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Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—marker design, verification, calibration and field validation

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Abstract

Quantitative real-time PCR (qPCR) is slowly becoming established as a tool to quantify abundance of different arbuscular mycorrhizal fungal (AMF) taxa in roots and in soil. Here, we describe the development and field validation of qPCR markers (i.e. primers with associated hydrolysis probes), targeting taxon-specific motifs in the nuclear large ribosomal subunit RNA genes. Design of such markers is complicated by the multinuclear and multigenomic cellular organization of these fungi and the high DNA sequence diversity within the smallest biologically relevant units (i.e. single-spore isolates). These limitations are further compounded by inefficient biomass production of these fungi, resulting in limited availability of pure genomic DNA (gDNA) of well-defined isolates for cross-specificity testing of the markers. Here we demonstrate, using a number of AMF isolates, the possibility to establish stringent qPCR running conditions allowing quantification of phylogenetically disjunctive AMF taxa. Further, we show that these markers can more generally be used to quantify abundance (i.e. number of target gene copies or amount of gDNA) of what is usually considered the level of AMF species, regardless of the isolate identities. We also illustrate the range of variation within qPCR signal strength across different AMF taxa with respect to the detected number of gene copies per unit amount of gDNA. This information is paramount for interpretation of the qPCR analyses of field samples. Finally, the field validation of these markers confirmed their potential to assess composition of field AMF communities and monitor the changes owing to agricultural practices such as soil tillage.

Keywords: calibration, field validation, marker design, nuclear large ribosomal subunit (nLSU, 28S), specificity, taxon-specific sequence motifs

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Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with the majority of land plant species. AMF colonize plant root systems and the surrounding soil, providing benefits to the plants in the form of nutrient acquisition, growth and/or tolerance to environmental stresses (Smith & Read 2008). In addition, AMF play important roles in ecosystem functioning and affect plant coexistence and soil structure (Read & Perez-Moreno 2003; Facelli *et al.* 2009). These fungi form the phylum Glomeromycota (Schüßler *et al.* 2001), a monophyletic group, in which all members are phylogenetically more closely related to each other than to any other living organisms. Although the Glomeromycota are widespread, occurring in virtually all soils and ecosystems on Earth, there are currently only about 230 species known to science thus far (Smith & Read 2008; Helgason & Fitter 2009). Global distribution patterns remain still rather unclear, although the influence of environmental factors on the AMF communities such as plant species identity, soil fertilization and soil disturbance have been documented (Johnson *et al.* 2004; Jansa *et al.* 2006; Treseder & Cross 2006; Öpik *et al.* 2010). Although traditionally AMF have been identified by the morphology of their soil-borne spores, it has been shown (e.g. Clapp *et al.* 1995) that AMF spore communities in the soil do not correctly reflect the composition of AMF communities in plant root. This observation was advocated for a method of direct identification/quantification of the AMF in roots to gain information with greater functional relevance to the plants.

In both natural and agricultural ecosystems, plant roots have been shown to be colonized simultaneously by more than one AMF taxon (Merryweather & Fitter

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1998; Jansa *et al.* 2003). However, the diversity of AMF communities in the root systems has been difficult to study, because of limited morphological features that could allow for microscopy discrimination of the different AMF taxa based in the anatomy of root colonization structures (Abbott 1982; Merryweather & Fitter 1998). Therefore, alternative approaches to microscopy have attracted more attention and investigation. A few of the following methods have been developed: AMF species-specific isoenzymes (Hepper *et al.* 1988; Tisserant *et al.* 1998), genera- or species-specific antibodies (Wilson *et al.* 1983; Friese & Allen 1991; Treseder & Allen 2002) and various DNA-based molecular methods (Clapp *et al.* 1995; van Tuinen *et al.* 1998; Jansa *et al.* 2003, 2008; Lee *et al.* 2008). Different molecular markers and approaches exist, and they have been proven to be unbeatable with respect to their capacity to discriminate between different sequence types used as biomarkers for AMF taxa such as genera, species or isolates (van Tuinen *et al.* 1998; Jansa *et al.* 2003; Börstler *et al.* 2008; Sýkorová *et al.* 2011). These markers and methods (see Krüger *et al.* 2009 for review) usually differ for their sensitivity and resolution and are easily transferable between laboratories around the globe. Despite these obvious advantages, molecular quantification of the AMF colonization in roots and soil has been slow to establish for two main reasons: (i) The need to precisely quantify the concentration of the target DNA sequence in environmental samples and (ii) Apparent sequence diversity within the gene pools of each of the AMF isolates coupled with a limited knowledge on the extent of sequence divergence between different species or like taxa of the AMF.

The first obstacle appeared to be solved by introduction of the real-time quantitative PCR (qPCR) to mycorrhizal research (Filion *et al.* 2003; Alkan *et al.* 2006), while the second issue remains a research challenge (Gamper *et al.* 2010). Overcoming this latter limitation requires not only a careful design of molecular markers targeting consistent differences between different fungal taxa but also the avoidance of differences between various sequence types within an AMF isolate. Marker verification then requires extensive cross-specificity assays, ensuring that no signal is generated by the presence of nontarget fungi, prokaryotes or other organisms. For this reason, use of qPCR quantification has thus far been mostly restricted to measuring abundance of AMF in simplified model systems, using two to four AMF isolates (Alkan *et al.* 2006; Jansa *et al.* 2008; Wagg *et al.* 2011a,b).

The qPCR is currently used as a well-established method to rapidly and precisely quantify concentration of DNA sequence motifs in a wide spectrum of biological samples (Bustin *et al.* 2009). It is currently possible to distinguish sequences that only differ in a single nucleotide position (Kianianmomeni *et al.* 2007). As noted earlier, wide exploitation of molecular methods in general, and the qPCR method in particular, has been slow to establish in mycorrhizal research, owing to difficulties in obtaining sequencing information about the AMF genes (Martin *et al.* 2008) and to high levels of variability of genes within each single AMF isolate (Kuhn *et al.* 2001; Jansa *et al.* 2002a). These limitations are a result of the multinuclear and multigenomic cellular organization (Sanders *et al.* 2003; Hijri & Sanders 2005), strict biotrophy and slow growth of these fungi, all of which, in turn, limit the availability of large amounts of pure genomic DNA (gDNA) of these fungi. Results of several recent studies under glasshouse conditions have now confirmed that design of qPCR markers and the establishment of specific cycling conditions are attainable to unequivocally distinguish and quantify the DNA of different AMF taxa (Alkan *et al.* 2006; Gamper *et al.* 2008; Jansa *et al.* 2008; Kiers *et al.* 2011). Only very recently, qPCR markers (primers with matching hydrolysis probes) have also been tested under field conditions to assess differences in indigenous AMF communities (König *et al.* 2010). In this study, however, the markers were developed for different sequence types rather than for real AMF taxa. This limits the comparison of abundance data across the different AMF taxa, and only provides limited confidence that biologically relevant units were discriminated by the different markers. Furthermore, the different markers were targeting different genes and gene regions, which may harbour different levels of genetic diversity within the AMF populations and communities (Sanders 2004; Gamper *et al.* 2010).

