicate *A. cochlearis* and *O. axillaris* plants (three cores per plant) in April (early autumn) 2009. This provided the source of an unknown assemblage of AM fungi within root systems of subterranean clover of a similar age for comparative analysis.

The soil cores were transferred to plastic–lined undrained pots and maintained in a temperature-controlled water tank at 20°C in a glasshouse. Basal nutrients (Abbott and Robson 1978) were added to the surface of the soil in each pot prior to maintaining the soil at 60% field capacity. Four subterranean clover plants were grown in each soil core and harvested after 6 weeks. Roots were washed to remove attached soil, cut into 1–2-cm fragments and frozen at −20°C for DNA extraction after sub-sampling for morphological assessment.

DNA extraction, PCR amplification and cloning

The remaining root fragments (excluding the subsamples for morphological analysis) were further cut into segments several mm long at the time of harvesting the bait plants and prepared for molecular analysis. Three replicates of 100–200 mg were collected randomly from well-mixed root fragments retained for the molecular analysis (Jansa et al. 2008). The total DNA was extracted from these roots using the PowerPlant™ DNA Isolation Kit (MO BIO, USA) and was also extracted from leaves of the subterranean clover plants as a control for non-specific amplification of eukaryotic host plant DNA.

Among several tested dilutions of the extracted DNA (1/5, 1/10, 1/20, 1/40, 1/100, 1/500 and 1/1000), the 1/40 dilution was selected for amplification with the primer pair ITS1 (White et al. 1990) and NDL22 (Van Tuinen et al. 1998) targeting eukaryotes. Reactions were performed in a final volume of 20 μl containing 1×NH_4 buffer (Bioline), 1 mM dNTP (Bioline), 0.4 μM of each primer, 0.5 U of BIOTAQ DNA polymerase (Bioline), 1.5 mM $MgCl_2$ solution and approximately 50 ng of total genomic DNA from roots. PCR was performed in a thermal cycler (Eppendorf Mastercycler, Germany), programmed as follows: initial denaturation cycle at 93°C (3 min), annealing at 55°C (40 s), extension at 72°C (50 s), followed by 30 cycles of

denaturation at 93°C (40 s), annealing at 55°C (40 s) and extension at 72°C (50 s). The last cycle was followed by a final extension at 72°C for 10 min. A 5-μl volume of the first PCR amplification was diluted 1/1,000 and used as a template for the second AM fungus-specific reaction with the primers pair LR1 (Van Tuinen et al. 1998), and FLR4 (Gollotte et al. 2004). Amplification conditions were as described above, except that 33 amplification cycles were run. Leaf DNA did not amplify with this nested PCR approach. Amplification products from the same root sample were pooled prior to cloning (Renker et al. 2006) using the p-GEM-T Easy Vector System (Promega).

Clone library screening by RFLP analysis and sequencing

Clones were checked for inserts of the correct size (approx. 700 bp) by PCR re-amplification with the primers LR1 and FLR4. RFLP analyses were carried out by independent digestions with *Mbo*I and *Taq*I, the restriction endonucleases found most suitable for discriminating AM fungi (Mummey and Rillig 2007). The extracted plasmids were digested by *Taq*I directly, while purified PCR products amplified by LR1 and FLR4 were digested by *Mbo*I (as the vector does not contain any *Mbo*I restriction sites).

The number of different RFLP patterns increased with the number of clones up to about 20 analysed clones. Therefore, 25–30 clones were analysed by RFLP analysis and RFLP patterns were classified by analyzing the restriction map on the electrophoretogram. Representatives of each RFLP pattern (65 clones from the spring bioassay and 89 clones from the autumn bioassay) were chosen for plasmid sequencing at MACROGEN (Korea) using the vector primers SP6 and T7 and BigDye v 3.1 chemistry (applied Biosystems). Then the RFLP patterns were defined by restriction on soft Primer 5 (Table S1).

Phylogenetic analyses

Per cent identities were determined using the BLASTN sequence similarity search tool (Altschul et al. 1997) on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were carried out on the newly generated sequences and corresponding closest match reference sequences from the public databases. Sequences were aligned in MEGA version 4 (Tamura et al. 2007) by ClustalW (Thompson et al. 2002) and analyzed by neighbour-joining (NJ) analyses (Saitou and Nei 1987). NJ trees and associated bootstrap branch support values were calculated from 1,000 pseudo-replicate datasets. All new sequences belonged to AM fungi and were deposited in GenBank with the accession numbers: GQ896283-GQ896331, HQ128635-HQ128705. Percent sequence identity cut-offs for the phylogenetic groups Glo1, Glo2 etc. (Fig. 1) were ≥94%. Phylogenetic groups of AM fungi were defined based on sequence similarity to known species, tree topology and a bootstrap value of >50%.

Mycorrhizal assessment using light microscopy

Approximately 0.5 g of randomly sub-sampled root fragments were cleared and stained with Trypan blue (Abbott and Robson 1981). Percentage of root length colonised by AM fungi was estimated using a line intersect method for assessing root length (Newman 1966). All of the colonised root fragments from each sample were separately mounted onto microscope slides (40–50 root fragments per slide). Each slide was scanned to intercept and score each root once under a compound microscope at ×200 and ×400 magnification. The identification of each morphotype of AM fungi was based on previously described morphological characteristics (Abbott 1982; Abbott and Robson 1978; Brundrett et al. 1996) or as observed in the single fungal species pot cultures. Percentage of root length colonised by different morphotypes was assessed to determine the relative abundance of AM fungal morphotypes within roots.

There is known similarity in morphotype characteristics for some species of *Archaeospora* and *Acaulospora* (Morton and Redecker 2001). Therefore, for verification of the morphological characteristics of AM fungi observed in roots, spores from *Archaeospora* and *Glomus* were isolated from soil collected adjacent to the intact soil cores and used to prepare pot cultures (Abbott 1982).

Comparison of molecular and morphological analyses of relative abundance of AM fungi in roots

For molecular analysis, the relative abundance of genera of AM fungi was based on the frequency of clones classified into three genera. For morphological analysis, the relative abundance of genera of AM fungi was based on the percentage of root length colonised by observed morphotypes. This included over-lapping colonisation in some root segments by different AM fungi.

Statistical analysis

A linear mixed model approach was used to determine if there were significant differences between the morphological and molecular genetic approaches used for estimating the relative abundance of *Archaeospora* and *Glomus* in roots. The comparison was made for each plant separately in order to determine whether differences occurred as either an increase or a decrease with respect to each other. Fixed effects of plant and assessment method, together with their interaction were used with the random effect of replicate. Analyses were performed on arc sine square root transformed data and p values are

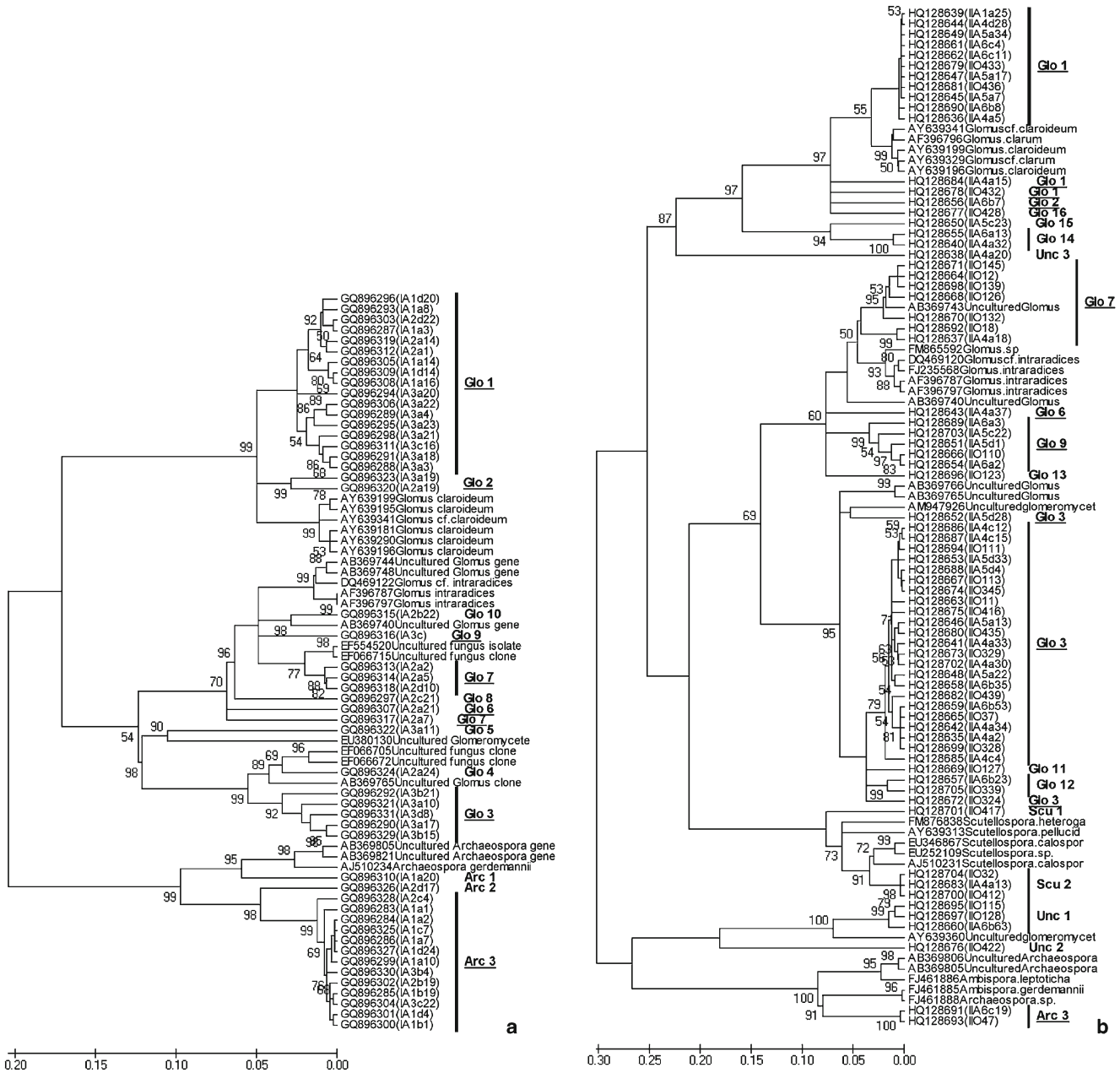

Fig. 1 Neighbour-joining (NJ) phylogenetic tree showing the relationships among the arbuscular mycorrhizal fungal phylotypes, inferred from partial nuclear ribosomal DNA sequences of the large subunit gene. Indicated bootstrap values of >50% are based on 1,000 replicates. Groups were defined as Glo 1–16, Arc 1–3, Scu 1–2 and Unc 1–3. The new sequences correspond to individual RFLPs, and their origin (behind the accession number), whose origins and numbers are indicated in parentheses. Groups with underline were found in both bioassays. *I* spring bioassay, *II* autumn bioassay, A. *A. cochlearis*, O. *O. axillaris*. **a** NJ tree with the AM fungal sequences obtained from the spring bioassay; **b** NJ tree with the AM fungal sequences obtained from the autumn bioassay. *Scale bars* indicate the average number of nucleotide substitutions per site

presented for differences of means between the two measurements (morphological and molecular genetic). Significance differences among data were examined separately for the two bioassays. For *Scutellospora*, there was very little colonisation (either no colonisation or low levels of colonisation). Therefore, only summary statistics for this genus could be included.