Currently, the development of qPCR protocols for analysing AMF communities faces the following dilemma: Despite the fact that the qPCR is being established within the mycorrhizal research (Robinson-Boyer *et al.* 2009), very little is still known about what information this method provides as compared to the traditional measurements of AMF abundance in roots and soil. Claims have been made that qPCR assesses different biological units than microscopy-based approaches (Corradi *et al.* 2007; Gamper *et al.* 2008) and that these approaches were not comparable. This fact limits our capacity to interpret current results of the qPCR experiments and to further develop this research discipline.

The objective of this study was to establish a qPCR toolbox using the combination of specific primers with fluorescently labelled hydrolysis probes for the rapid and specific quantification of several AMF isolates (here defined as laboratory cultures of the AMF originally derived from one single spore each). We decided to use markers targeting the nLSU region (nuclear large ribosomal subunit, 28S), because they provide a good resolution and, at the same time, rely on a solid sequences database (Krüger *et al.* 2009). We then tested whether these tools could also be used as broader taxa (e.g. spe-

cies)-specific markers for the quantification of AMF taxa in field samples. This necessitated the inclusion of several AMF isolates per AMF species. For the first time, we estimated the absolute numbers of the target gene (nLSU) copies per unit of gDNA of the different AMF species to provide a baseline for the interpretation of the qPCR results. Finally, the markers were validated using root samples from a field site, where the composition of AMF communities had previously been well characterized by independent approaches.

Material and methods

Primers

Several sequences of the nuclear large ribosomal subunit (nLSU) gene were obtained and reported during several years of research (Jansa *et al.* 2003, 2008) for each of the five target AMF isolates (using the traditional nomenclature and BEG standing for the International Bank for the Glomeromycota, http://www.kent.ac.uk/bio/beg): *Glomus intraradices* BEG 158, *Glomus claroideum* BEG 155, *Glomus mosseae* BEG 161, *Gigaspora margarita* BEG 152 and *Scutellospora pellucida* BEG 153. These AMF were all previously isolated from a single field site in Switzerland (Jansa *et al.* 2002b) and seem to be the dominant taxa in this field, as well as in many agricultural soils, in (central) Europe (Jansa *et al.* 2002b; Oehl *et al.* 2005; Öpik *et al.* 2010). Furthermore, these AMF isolates are extensively being used in our functional diversity experiments, where quantification of their development alone or in mixtures is required (e.g. Lendenmann *et al.* 2011; Wagg *et al.* 2011a,b). The sequences are all available at the GenBank (http://www.ncbi.nlm.nih.gov) under the following accession numbers: AF396782–AF396788, AF396790, AF396791, AF396793, AF396795, AF396796, AF396798 and HM625883–HM625903. For the design of specific primers, these sequences were aligned with nLSU gene sequences of the same or other AMF taxa (e.g. *Acaulospora* spp.), as well as other (nonmycorrhizal) fungal species and a plant (*Sinapis alba*), all downloaded from the GenBank (see Appendix S1, Supporting information). The nontarget AMF and other fungal and plant species were included to avoid cross-reactivity of the primers with undesired taxa.

As a first step, multiple specific primers for each of the target isolates were designed using the AlleleID version 4 software (Premier Biosoft International, Palo Alto, CA, USA). Care was taken to target conserved sequence motifs, which showed little variability across different gene variants cloned from the same AMF isolate. More than 30 primer pairs were designed (4–7 alternative pairs per each target AMF taxon), synthesized and purified by polyacrylamide gel electrophoresis (PAGE) at Microsynth AG (Balgach, Switzerland). These were subsequently tested for cross-reactivity using gDNA from spores of the five target AMF isolates. Large numbers of spores of the different AMF isolates were needed for these tests (3000–300 000 spores per sample, depending on the spore size). The spores were purified from open-pot cultures planted with leek for 12 months, by using wet-sieving and sucrose-gradient centrifugation, as described previously (Jansa *et al.* 1999, 2002b). DNA was extracted from the spores by using the DNeasy Plant Mini kit (Qiagen, Hombrechtikon, Switzerland), following spore homogenization by hand-held micropestle in 1.5-mL Eppendorf vials. DNA concentration in the final extracts was assessed by UV spectrophotometry (260 nm) and diluted to reach 1 μg/mL in all samples. Cross-specificity of the designed primer pairs was tested with both ordinary PCR coupled with agarose gel electrophoresis and by qPCR (using the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit and LightCycler 2.0 instrument, both from Roche Diagnostics, Rotkreuz, Switzerland). Low-stringency conditions (annealing time 90 s, annealing temperature 48 °C, elongation time 60 s, 40 cycles) were employed for both approaches. Based on these tests, one primer pair per each of the target AMF isolates was selected for further optimization. These primer pairs showed both strong amplification with the target AMF isolates and either a complete absence of amplification with nontarget AMF isolates or the greatest difference in quantification cycle (Cq) values between target and nontarget AMF isolates.

Hydrolysis probes and cross-specificity tests

The selected primers were further complemented by hydrolysis probes, designed using the Allele ID software version 6. The probes were synthesized and labelled with fluorescein (5′) and BHQ-1 quencher (3′), and subsequently purified by PAGE in Microsynth. The resulting five sets of specific markers (i.e. primers and hydrolysis probes) for the different AMF taxa (Table 1) were subjected to specificity testing with medium-stringency cycling conditions (Table 2) by using the gDNA of the five AMF isolates listed earlier, and a qPCR using the LightCycler® TaqMan® Master kit and LightCycler 2.0 (Roche). In addition, gDNA was prepared from various plant species (maize, *Medicago*, leek) and spores of 19 other AMF isolates available in the local culture collection at the ETH Zürich (Table 2) and included in specificity testing of the markers. Admittedly, the selection of AMF isolates was far from extensive and covered mainly cultures isolated from European agricultural soils. However, it contained members of all main clades of Glomeromycota, thus providing the possibility to check for cross-specificity amongst both closely and distantly related AMF isolates. Absence of cross-reactivity of a

Table 1 Sequences of primers and hydrolysis probes used for the quantitative real-time PCR quantification of large ribosomal subunit gene copies of the different arbuscular mycorrhizal fungal (AMF) taxa

Target AMF species	Target AMF isolate	Abbreviation of the specific primers/ probe set	Sequences (5′ → 3′) (primer forward, primer reverse, hydrolysis probe)	Amplicon size (base pairs)	Optimal annealing temperature (°C)
Glomus intraradices Schenck & Smith	BEG 158*	*intra*	TTCGGGTAATCAGCCTTTCG TCAGAGATCAGACAGGTAGCC TTAACCAACCACACGGGCAAGTACA	250	52
Glomus claroideum Schenck & Smith	BEG 161	*clar*	GCGAGTGAAGAGGGAAGAG TTGAAAGCGTATCGTAGATGAAC AACAGGACATCATAGAGGGTGACAATCCC	177	52
Glomus mosseae Gerd & Trappe	BEG 155	*moss*	GGAAACGATTGAAGTCAGTCATACCAA CGAAAAAGTACACCAAGAGATCCCAAT AGAGTTTCAAAGCCTTCGGATTCGC	122	54
Gigaspora margarita Becker & Hall	BEG 152	*gig*	CTTTGAAAAGAGAGTTAAATAG GTCCATAACCCAACACC TAACCTGCCAAACGAAGAAGTGC	272	48
Scutellospora pellucida Walker & Sanders	BEG 153	*scut*	AGAAACGTTTTTTACGTTCCGGGTTG CCAAACAACTCGACTCTTAGAAATCG CCGTGTATACCAACCACTGGAATGTTATT	127	54

Location of the primers and probes on sequences is given in Appendix S2.
*International Bank for the Glomeromycota (http://www.kent.ac.uk/bio/beg)

taxon-specific molecular marker within Glomeromycota will namely indicate very low probabilities of cross-reactivity beyond this monophyletic group, thus saving resources that would otherwise be needed to test cross-reactivity with other organisms (animals, bacteria, etc). Different cycling conditions (data not shown) were tested to optimize the qPCR conditions for each set of markers with respect to the amplification efficiency (i.e. deviation from optimal cycling conditions postulating duplication of DNA amount every cycle) and specificity (Table 2). Optimized cycling conditions were established as follows: initial DNA denaturation and DNA polymerase activation at 95 °C for 15 min, then 45 cycles each with denaturation at 95 °C for 10 s, annealing at temperature optimized for each marker (Table 1) for 30 s and elongation at 72 °C for 1 s. Reaction volume was 9 μL (4.52 μL H_2O + 0.18 μL each primer (25 μM) + 0.07 μL hydrolysis probe (25 μM) + 1.8 μL Roche Master Mix TaqMan + 2.25 μL template).

Calibration

The calibration of the qPCR analyses was carried out in two ways, to allow for the conversion of the qPCR output (i.e. Cq) into (i) nLSU gene copy numbers and (ii) amount of gDNA of each of the AMF taxa. The qPCR quantification protocol for nLSU copy numbers was established following the outline presented before (Jansa *et al.* 2008). Briefly, DNA was extracted from single spores of the five target AMF isolates listed earlier (Table 1) and further used as templates for normal PCR with LR1 (5′-GCATATCAATAAGCGGAGGA-3′) and FLR2 (5′-GTCGTTTAAAGCCATTACGTC-3′) primers, using the following cycling conditions: initial denaturation at 95 °C for 5 min; 35 cycles: denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min; final elongation at 72 °C for 5 min (Jansa *et al.* 2002b). The concentration of nLSU copy numbers (NC, copies/L) in each of these PCR products was calculated using the amplicon length, *L* (761, 759, 767, 702 and 700 bp for *Glomus intraradices*, *Glomus claroideum*, *Glomus mosseae*, *Gigaspora margarita* and *Scutellospora pellucida*, respectively), the DNA concentration of the sample (*K*, g/L, determined by UV spectrophotometry) and molecular weight of DNA (660 Da/bp) in the following equation, where N_a is Avogadro's constant (6.022×10^{23}).

$$\mathrm{NC} = \frac{K \times N_a}{660 \times L} \qquad (eqn1)$$

Then, the PCR products, harbouring the different sequence variants occurring in each individual AMF isolate, were serially diluted to obtain billions to dozens of nLSU copies/μL. These dilution series were run through the qPCR with their respective markers under the optimized cycling conditions for each marker. Based on these analyses, calibration curves were established allowing the conversion of qPCR output into the concentration of nLSU copies of each AMF taxon in a sample. Similarly, calibration curves were established for the conversion of the qPCR output to concentration of gDNA of each

Table 2 Cross-specificity of the quantitative real-time PCR markers (primers with hydrolysis probes) as assessed with genomic DNA obtained from spores of the different arbuscular mycorrhizal fungal (AMF) isolates

Templates used for the cross-specificity test				Cq values (qPCR with AMF taxa-specific markers)				
AMF species	Accession number	Isolate origin	DNA concentration used for real-time PCR tests (ng/μL)	*gig*	*scut*	*clar*	*moss*	*intra*
Glomus intraradices	BEG 158*	Tänikon, Switzerland	1			*27.12*		**19.51**
Glomus intraradices	BEG 75	Wädenswil, Switzerland	1					**19.88**
Glomus intraradices	MN 181	Changins, Switzerland	1				*35.91*	**20.14**
Glomus 'cluster-forming' sp.	BEG 140	Chvaletice, Czech Rep.	0.32					*27.07*
Glomus mosseae	BEG 161	Tänikon, Switzerland	1				**23.83**	
Glomus mosseae	BEG 95	Most, Czech Rep.	0.84				**22.98**	
Glomus mosseae	BEG 76	Wädenswil, Switzerlan	0.13			*27.97*	**24.35**	
Glomus caledonium	JJ658	Tänikon, Switzerland	1					
Glomus geosporum	BEG 11	Kent, UK	0.58			*27.75*		
Glomus geosporum	24A	Tänikon, Switzerland	1					
Glomus hoi	BEG 48	Finland	1					
Glomus claroideum	BEG 155	Tänikon, Switzerland	1			**18.13**		
Glomus claroideum	BEG 210	Estarreja, Portugal	0.43			**22.25**		
Glomus claroideum	BEG 96	Kolín, Czech Rep.	1			**18.5**		
Glomus claroideum	BEG 23	Praha, Czech Republic	1			**18.21**		
Glomus etunicatum	Tä 96	Tänikon, Switzerland	1			**17.94**		
Gigaspora margarita	BEG 152	Tänikon, Switzerland	1	**23.63**				
Gigaspora margarita	BEG 34	New Zealand	1	**22.85**				
Gigaspora rosea	BEG 9	USA	1	*27.19*				
Scutellospora pellucida	BEG 153	Tänikon, Switzerland	1		**24.21**			
Scutellospora heterogama	BEG 35	USA	0.05		*32.57*			
Acaulospora mellea	n.a.	Colombia	1					
Acaulospora delicata	n.a.	Bhutan	1					
Acauspora scrobiculata	n.a.	Maseno, western Kenya	1					
Water control			n.a.					

Abbreviations of the specific primers/probe sets are as in Table 1. Quantification cycle (Cq) values are given of the real-time PCR (Lightcycler 2.0) for reactions with volume of 9 μL and subjected to medium-stringency cycling conditions (initial denaturation at 95 °C for 15 min, 45 cycles: denaturation at 95 °C, 10 s; annealing at 48 °C (gig) or at 54 °C (all others), 20 s; elongation at 72 °C, 5 s). Missing values indicate absence of amplification, i.e. signal below the detection limit. Values in bold indicate Cq values of amplification with expected shape of the amplification curve (S-form) and with a curve plateau above 0.1 fluorescence units (LightCycler 2.0). Values in italics indicate amplification with low-intensity curves (plateau ≤0.1 fluorescence unit) or not reaching a plateau within 45 cycles.
*International Bank for the Glomeromycota (http://www.kent.ac.uk/bio/beg); n.a., not applicable.

particular AMF taxon per sample by using serial dilution of gDNA extracted from the five target AMF isolates (see Table 1). This calibration with gDNA was subsequently also carried out for all other AMF isolates for which sufficient amounts of gDNA were available.