Results

Molecular genetic analysis of mycorrhizal roots

In the first bioassay (spring), 314 positive clones from root systems of 12 soil cores were used to determine

the richness of AM fungi in roots, resulting in 50 RFLP patterns after digestion by *Taq*I and *Mbo*I separately (Table S1). In the second bioassay (autumn), 376 positive clones from 18 soil cores resulted in 60 RFLP patterns after digestion (Table S1).

AM fungi detected in roots of bait plants grown in soil from under *A. cochlearis* with molecular analysis of ribosomal RNA genes belonged to DNA sequences affiliated with two genera, *Glomus* and *Archaeospora* (first bioassay; Fig. 1a). DNA sequences corresponding with *Scutellospora* were detected only in the second bioassay (Fig. 1b). Most sequences belonged to *Glomus* (Table S2), based on a phylogenetic analysis (alignment with the most similar sequences deposited in GenBank; Fig. 1). *Glomus* phylotypes, labelled as "Glo", were more diverse than those of *Archaeospora* (Arc) and *Scutellospora* (Scu). The community of AM fungi from the first bioassay was dominated by two phylotypes (Glo1 and Arc3), one from *Glomus* and the other from *Archaeospora* (Table S2). The dominant phylotypes of AM fungi in the second bioassay were Glo1 and Glo3, which were also detected in the roots from the first bioassay. In contrast to the first bioassay, few sequences belonging to *Archaeospora* were obtained in the second bioassay (Table S2). Three sequence groups detected in the second bioassay could not be ascribed to a genus and thus were labelled as unclassified (Unc).

Only a few sequence groups in our investigation clustered with sequences of defined species or isolates. Sixteen phylogenetic groups matched *Glomus* and three corresponded with *Archaeospora* (Fig. 1). Seven phylogenetic groups were detected in both bioassays. Five phylogenetic groups (three in *Glomus* and one in *Scutellospora*) were obtained only in the second bioassay plants grown in the soil collected under *O. axillaris* (Fig. 1b). In spring bait plants, *Glomus* sp. (Glo1) resembling *Glomus claroideum* and *Archaeospora* (Arc3) resembling *Archaeospora gerdemanii* were dominant (Table S2). In autumn bait plants, *Glomus* sp. (Glo3) with no close relative in the databases and *Glomus* sp. (Glo1) resembling *Glomus claroideum* were dominant (Table S2).

Microscopic root colonisation measurements

The dominant morphotypes of AM fungi within roots from both bioassays corresponded with those of *Glomus* and *Archaeospora* (Fig. 2). *Glomus* and *Scutellospora* morphotypes resembled those of AM fungi previously characterised (Abbott 1982; Abbott and Robson 1978; Merryweather and Fitter 1998a). The *Archaeospora* morphotype resembled an *Acaulospora* morphotype described by Abbott (1982) but without vesicles, as recorded for *Archaeospora* by Morton and Redecker (2001, their comparative Table 1). Missing vesicles were also verified for pot cultures established from field-collected spores resembling *Archaeospora* (acaulosporoid form).

Based on colonisation of roots from the soil collected for the first bioassay, infective hyphae of the *Archaeospora* morphotype were dominant under one of the *A. cochlearis* plants (Fig. 3a), whereas infective hyphae of the *Glomus* morphotype were dominant under the other *A. cochlearis* plants (Fig. 3a). A low level of co-colonisation by morphotypes was observed (Fig. 3a).

In the second bioassay, the *Glomus* morphotype was less abundant than in the first bioassay with more frequent occurrence of the *Archaeospora* morphotype, not only under *A. cochlearis* but also under *O. axillaris* (Fig. 3b). Co-colonisation of both morphotypes was more frequently found in the second bioassay compared with the first bioassay (Fig. 3b).

Comparison of the estimation methods for relative abundance of AM fungi in roots

There were instances where molecular genetic analysis did not detect the presence of an AM fungus which was observed under the microscope (Table 2, see e.g. *Glomus* in core IA1c, *Archaeospora* in cores IA2a, IA3a, IIA4a–6b and *Scutellospora* in cores IA1d, IA2b, IA3a–d, IIA5b and IIA6a).

Overall, there was no consistent correspondence between the relative abundance of AM fungi inside roots based on a comparison of the two approaches used (Tables 2 and 3). Marked differences were found in the estimated relative abundances of the two dominant genera, *Archaeospora* and *Glomus*, in some but not all of the bait plants (Table 2). The dominance of genera of AM fungi determined by either morphological or molecular genetic approaches differed considerably (Table 2). For example, microscopic assessment showed that the *Archaeospora* morphotype was sub-dominant (<10%) in bait plants from cores IA2b, IA3b, and IA3d, whereas molecular genetic analysis found it to be dominant (>64%) in roots from the same soil cores.

Differences in relative abundance of the two dominant AM fungal genera as determined by these two methods (microscopic quantification and clone enumeration) were significant in most cases (Table 3), illustrating a degree of methodological inconsistency. For example, the two methodologies differed in five of the six instances for *Archaeospora*. In the first bioassay root samples, microscopy underestimated the abundance of *Archaeospora* relative to the molecular genetic quantification, but in second bioassay, the abundance of *Archaeospora* was overestimated in comparison. For *Glomus*, three out of six comparisons were significantly different. Furthermore, the relative quantification biases in the two seasonal bioassays for *Glomus* were opposite to those for *Archaeospora*.