Detected nLSU gene copies per unit amount of gDNA

For the AMF isolates, for which enough gDNA was available to carry out the calibration, the ratio of detected nLSU gene copies per unit amount of gDNA was calculated. Namely, the calibration curves for nLSU copy numbers (Fig. 1) were used to calculate Cq values for theoretical 10, 100, 1000 and 10 000 detected nLSU copies per microlitre of template. These four Cq values per AMF isolate were subsequently used to calculate the amount of gDNA per microlitre of template using the equations given in Fig. 2. Resulting four ratios (nLSU copies per ng gDNA) per AMF isolate were used to calculate mean ratio of nLSU copies/ng gDNA and to analyse the associated variation of estimation, either because of different slopes of the calibration curves for nLSU copies and gDNA concentrations or variability between different AMF isolates within AMF species (Table 3).

Field validation

To verify whether the qPCR markers also worked in the field settings, we quantified the different AMF taxa in

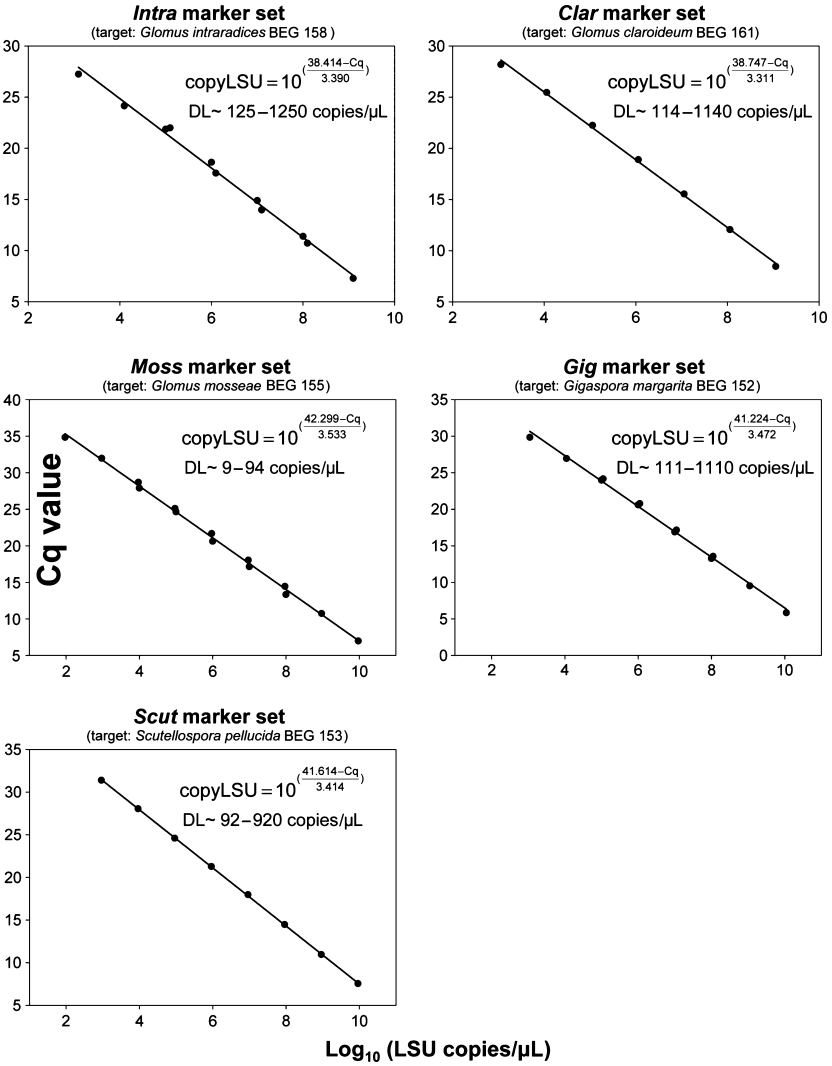

Fig. 1 Calibration curves for conversion of the quantification cycle (Cq) of the quantitative real-time PCR (qPCR) assay to nLSU gene copy concentration (copy LSU) of a particular arbuscular mycorrhizal fungal (AMF) taxon in a sample. PCR amplicons from the target AMF isolates (see Table 1 for details) were diluted with water to reach different concentrations of nLSU gene copies and then used as templates for the qPCR. Arbitrary detection limits (span of tested gene concentrations between which the response of the procedure either vanished completely or lost its linearity, DL) are given for each of the marker sets and calibration procedure; abbreviations as in Table 1. R^2 for the regressions were above 0.99 in all cases.

maize roots from a field site (i.e. long-term soil tillage experiment, Tänikon, Switzerland) with previously well-characterized AMF communities (Jansa *et al.* 2002b). At this site, independent AMF community profiling of the roots had already been carried out (Jansa *et al.* 2003). The maize roots used for this validation were collected in July 2000. This is the same site from which the target AMF isolates (Table 1) were originally obtained. Three individual root samples per field plot were analysed, with four replicate plots per each of the three tillage treatments (conventional, chisel and no tillage). The DNA was extracted from ~30 mg of lyophilized roots using the Qiagen Plant DNeasy kit, following manufacturer's recommendations. Each sample was spiked with an internal DNA standard before the extraction to normalize rates of DNA extraction efficiencies between the samples and also to test for presence of PCR inhibitors. To this end, 2×10^{10} copies of the linearized plasmid carrying fragment of cassava mosaic virus DNA (GenBank accession number AJ427910) was used, and its recovery after the extraction was quantified by a qPCR. The following qPCR marker system was used for the internal standard: forward primer (5′–3′): CGAACCTGGACTGTTATGATG, reverse primer: AATAAACAATCCCCTGTATTTCAC, and a hydrolysis probe: fluorescein-5′-CACCAGGCAC CAACAACGACCATT-3′-BHQ1 quencher. Cycling conditions were as earlier, with the annealing temperature being 50 °C. The DNA recovery rates of the internal standard for each individual DNA sample (spanning 30% through 70% in this study) were used to correct the qPCR results obtained with the AMF taxa-specific markers, as described previously (von Felten *et al.* 2010).

A diagram showing the important steps for the quantification of AMF abundance in root samples is presented in Fig. 3. For convenience, a more detailed bench protocol with technical remarks is provided in the Appendix S3 (Supporting information).

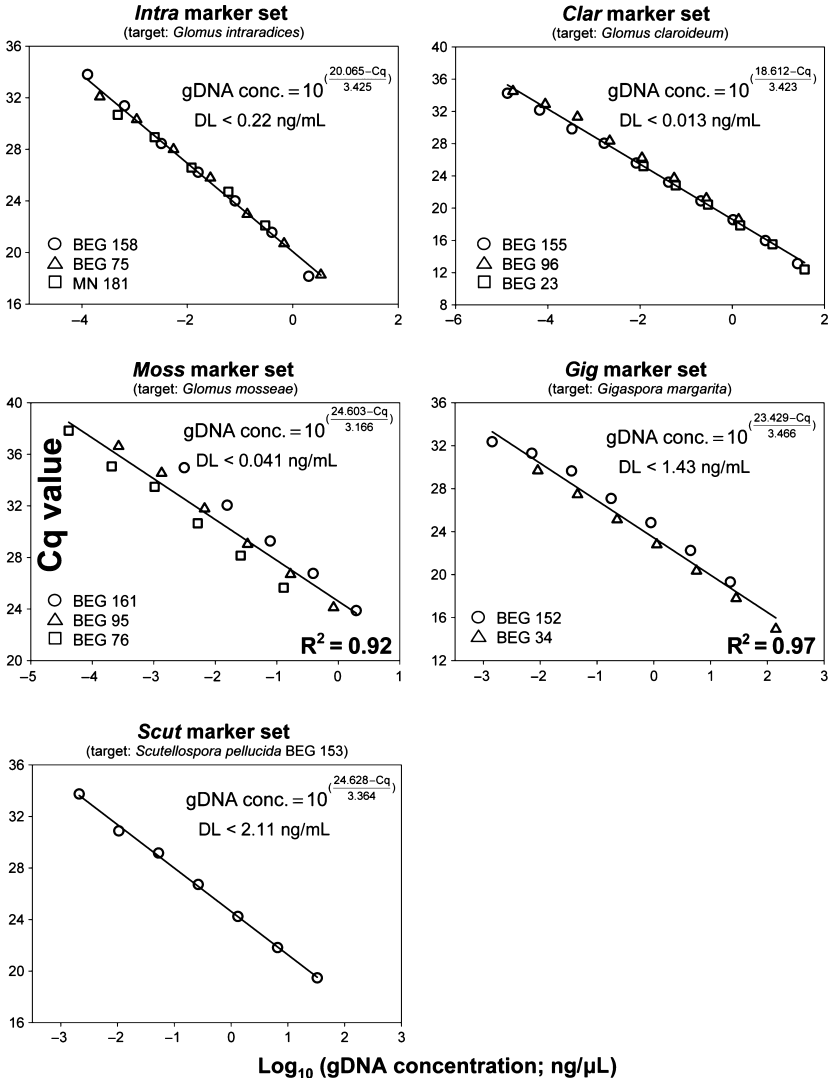

Fig. 2 Calibration curves for conversion of the quantification cycle (Cq) of the quantitative real-time PCR assay to the genomic DNA (gDNA) concentration of a particular arbuscular mycorrhizal fungal (AMF) taxon. Arbitrary detection limits (span of tested gDNA concentrations between which the response of the procedure either vanished completely or lost its linearity, DL) are given for each of the marker sets and calibration procedure; abbreviations as in Table 1. R^2 for the regressions were above 0.99 unless specified otherwise. Identity of the AMF isolates is given as in Table 2.

Results

The qPCR markers described in this stucy allow very specific detection and quantification of the DNA sequence motifs of target AMF isolates (Table 1). Moreover, these markers appear to systematically discriminate groups of isolates classified as the same AMF species, from the other AMF taxa. This was confirmed by using a broad selection of biological materials (Table 2), showing either complete absence of cross-amplification with nontarget AMF taxa or a very weak signal, with the quantification cycle (Cq) values for nontarget AMF taxa being about 10 or more cycles beyond those obtained for the target AMF taxon (i.e. about 1000 lower reactivity for nontarget DNA, or 0.1% probability of a false positive). Furthermore, cross-amplification tests with gDNA extracted from various plant species (maize, *Medicago*, leek) showed no signal whatsoever with any of the markers described here (data not shown). Because of extensive optimization of the qPCR conditions for each of the markers, cycling conditions close to theoretical optimum were eventually achieved, without compromising specificity (see equations in Figs 1 and 2). The theoretical optimum is achieved when the amount of target DNA duplicates exactly during each PCR cycle. This will correspond to a denominator in the composite exponent in Figs 1 and 2 equalling $3.32 (= \frac{\log(10)}{\log(2)})$.

Calibration of the qPCR markers both with nLSU gene amplicons of the target AMF isolates (Fig. 1) and with the gDNA of different AMF isolates (Fig. 2) showed a linear response over 7 to 10 orders of magnitude in template concentrations. Apparent detection limits (samples with no positive signal or with a signal violating the assumption of response linearity) were usually between 100 and 1000 target gene copies per microlitre of template, depending on the marker (Fig. 1).

Overlaying the calibration curves for nLSU gene copies and the gDNA amounts (Figs 1 and 2) allowed the

Table 3 Numbers of nuclear large ribosomal subunit (nLSU) gene copies per unit weight of the fungal genomic DNA (gDNA) as derived from the quantitative real-time PCR results

AMF species	Isolates used for gDNA calibration	Ratio of nLSU copies/ng of gDNA (mean of 4 values)	Standard deviation of the estimation (4 values)	Coefficient of variation, % (100 × SD/mean nLSU copies/ng gDNA)
Glomus intraradices	3	201131	6109	3.04
G. claroideum	3	923860	89765	9.72
G. mosseae	3	208322	70865	34.02
G. mosseae	1 (BEG 161 only)	142318	11512	8.09
Gigaspora margarita	2	134846	694	0.51
Scutellospora pellucida	1	102921	4546	4.42

The equations given in Figs 1 and 2 have been used to calculate the ratios for hypothetical 10, 100, 1000 and 10 000 target nLSU copies per microlitre of a sample. Standard deviations and coefficients of variation are given for each model.

calculation of the number of detected nLSU copies per unit of gDNA for the different AMF species (Table 3). The highest number of nLSU copies per unit amount of gDNA was found in *Glomus claroideum* and the lowest in *Scutellospora pellucida*. The variability in qPCR results between different isolates of *G. mosseae* contributed substantially to the variability of the estimates for nLSU copy numbers per unit amount of gDNA within the group of *G. mosseae* isolates (Table 3). Despite evidence for variability in the number of nLSU copies per unit of gDNA between different isolates of *G. mosseae*, the strength of the qPCR signal (Cq value) varied by a maximum of 3.3 cycles for the same gDNA concentration between the isolates of *G. mosseae*. This indicates the variation in nLSU copies per unit of gDNA within this one AMF species was restricted to one order of magnitude ($2^{3.3}$ = 9.75fold). In contrast, qPCR results obtained with the sequentially diluted gDNA of the different isolates of *G. intraradices* and *G. claroideum* indicated that the strength of the qPCR signal could easily be used as a proxy for the abundance of these AMF taxa, irrespective of the isolate identity, although matching of markers with local AMF genotypes in soils beyond Europe or from nonagricultural settings shall always be re-confirmed.