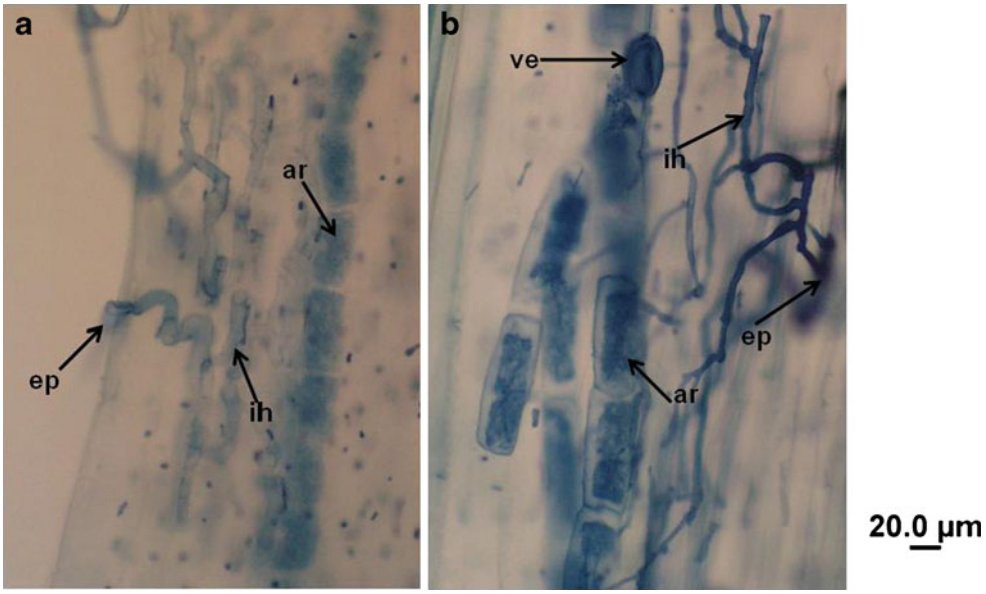

Fig. 2 AM fungal colonisation of roots of subterranean clover (*T. subterraneum*) bioassay plants grown in cores around *A. cochlearis* at Yalgorup, Western Australia. *Scale bar*, 20 µm. **a** *Archaeospora* morphotype; **b** *Glomus* morphotype. Differences in the characteristics of both morphotypes are indicated in structures such as entry points (*ep*), intercellular hyphae (*ih*), arbuscules (*ar*) and vesicles (*ve*)

Discussion

Contrary to our hypothesis, estimates of the relative abundance of AM fungal genera in roots of bait plants colonised by unknown assemblages from field soil were not consistent when they were assessed using either nested PCR amplicons of ribosomal RNA genes or light microscopy approaches. Furthermore, in some cases, even the detection of some AM fungal genera and the dominant genus present in roots according to these two methods were not consistent. Discrepancies occurred for roots examined in spring and autumn. A similar finding was found in a study of ectomycorrhizal fungi (Horton and Bruns 2001) in which use of morphological and molecular approaches demonstrated deficiencies in use of either approach alone, limiting understanding of complex ecological questions.

Our investigation further highlights the risk that estimates of the relative abundance of AM fungi in roots (even at a genus level) using molecular analysis of ribosomal RNA genes including nested PCR, cloning and RFLP may need to be verified. Molecular genetic analysis of ribosomal RNA genes have usually be used to estimate relative abundance of AM fungi in roots at species level. Our study has shown that even when the species are aggregated at genus level, quantification of the relative abundance of AM fungi may not consistently match the relative proportions visible within stained roots from the same root systems.

Although molecular tools (cloning, RFLP, T-RFLP, etc.) are now considered robust for identifying AM fungi diversity (Schechter and Bruns 2008), the application of these methodologies to quantification of AM fungi in roots and soil needs to be approached with caution, especially with respect to the adequacy of sampling. Studies employing this approach commonly use up to 30 cm of root fragments for DNA extraction, but this may not always be sufficient. Sampling inadequacies have previously been recognised when small amounts of root are used for DNA extraction in order to avoid interference by polysaccharides, phenolics and other secondary metabolites (Robinson-Boyer et al. 2009). Although in our study, the total amount of root used for DNA extraction was nearly equal to that used for morphological analysis, other bias may also exist. For example, a sampling bias may be introduced when there is an uneven fungal distribution within roots (Gamper et al. 2008). This also applies to real time PCR assays which have been shown to be effective for quantification of AM fungal nucleic acids in roots (Gamper et al. 2008). An uneven distribution of AM fungi in roots can introduce a similar bias for visual estimates of AM fungi, and this could be avoided by assessing a larger root sample with adequate replication.

PCR amplification of DNA extracted from environmental samples with diverse and unknown sequences and unknown concentrations may be exposed to several kinds of bias (Lueders and Friedrich 2003). The amount of AM fungal DNA in different kinds of extracts could lead to substantial differences in the amplification of target regions (Hempel et al. 2007). Thereafter, sequences may be more readily amplified if they are common and have an optimum sequence match. This can lead to bias in estimates of their relative abundance. Alternatively, when the amount of DNA from an organism is low and has a low homology to the primers used, it is likely to be kinetically out-competed during PCR (Polz and Cavanaugh 1998).