All five AMF taxa, for which the specific markers were established, were also detected in maize root samples from the field experiment. Of the 36 samples used in this analysis, *G. claroideum* tested positive in 35 samples, *G. intraradices* in 31 samples, *G. mosseae* in 28 samples, *Scutellospora pellucida* in 11 samples and *Gigaspora margarita* in 16 samples. The results presented in Fig. 4 indicate apparently high levels of root colonization by both *G. mosseae* and *G. intraradices*, whereas the colonization levels of *S. pellucida* and *G. claroideum* were comparably low. The total level (sums) of detected fungal DNA belonging to the five AMF taxa per unit weight of roots was significantly lower for the no-tillage treatment than under the conventional tillage (Fig. 4). Clearly, soil tillage induced shifts in composition of AMF communities in maize roots. Namely, the roots harboured greater amounts of non-*Glomus* fungi under no tillage, whereas inverse trends were observed for the *Glomus* spp. (Fig. 4).

Discussion

The qPCR markers described here offer the possibility to quantify the development of the specific (target) AMF isolates, for which the markers have been designed and calibrated. This approach is particularly objective as compared to rather subjective microscopy, where the results, to a great extent, depend on the training/experience of the person carrying out the analyses, and which generally does not allow for the differentiation of different AMF taxa in mixtures. Furthermore, during traditional microscopic estimation of root AMF colonization, using the approach of McGonigle *et al*. (1990), neither the intensity of colonization (i.e. number of hyphae crossing a root intersection) nor the vitality of the colonizing structures is assessed. In addition, the sequence-specific primers combined with hydrolysis probes provide enormous specificity as compared to the commonly used SYBR Green real-time PCR approach, which normally requires a melting curve analysis to distinguish the real positives from false positives (Alkan *et al*. 2006; Jansa *et al*. 2008; Lendenmann *et al*. 2011). Therefore, the molecular markers described here are particularly well suited for rapid quantification of the abundance of different AMF taxa in mixtures (e.g. Wagg *et al*. 2011a,b).

The reliability of the proposed method was further re-inforced, as cross-tests with different isolates belonging to the same AMF species showed that the qPCR markers originally developed for AMF isolates are, in fact, discriminating between AMF species without much preference to a specific isolate (Table 2). To our knowledge, this is the first experimental evidence for AMF taxa-specific qPCR markers being useful for the quantification of the abundance of AMF species (i.e. their specific DNA sequence motifs) under field conditions.

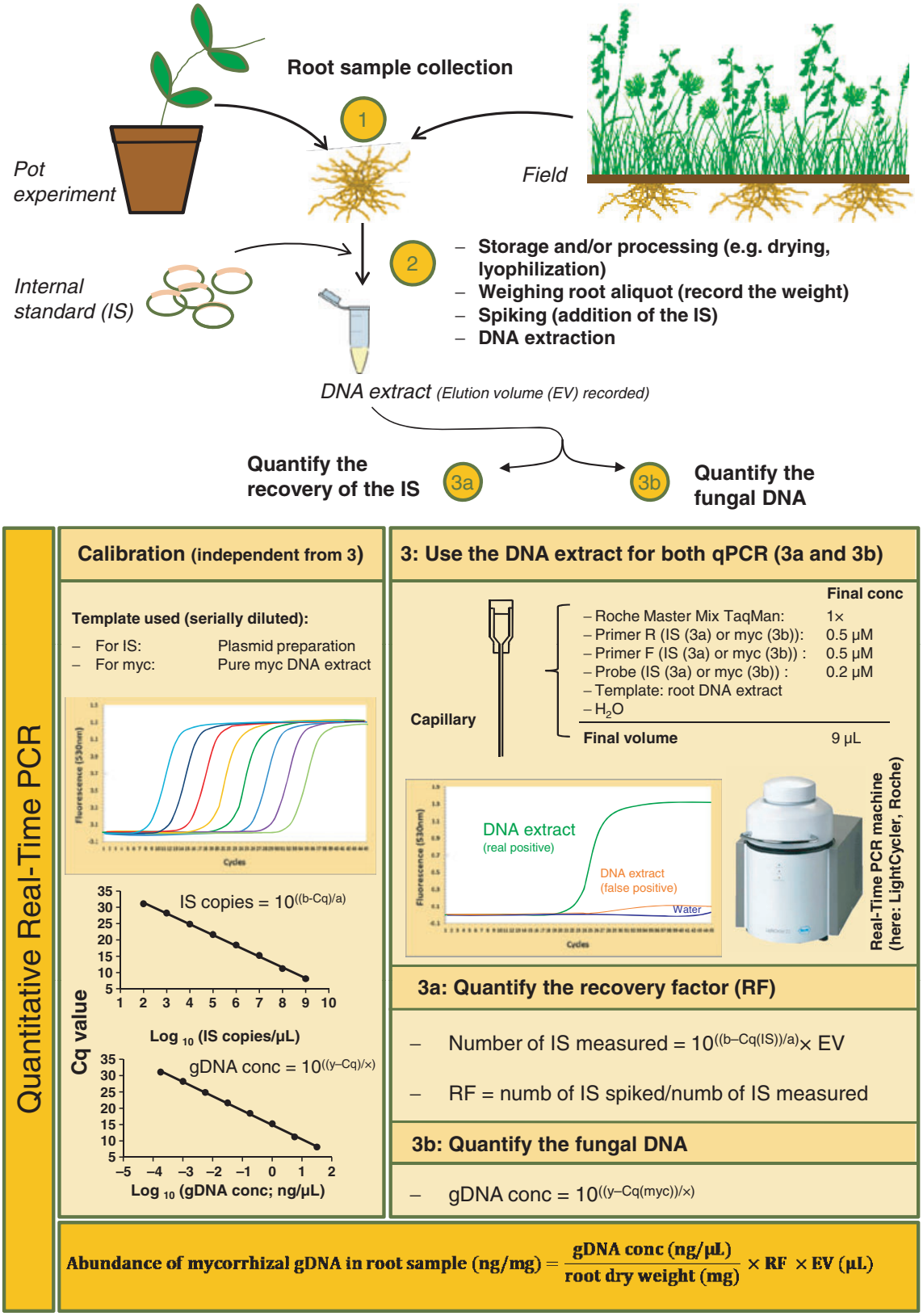

Fig. 3 Diagram showing the important steps for the quantification of arbuscular mycorrhizal fungal abundance in root samples by using the qPCR with hydrolysis probes. More details and technical notes are provided in Appendix S3.

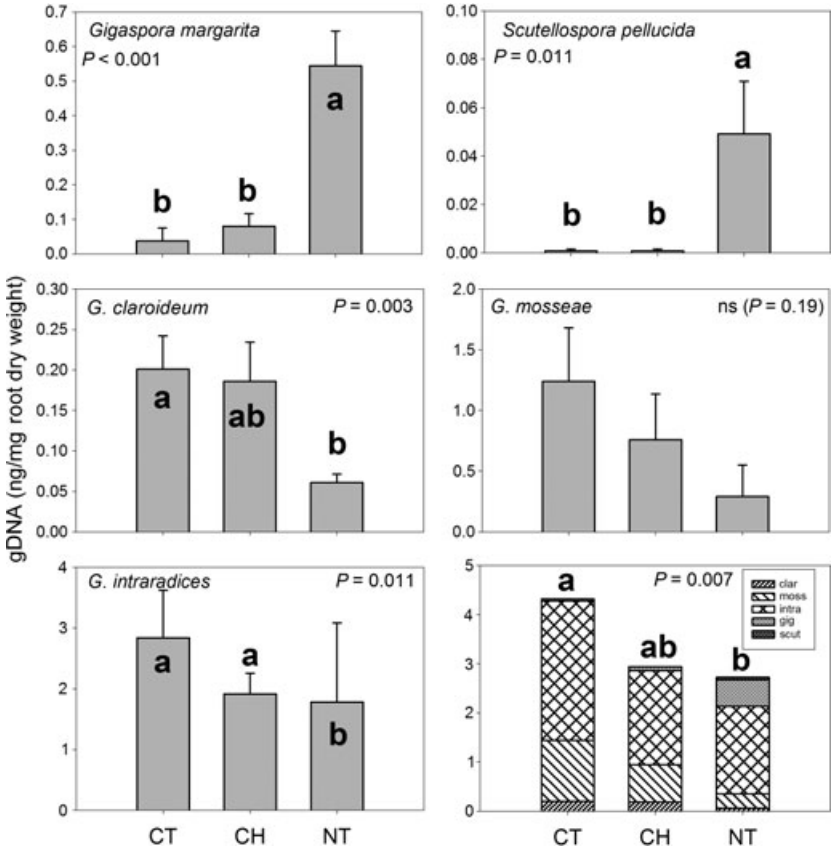

Fig. 4 Quantification of maize root colonization by different arbuscular mycorrhizal fungal (AMF) taxa in the field experiment in Tänikon, Switzerland, as of July 2000. The levels of AMF colonization were assessed by using the quantitative real-time PCR (qPCR) markers described in this study. Three individual root samples were analysed per each field plot, with four replicate plots per tillage treatment being included in the experimental design (randomized blocks). Means of twelve values and associated standard errors of means are shown. Levels of statistical significance refer to Kruskal–Wallis nonparametric test comparing treatment medians. Different letters indicate significant differences between treatments as of Bonferroni's multiple mean comparison procedure ($P < 0.05$), computed with log-transformed values (+0.00000000001) to counteract data heteroscedasticity. CT, conventional tillage; CH, chisel tillage; NT, no tillage; ns, not significant ($P \geq 0.05$). Results of analyses and associated statistics are shown for the individual qPCR markers and then a cumulative picture is provided, demonstrating the composition of mycorrhizal communities as assessed by the different molecular markers (bottom right).

Here we also provide, for the very first time, conversion factors allowing for the calculation of the amount of gDNA of specific AMF taxa from the qPCR results, rather than gene copy concentrations. However, we recognize that most of the isolates used in this study originated from agricultural soils in Europe. It is, thus, possible that different AMF genotypes abundant in environmental samples from other parts of the world and/or from different (nonagricultural) ecosystems will not match the markers described here. The traditional molecular profiling methods for AMF communities (e.g. Redecker *et al.* 2003) are still important as a first approach to characterize local AMF communities in these (and also other) cases, whereas tailored qPCR may prove useful for precise quantification of abundance of specific taxa.

The fact is that there is still very little known about the genetic structure of the AMF, variation in genome size across taxa, levels of genetic variation within individual fungus (such as a single-spore isolate), copy numbers of specific genes per unit of fungal gDNA and/or unit of living biomass. Therefore, molecular approaches to dissect community and population structures of AMF such as T-RFLP profiling, pyrosequencing or qPCR all suffer from exactly the same shortcomings with respect to interpretation of what the number of copies of one or another gene may mean for fungal abundance, vitality and/or biomass (Gamper *et al.* 2008). In this respect, qPCR is not less or more valuable than any of the alternative molecular genetic methods based on abundance of different sequence motifs. However, qPCR has a number of advantages. It is extremely fast (analysis takes usually <1 h, excluding DNA extraction), rather cheap (1–3 USD per analysis, depending on the platform), very easy to evaluate with respect to computing power and very

reproducible on the established molecular diagnostics platform, such as the LightCycler from Roche (own unpublished observation). In addition, in contrast to the methods that include end-point PCR (pre-) amplification, it is unaffected by common technical limitations, such as PCR bias (e.g. PCR selection and/or drift). It is also important to note here that all the next-generation sequencing studies that use pre-amplification of DNA samples with group-specific primers suffer from PCR bias in the same way as earlier studies (e.g. Lueders & Friedrich 2003; Frey *et al.* 2006). This, however, is not the case with the qPCR (Smith & Osborn 2009).

Nonspecific PCR amplifications with nontarget AMF isolates, occasionally recorded in this study (Table 2), seem to be of little importance. These would, in the worst-case scenario, only generate false positives with <0.1–0.5% strength of the signal seen with the target AMF. Moreover, the false positives recorded in the cross-tests here could be due to possible (but visually undetected) contaminations of the pot cultures used for AMF spore isolation or contaminations during spore purifications (sieves, filters, etc.) or to insufficient purity of the primers/hydrolysis probes. The easiest way to overcome this source of irregularities appears to be to ignore very flat amplification curves with plateaus below 0.1 of the arbitrary fluorescence units on the LightCycler (Table 2).

The unusual qPCR reactivity requires careful consideration of two of the AMF isolates included in this study, namely the BEG140 '*Glomus* cluster-forming' and BEG210 quoted as '*Glomus claroideum*' (Table 2). Based on both morphological and DNA sequence data, BEG140 appears to be related to *G. intraradices* (Malcová *et al.* 2003) or to *Rhizophagus* (formerly *Glomus*) *irregularis* (Sýkorová *et al.* 2011). This isolate was originally obtained from a pyrite waste deposit (Sýkorová *et al.* 2011), and the '*G. claroideum*' BEG210 originates from extremely alkaline soil with pH above 12 (Oliveira *et al.* 2006). Thus, both of these isolates originate from rather extreme environments and may well be genetically different (qualitatively or quantitatively) from other strains of the same species. There is, however, not sufficient information on the sequence composition of the nLSU genes in these two isolates, and sequencing of the nLSU of these two (and other) isolates was beyond the scope of the study described here. Apart from these two exceptions, in all other cases, the strength of the qPCR signal is well comparable between different AMF isolates if using equal concentrations of the fungal gDNA (Table 2, Fig. 2). Our results, thus, suggest that the numbers of detected nLSU gene or gene variant copies—as we know that there might be a diversity of sequence motifs within each isolate, e.g. see Jansa *et al.* (2002a)—per unit of gDNA amount is rather constant within the tested AMF species, but variable amongst the different species (Table 3). In addition, elucidation of the irregularities within the group of *Glomus mosseae* (Table 3) deserves future efforts, which will inevitably require large amounts of fungal materials (i.e. different pure isolates of *G. mosseae*) and substantial amounts of pure fungal DNA extracted from each of the isolates.