For root samples, the amount of AM fungal DNA is very small compared with plant DNA and the use of a nested PCR approach is usually necessary for AM fungal molecular assays, although this may compound potential PCR-bias (Junier et al. 2008). Quantitative (real-time) PCR, which

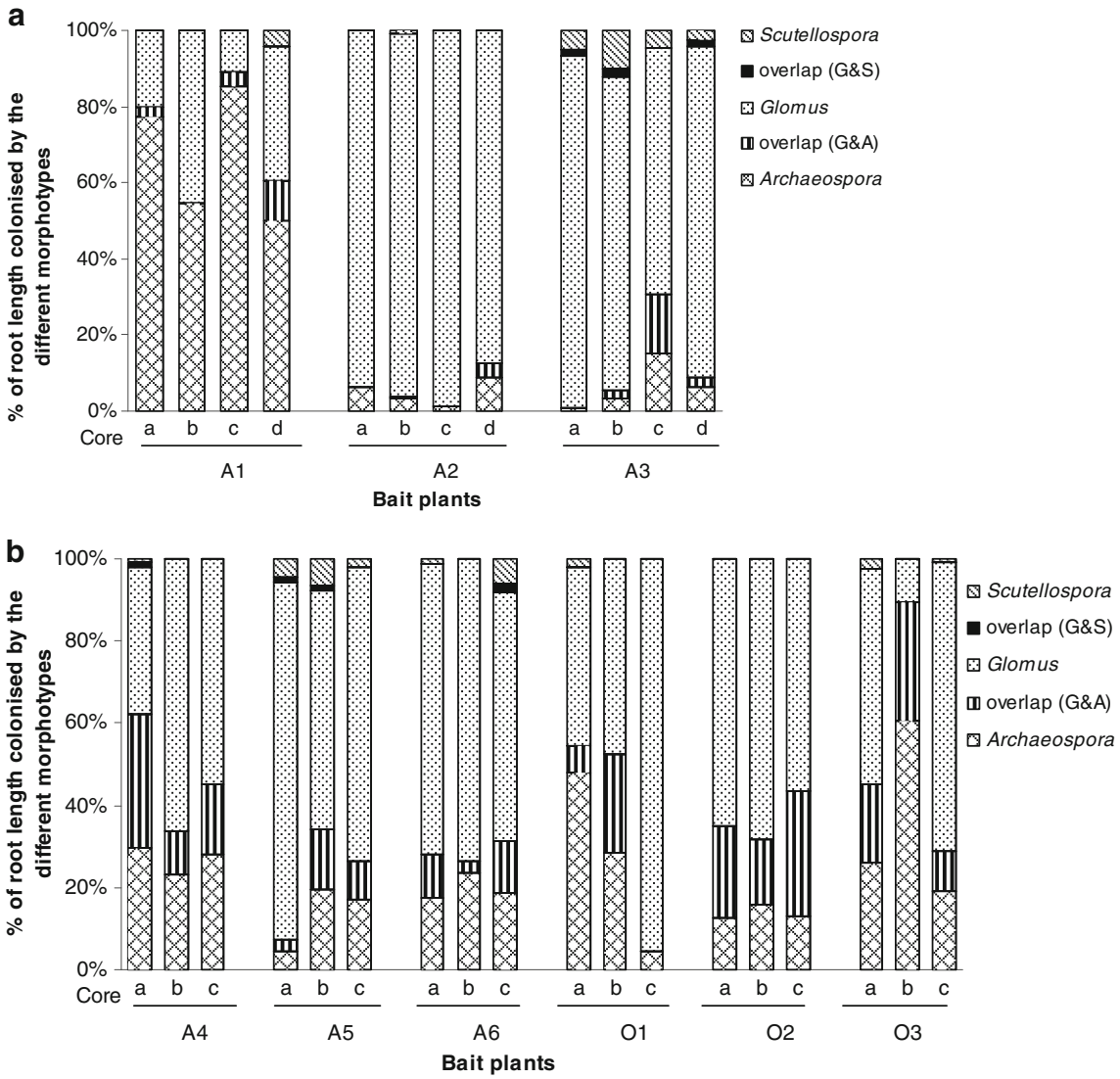

Fig. 3 Relative abundance of AM fungal morphotypes within roots of subterranean clover (*T. subterraneum*) bioassay plants grown in soil cores collected under either *A. cochlearis* (*A1–6*) or *O. axillaris* (*O1–3*) at Yalgorup, Western Australia. **a** Spring bioassay and **b** Autumn bioassay. Overlap (*G&S*): roots co-colonised by both *Glomus* and *Scutellospora*. Overlap (*G&A*): roots co-colonised by both *Glomus* and *Archaeospora*

detects amplicons during the early exponential phase of PCR, can be used to ensure there is a better proportional relationship between the amount of amplified product and the starting template (Jansa et al. 2008). Indeed, Alkan et al. (2004) validated the use of qRT-PCR using *G. intraradices* by comparison with microscopic assessment of colonisation. Good correlations were obtained between the two methods. The extension of this approach to field samples of unknown AM fungal communities would depend on the availability of suitable primers, but it also has the advantage of developing molecular quantitative methods for distinguishing between living and dead fungal components (Alkan et al. 2006).

Another potential bias applicable to all ribosomal DNA-based analyses is the existence of multiple variants in ribosomal gene copy number within an AM fungal population (Corradi et al. 2007). The PCR-cloning approach used in our study is reliant on adequate coverage of the primers used and all PCR-based approaches require continual improvement in primer design as more sequences are deposited into the database. A lack of AM fungal LSU rDNA in the databases has limited primer design, and indeed, primer FLR4 has been shown to have multiple mismatches to *Archaeospora gerdemannii* (Mummey and Rillig 2007). However, potential differences in primer coverage of AM fungi from *Archaeospora* and *Glomus* detected in our study did not appear to explain the discrepancy with morphological observations.

Recently, massively parallel pyrosequencing has been used to estimate the relative abundance of AM fungi without the process of cloning (Opik et al. 2009; Verbruggen et al.