Estimation of detection limits (Figs 1 and 2) indicate that the use of the markers described here is constrained by very low target concentrations in the samples and/or upon high DNA losses during DNA extraction/purification and/or presence of PCR inhibitors in the samples. The two latter sources of irregularities can be addressed and corrected for by using internal DNA standard (Jansa *et al.* 2008; von Felten *et al.* 2010, Appendix S3). However, interpretation of zeros in the qPCR analyses should take into account the detection limits of the markers rather than stating complete absence of the respective taxa. To our knowledge, both the issue of detection limits of the different qPCR markers for the AMF taxa and the influence of PCR inhibitors on the molecular quantification assays for measuring AMF abundance have been nearly ignored in the literature thus far and deserve particular attention in the future.

Results of the qPCR analyses on the field roots of maize (Fig. 4) are congruent with the previous AMF community profiling using hierarchic sampling design and end-point amplification with AMF taxa-specific PCR primers and SSCP profiling (Jansa *et al.* 2003). In particular, the previously recorded higher abundance of non-*Glomus* AMF species in no-tilled soil has now been confirmed for both of the analysed AMF taxa. The results of the qPCR analyses resemble the genotype-specific results (e.g. *Scutellospora* type A as reported in our previous paper, Jansa *et al.* 2003), more so than the results obtained with genus (*Scutellospora*)-specific primers. Further, we have confirmed here inverse trends (i.e. higher abundance in conventionally tilled than in the no-tilled treatments) for the same two *Glomus* spp. (*G. claroideum* and *G. intraradices*) as reported previously (Jansa *et al.* 2003). For the first time, we are now able to compare the absolute levels of root colonization (as of DNA concentration per unit weight of roots, not percentages of root length colonized) between the different AMF taxa. Based on the qPCR results, the (active) AMF communities in the field site were dominated by *G. intraradices* (Fig. 4). This is congruent with previous reports based on a very detailed AMF cultivation and spore microscopy analyses (Jansa *et al.* 2002b). The apparently lower sum of AMF gDNA in the roots from the no-till soils as compared to conventional tillage needs a careful consideration. This is because the levels of root AMF colonization as assessed by staining/microscopy were not different between the different tillage treatments, reaching in all treatment ~80% of the root length colonized by the AMF structures

(data not shown). Most likely, the observed phenomenon is because of presence of other AMF taxa, for which qPCR markers are not yet available (e.g. *Acaulospora*, *Entrophospora*, etc.), but which were also found in this particular field and tend to be more abundant in no-till as compared to conventionally tilled soils (Jansa *et al.* 2002b). Alternatively, some mycorrhizal structures detected by microscopy may have recently died or (at least) were devoid of cytoplasm and nuclei, thus not contributing any substantial amounts of DNA. Although the traditional staining/microscopy assessment of root colonization levels do often correlate well with qPCR for young root samples (e.g. Wagg *et al.* 2011b and our own unpublished observations), the correlations are usually less good for older roots or hyphal structures (Gamper *et al.* 2008). Careful comparisons of traditional estimates of development of AMF in roots and in soils (e.g. microscopy, signature fatty acids and chitin content) with qPCR should be urgently carried out.

Conclusions

The qPCR markers developed here were specific for the different AMF isolates; no cross-reactivity was detected. Thus, these markers are well suitable for tracking and quantification of the target AMF isolates in fungal communities. These markers also allowed quantification of the nLSU gene carrying the specific sequence motifs over several orders of magnitude, with detection limits for the individual markers set between several dozen through a few thousand gene copies per sample. In addition to the demonstrated isolate specificity, the markers described here also demonstrated remarkable species specificity, rendering them suitable as AMF species-specific markers under natural settings, at least in the context of agricultural ecosystems in (central) Europe. However, some differences in the strength of the qPCR signal were observed between some isolates of the same species, when comparing equal amounts of gDNA of the different AMF isolates. This advocates careful testing of the qPCR markers using a range of real biological materials as outlined here, not only in *in-silico* tests with sequence information as in another recent report (e.g. König *et al.* 2010). Our results suggest that abundance of some AMF taxa can be, to a limited extent, over- or under-estimated if samples are compared harbouring different genotypes (e.g. ecotypes) of the same AMF species. Similar evidence for a single AMF species has been provided previously (Corradi *et al.* 2009). The variation in abundance of a specific gene motif per unit amount of gDNA between different isolates seems, however, restricted to about 1 order of magnitude, based on the data reported in this study and previously (Corradi *et al.* 2007). In addition to DNA-based assays described here, these markers will also be suitable for monitoring ribosomal RNA abundances, as they are targeting transcribed regions of the fungal nLSU genes (own unpublished observations). Finally, these markers have been shown to successfully detect and quantify the abundance of the target AMF taxa in environmental samples such as field roots. Using qPCR with these markers seems particularly well suited to monitor how environmental factors or anthropogenic actions influence the changes in AMF communities. Moreover, the methodology proposed in this work can be extended and adapted to other AMF taxa (e.g. *Diversispora celata*, as reported by Wagg *et al.* 2011a) to broaden the range of potentially traceable AMF taxa in environmental samples. Thus, the qPCR quantification of the abundance of various AMF taxa, as described here, has the potential to become an objective, accurate, cheap and easy-to-use standard approach in mycorrhizal ecology, potentially replacing the traditional staining/microscopy, as well as the bias-prone conventional PCR with group-specific markers (e.g. Krüger *et al.* 2009), followed by cloning and sequencing or by next-generation sequencing.

The next step in developing this method is to relate the qPCR signal generated by the markers described here to the traditional estimates of AMF colonization of roots (microscopy, signature fatty acids, chitin content, etc), as proposed by Gamper *et al.* (2008). Previous observations indicate that age and vitality of colonization structures could play important roles at least in some AMF taxa (Jansa *et al.* 2008). This now needs to be addressed using the new markers described here and carefully designed experiments such as described recently for plant-bound qPCR markers (Riley *et al.* 2010).

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This research was carried out in the Group of Plant Nutrition, ETH Zürich. The authors aim at linking the composition of AMF communities with their functions, using both molecular and radio- and stable isotopic approaches. They study the competition between different AMF species in synthetic AMF communities, established by mixing pure cultures of the different AMF taxa under controlled laboratory conditions. Further, they work on tracing industrial AMF inoculants in nonsterile field soils using quantitative PCR. The impact of realized AMF community diversity and composition is linked to changes in P and C fluxes between the AMF and plants.

Data Accessibility

DNA sequences: GenBank accessions AF396782–AF396788, AF396790, AF396791, AF396793, AF396795, AF396796, AF396798, HM625883–HM625903. Final DNA sequence alignment uploaded as Appendix S1. Locations of the qPCR markers on the nLSU sequences uploaded as Appendix S2.

Supporting Information

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

Appendix S1 Identity and alignment of nLSU sequences used for design of AMF taxon-specific qPCR primers and hydrolysis probes.

Appendix S2 Locations of the specific primers (red arrows, 5′ → 3′) and hydrolysis probes (blue arrows, 5′ → 3′) on the alignment of nLSU sequences of the target AMF isolates.

Appendix S3 Detailed bench protocol for quantification of AMF abundance in root samples by using the qPCR with hydrolysis probes.

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