Table 2 Colonisation of roots by arbuscular mycorrhizal fungal genera in bioassay plants

Core	Morphological analysis			Molecular analysis			
	Archaeospora	*Glomus*	*Scutellospora*	*Archaeospora*	*Glomus*	*Scutellospora*	Unclassified
IA1a	80	23	0	76	24	0	0
IA1b	55	45	0	96	4	0	0
IA1c	89	15	0	100	0	0	0
IA1d	60	46	4	84	16	0	0
SEM	4.0	3.9	0.5	2.8	2.8		
IA2a	6	94	0	0	100	0	0
IA2b	4	96	1	84	16	0	0
IA2c	1	99	0	52	48	0	0
IA2d	13	91	0	80	20	0	0
SEM	1.3	0.8	0.1	9.7	9.7		
IA3a	1	94	7	0	100	0	0
IA3b	5	86	12	64	36	0	0
IA3c	31	80	5	76	24	0	0
IA3d	8	91	4	88	12	0	0
SEM	3.4	1.5	0.9	9.8	9.8		
IIA4a	64	70	1	0	86	10	4
IIA4b	34	77	0	0	100	0	0
IIA4c	45	72	0	0	100	0	0
SEM	5.1	1.2	0.2		2.7	1.9	0.8
IIA5a	7	91	6	0	96	4	0
IIA5b	34	74	8	0	100	0	0
IIA5c	26	81	2	0	96	4	0
SEM	4.6	2.8	1.0		0.8	0.8	
IIA6a	28	81	1	0	96	0	4
IIA6b	26	76	0	0	96	0	4
IIA6c	32	75	8	3	84	13	0
SEM	1.0	1.1	1.5	0.6	2.3	2.5	0.8

For morphological analysis, data are percentage of root length colonised. For molecular genetic analysis, data are frequency of clones classified into three genera

I Data from the spring bioassay (November 2008); *II* data from the autumn bioassay (April 2009), *SEM* standard error of the mean

2010). It has been claimed that this technique has the potential to overestimate diversity as a result of sequencing errors and inappropriate bioinformatics analyses that may fail to remove the high number of singletons arising from these errors (Dumbrell et al. 2011; Opik et al. 2009).

Table 3 Mean percentage difference and *p* value for significance of difference between morphological and molecular measurements

Bioassay	Plant	Genera of AM fungi		
		Archaeospora	*Glomus*	*Scutellospora*
I	A1	−18.0 (0.219)	21.3 (0.193)	1
	A2	−48 (0.024*)	49 (0.034*)	0.25
	A3	−45.8 (0.033*)	44.8 (0.073)	7
II	A1	47.7 (<0.001**)	−22.3 (0.006**)	−3
	A2	22.3 (0.001**)	−15.3 (0.028*)	2.7
	A3	22.7 (0.001**)	−14.7 (0.071)	−1.3

*p value significant at 5% level; **p value significant at 1% level (n=4)

Furthermore, bias may still occur because PCR or nested PCR are applied before pyrosequencing analysis.

The use of microscopy to quantify morphotypes of AM fungi inside roots also has limitations (Abbott 1982). For example, hyphae of AM fungi may differ in staining intensity (Brundrett et al. 1996), leading to difficulties in their detection, especially when co-colonised with strongly staining fungi (Merryweather and Fitter 1991). As already mentioned, further discrepancy can arise because the density of hyphae of AM fungi is usually ignored when the length of root colonised is assessed (Abbott and Gazey 1994; Gazey et al. 1992; Jansa et al. 2008). In addition, hyphae attached to the surface of roots, which may be included in the total DNA quantified, are likely to be removed during preparation for microscopy leading to a quantitative difference in measurement of the relative abundance of AM fungi. More accurate techniques for enumeration of all structures present in the roots (hyphae, vesicles, spores and arbuscules) and the use of large samples composed of root fragments smaller than 1 cm long could be considered (see Gamper et al. 2008).

Usually, there is no independent evaluation of estimates of the relative abundance of AM fungi in roots using either molecular genetic analysis of ribosomal RNA genes for fungi inside roots or microscopy. Our microscopic vs. molecular generic comparison of relative abundance of AM fungi from a field community supports previous conclusions (Robinson-Boyer et al. 2009) that there are limitations in use of nested PCR amplicons and cloning approach for quantifying the relative abundance of AM fungi in roots. Where bias is related to adequacy of sampling, limitations will also apply to other molecular approaches unless sampling errors are identified and specifically excluded.

Quantification of different AM fungi in roots is a precursor to understanding their dynamics and roles under field conditions, including their influence on plant physiology and community structure. Previous field studies have demonstrated spatial heterogeneity of AM fungi based on either morphological (e.g. Brundrett and Abbott 1995) or molecular (e.g. König et al. 2010) approaches. Therefore, we specifically sourced roots with expected natural variability in our comparison of two commonly reported approaches to quantification of AM fungi in roots. We showed that an estimate of dominance of both *Archaeospora* and *Glomus* in root samples from uniformly aged plants growing in the same soil core may vary according to the method of analysis. In our investigation of the fundamental question of the accuracy of quantification of relative abundance of AM fungi in roots grown in field soils, our data highlight the need for caution and / or verification. As discussed above, there are several sources of variability in quantification of AM fungi within roots using both molecular and morphological methods. These sources of variability are of particular concern when quantifying the relative abundance of different AM fungi within roots and supports the conclusion of König et al. (2010) that abundance data based on molecular techniques need to be interpreted carefully in relation to estimations of the diversity and community structure of arbuscular mycorrhizal fungi.

Acknowledgements This research was enabled by the support of the Australian Research Council and Linkage grant partner Alcoa of Australia under project LP07765963 lead by Associate Professor Daniel Murphy and the China Scholarship Council under the State Scholarship Fund. We thank Drs. Yoshi Sawada and Mark Dobrowlski for assistance during the sampling, Dr. Zakaria Solaiman for technical advice on mycorrhizal morphology, Dr. Kevin Murray for statistical analysis and Dr. Ian Phillips for project support. A sampling permit was provided by the Department of Environment and Conservation, Western Australia. We appreciate the valuable comments on our manuscript made by Drs. Hayley Ridgeway, Deirdre Gleeson, Ian Abbott and anonymous reviewers.

References

Abbott LK (1982) Comparative anatomy of vesicular arbuscular mycorrhizas formed on subterranean clover. Aust J Bot 30:485–499

Abbott LK, Gazey C (1994) An ecological view of the formation of VA mycorrhizas. Plant Soil 159:69–78

Abbott LK, Robson AD (1978) Growth of subterranean clover in relation to the formation of endomycorrhizas by introduced and indigenous fungi in a field soil. New Phytol 81:575–587

Abbott LK, Robson AD (1981) Infectivity and effectiveness of 5 endomycorrhizal fungi—competition with indigenous fungi in field soils. Aust J Agr Res 32:621–630

Abbott LK, Robson AD (1984) Colonization of the root system of subterranean clover by three species of vesicular–arbuscular mycorrhizal fungi. New Phytol 96:275–281

Alguacil MM, Torres MP, Torrecillas E, Díazb G, Roldána A (2011) Plant type differently promote the arbuscular mycorrhizal fungi biodiversity in the rhizosphere after revegetation of a degraded, semiarid land. Soil Biol Biochem 43:167–173

Alkan N, Gadkar V, Coburn J, Yarden O, Kapulnik Y (2004) Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. New Phytol 161:877–885

Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Appl Environ Microb 72:4192–4199

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Beck A, Haug I, Oberwinkler F, Kottke I (2007) Structural characterization and molecular identification of arbuscular mycorrhiza morphotypes of *Alzatea verticillata* (Alzateaceae), a prominent tree in the tropical mountain rain forest of South Ecuador. Mycorrhiza 17:607–625

Bell J, Wells S, Jasper DA, Abbott LK (2003) Field inoculation with arbuscular mycorrhizal fungi in rehabilitation of mine sites with native vegetation, including *Acacia* spp. Aust Syst Bot 16:131–138

Brundrett MC, Abbott LK (1995) Mycorrhizal fungus propagules in the jarrah forest.2. Spatial variability in inoculum levels. New Phytol 131:461–469

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. Australian Centre for International Agricultural Research, Canberra, A.C.T.

Cesaro P, van Tuinen D, Copetta A, Chatagnier O, Berta G, Gianinazzi S, Lingua G (2008) Preferential colonization of *Solanum tuberosum* L. roots by the fungus *Glomus intraradices* in arable soil of a potato farming area. Appl Environ Microb 74:5776–5783

Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265

Corradi N, Croll D, Colard A, Kuhn G, Ehinger M, Sanders IR (2007) Gene copy number polymorphisms in an arbuscular mycorrhizal fungal population. Appl Environ Microb 73:366–369

Djuuna IAF, Abbott LK, Solaiman ZM (2009) Use of mycorrhiza bioassays in ecological studies. In: Varma A (ed) Symbiotic fungi, vol. 18. Soil biology. Springer, Berlin, pp 41–50

Dumbrell AJ, Ashton PD, Aziz N, Feng G, Nelson M, Dytham C, Fitter AH, Helgason T (2011) Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. New Phytol 190:794–804

Gamper HA, Young JPW, Jones DL, Hodge A (2008) Real-time PCR and microscopy: are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance? Fungal Genet Biol 45:581–596

Gamper HA, Walker C, Schüßler A (2009) *Diversispora celata* sp. nov: molecular ecology and phylotaxonomy of an inconspicuous arbuscular mycorrhizal fungus. New Phytol 182:495–506

Gamper HA, van der Heijden MGA, Kowalchuk GA (2010) Molecular trait indicators: moving beyond phylogeny in arbuscular mycorrhizal ecology. New Phytol 185:67–82

Gazey C, Abbott LK, Robson AD (1992) The rate of development of mycorrhizas affects the onset of sporulation and production of external hyphae by 2 species of *Acaulospora*. Mycol Res 96:643–650

Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. Mycorrhiza 14:111–117

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JP (1998) Ploughing up the wood-wide web? Nature 394:431

Helgason T, Fitter AH, Young JPW (1999) Molecular diverisity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. Mol Ecol 8:659–666

Hempel S, Renker C, Buscot F (2007) Differences in the species composition of arbuscular mycorrhizal fungi in spore, root and soil communities in a grassland ecosystem. Environ Microbiol 9:1930–1938

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Huber JA, Morrison HG, Huse SM, Neal PR, Sogin ML, Welch DBM (2009) Effect of PCR amplicon size on assessments of clone library microbial diversity and community structure. Environ Microbiol 11:1292–1302

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B (2005) Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. J Plant Physiol 139:1401–1410

Jansa J, Smith FA, Smith SE (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytol 177:779–789

Junier P, Kim OS, Hadas O, Imhoff JF, Witzel KP (2008) Evaluation of PCR primer selectivity and phylogenetic specificity by using amplification of 16S rRNA genes from betaproteobacterial ammonia-oxidizing bacteria in environmental samples. Appl Environ Microb 74:5231–5236

König S, Wubet T, Dormann CF, Hempel S, Renker C, Buscot F (2010) TaqMan real-time PCR assays to assess arbuscular mycorrhizal responses to field manipulation of grassland biodiversity: effects of soil characteristics, plant species richness, and functional traits. Appl Environ Microbiol 76:3765–3775

Krüger M, Stockinger H, Kruger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223

Liu Y, Mao L, He X, Cheng G, Ma X, An L, Feng H (2011) Rapid change of AM fungal community in a rain-fed wheat field with short-term plastic film mulching practice. Mycorrhiza. doi:10.1007/s00572-011-0378-y

Lueders T, Friedrich MW (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl Environ Microb 69:320–326

Mandyam K, Jumpponen A (2008) Seasonal and temporal dynamics of arbuscular mycorrhizal and dark septate endophytic fungi in a tallgrass prairie ecosystem are minimally affected by nitrogen enrichment. Mycorrhiza 18:145–155

Merryweather JW, Fitter AH (1991) A modified method for elucidating the structure of the fungal partner in a vesicular–arbuscular mycorrhiza. Mycol Res 95:1435–1437

Merryweather J, Fitter A (1998a) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* I. Diversity of fungal taxa. New Phytol 138:117–129

Merryweather J, Fitter A (1998b) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* II. Seasonal and spatial patterns of fungal populations. New Phytol 138:131–142

Morton JB, Redecker D (2001) Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia 93:181–195

Mummey DL, Rillig MC (2007) Evaluation of LSU rRNA-gene PCR primers for analysis of arbuscular mycorrhizal fungal communities via terminal restriction fragment length polymorphism analysis. J Microbiol Meth 70:200–204

Newman EI (1966) A method of estimating total length of root in a sample. J Appl Ecol 3:139–145

Opik M, Metsis M, Daniell TJ, Zobel M, Moora M (2009) Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. New Phytol 184:424–437

Palatinszky M, Nikolausz M, Svába D, Márialigeti K (2011) Preferential ligation during TA-cloning of multitemplate PCR products—a factor causing bias in microbial community structure analysis. J Microbiol Meth 85:131–136

Pearson JN, Abbott LK, Jasper DA (1993) Mediation of competition between two colonizing VA mycorrhizal fungi by the host plant. New Phytol 123:93–98

Plenchette C, Morel C (1996) External phosphorus requirement of mycorrhizal and non-mycorrhizal barley and soybean plants. Biol Fert Soils 21:303–308

Polz MF, Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microb 64:3724–3730

Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921

Redecker D, Hijri I, Wiemken A (2003) Molecular identification of arbuscular mycorrhizal fungi in roots: perspectives and problems. Folia Geobot 38:113–124

Renker C, Weißhuhn K, Kellner H, Buscot F (2006) Rationalizing molecular analysis of field-collected roots for assessing diversity of arbuscular mycorrhizal fungi: to pool, or not to pool, that is the question. Mycorrhiza 16:525–531

Robinson-Boyer L, Grzyb I, Jeffries P (2009) Shifting the balance from qualitative to quantitative analysis of arbuscular mycorrhizal communities in field soils. Fungal Ecol 2:1–9

Rosendahl S (2008) Communities, populations and individuals of arbuscular mycorrhizal fungi. New Phytol 178:253–266

Rosendahl S, Matzen HB (2008) Genetic structure of arbuscular mycorrhizal populations in fallow and cultivated soils. New Phytol 179:1154–1161

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Santos-Gonzalez JC, Finlay RD, Tehler A (2007) Seasonal dynamics of arbuscular mycorrhizal fungal communities in roots in a semi-natural grassland. Appl Environ Microb 73:5613–5623

Schechter SP, Bruns TD (2008) Serpentine and non-serpentine ecotypes of *Collinsia sparsiflora* associate with distinct arbuscular mycorrhizal fungal assemblages. Mol Ecol 17:3198–3210

Scheltema MA, Abbott LK, Robson AD (1987) Seasonal variation in infectivity of VA mycorrhizal fungi in annual pastures in a Mediterranean environment. Aust J Agr Res 38:707–715

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, Cambridge

Stockinger H, Kruger M, Schüßler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytol 187:461–474

Sýkorová Z, Ineichen K, Wiemken A, Redecker D (2007) The cultivation bias: different communities of arbuscular mycorrhizal fungi detected in roots from the field, from bait plants transplanted to the field, and from a greenhouse trap experiment. Mycorrhiza 18:1–14

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tawaraya K, Imai T, Wagatsuma T (1999) Importance of root length in mycorrhizal colonization of welsh onion. J Plant Nutr 22:589–596

Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Current Protoc Bioinformatics. John Wiley & Sons, Inc.

Van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Verbruggen E, Röling WFM, Gamper HA, Kowalchuk GA, Verhoef HA, van der Heijden MGA (2010) Positive effects of organic farming on below-ground mutualists: large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytol 186:968–979

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315-322

Wilde P, Manal A, Stodden M, Sieverding E, Hildebrandt U, Bothe H (2009) Biodiversity of arbuscular mycorrhizal fungi in roots and soils of two salt marshes. Environ Microbiol 11:1548–1561

Wu BY, Hogetsu T, Isobe K, Ishii R (2007) Community structure of arbuscular mycorrhizal fungi in a primary successional volcanic desert on the southeast slope of Mount Fuji. Mycorrhiza 17:495–506

Zarei M, Hempel S, Wubet T, Schäferb T, Savaghebi G, Jouzani GS, Nekouei MK, Buscot F (2010) Molecular diversity of arbuscular mycorrhizal fungi in relation to soil chemical properties and heavy metal contamination. Environ Pollut 158:2757–2765

Zhao B, Trouvelot A, Gianinazzi S, Gianinazzi-Pearson V (1997) Influence of two legume species on hyphal production and activity of two arbuscular mycorrhizal fungi. Mycorrhiza 7:179–185