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Note

Evaluation of LSU rRNA-gene PCR primers for analysis of arbuscular mycorrhizal fungal communities via terminal restriction fragment length polymorphism analysis

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Abstract

The efficacy of the LSU rDNA PCR primers FLR3 and FLR4 for discrimination of arbuscular mycorrhizal fungi communities via T-RFLP analysis was examined. Analysis of both public database and site-specific derived DNA sequences suggesting LSU rDNA-based T-RFLP analysis represents a valuable alternative for analysis of AMF communities.

Keywords: Arbuscular mycorrhizal fungi; AMF; Glomeromycota; T-RFLP; Terminal restriction fragment polymorphism; LSU rRNA

Terminal restriction fragment length polymorphism (T-RFLP) analysis is becoming increasingly popular for examination of arbuscular mycorrhizal fungi (AMF) communities in environmental samples (Johnson et al., 2003; Vandenkoornhuyse et al., 2003; Mummey et al., 2005; Mummey and Rillig, 2006; Wolfe et al., 2007).

These methods involve end-labeling PCR amplicons with fluorescent molecules attached to the 5′-end of one or both PCR primers. Sequence heterogeneity between rRNA genes of different species or phylogenetic groups results in different terminal restriction fragment (T-RF) sizes when PCR amplicons are digested with select restriction enzymes. After electrophoretic separation of the resulting fragments on polyacrylamide gel or capillary DNA sequencers, T-RF size distributions are typically analyzed by laser excitation and visualization of the fluor. T-RF size distributions can be compared between samples to yield measures of community similarity amenable to analysis using a variety of multivariate statistical methods (e.g. Blackwood et al., 2003).

Optimal application of these methods requires that the PCR amplicons examined 1) be specific to the Glomeromycota 2) broadly represent all of the diversity within the Glomeromycota and 3) contain sufficient information for discrimination of this diversity. In this study sequence data derived from both public databases and specific sites were examined to determine the efficacy of LSU rRNA-gene PCR primers FLR3 and FLR4 (Gollotte et al., 2004) with regard to these criteria.

To determine primer homology to public database sequences, nucleotide–nucleotide BLAST searches (for short nearly exact matches; NCBI; Altschul et al., 1990) was conducted using the sequences of FLR3 and FLR4 as search strings. For primer FLR4 a search returning 1000 “hits” yielded only sequences affiliated with the Glomeromycota. Among these sequences *Glomus* groups A and B, the Gigasporaceae and the Acaulosporaceae were well represented. Since Archaeosporaceae LSU rRNA gene sequences are poorly represented in GenBank, representative analysis of primer homology to this phylogenetic group was not possible. However, primer FLR4 was found to have multiple mismatches to *Archaeospora gerdemannii* (acc. AJ271712). For primer FLR3 the results indicated not only complete homology to broad groups within the Glomeromycota, but also, as reported previously (Gollotte et al., 2004), to representatives of the Basidiomycetes.

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We additionally obtained Glomeromycota sequences by querying GenBank using the search phrase [arbuscular AND LSU]. The resulting sequences were aligned using Clustal W (Chenna et al., 2003) and imported into GeneDoc (Nicholas et al., 1997). All sequences found not to contain the complete FLR3-FLR4 amplicon were removed from the dataset. All sequence data outside the FLR3-FLR4 amplicon were also removed. This dataset (216 sequences) was queried for primer homology by allowing 0, 1 or 2 mismatches to each of the primer sequences. These analyses indicated that 175, 204 and 206 of these sequences had no, one or less, or two or less mismatches to the FLR3 primer sequence, respectively. Analysis of where discrepancies occurred between primer FLR3 and these sequences showed that 11 sequences had mismatches in the first 11 bases from the 5′ end, and 30 had mismatches in the last 11 bases. Similar analysis of the FLR4 priming site indicated that 126, 168 and 180 of these sequences had no, one or less, or two or less mismatches to the FLR4 primer sequence, respectively. Analysis of where discrepancies between this primer and database sequences occurred indicated that 87 sequences had mismatches in the first 10 bases on the 5′ end, while only 10 mismatches were found in the 11 bases on the 3′ end.

These analyses demonstrate that homology between FLR3 and FLR4 and all potential target sequences is not perfect, highlighting the difficulty in developing primers with complete homology to all the Glomeromycota (e.g. Douhan et al., 2005; Van Tuinen et al., 1998; Clapp et al., 2001) and potentially biasing the composition of amplification products. However, these analyses also show that this primer pair is generally applicable to at least the four major AMF lineages for which substantial sequence data are available; *Glomus* groups A and B, the Acaulosporaceae and the Gigasporaceae. Moreover, most mismatches between FLR4 and database sequences were found to occur on the primer's 5′ end. Since mismatches between primer and target sequences are generally more important to primer specificity if they occur on the 3′ end (e.g. Sommer and Tautz, 1989), primer FLR4 may have broader coverage than results of homology analyses imply.

We also conducted site-specific analyses of AMF communities associated with roots and soils of a field site (described previously; Mummey and Rillig, 2006), and roots of glasshouse-grown plants to examine primer specificity and amplicon information content and suitability for T-RFLP analysis. Genomic DNA from soils was extracted using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) and from roots using either a modification of the method described in Edwards et al. (1991), incorporating a bead-beating step in place of grinding, or the DNeasy® Plant DNA extraction kit (Qiagen, Valencia, CA).

PCR amplification consisted of two PCR rounds, the first employing the primer pair LR1 and FLR2 (Trouvelot et al., 1999;

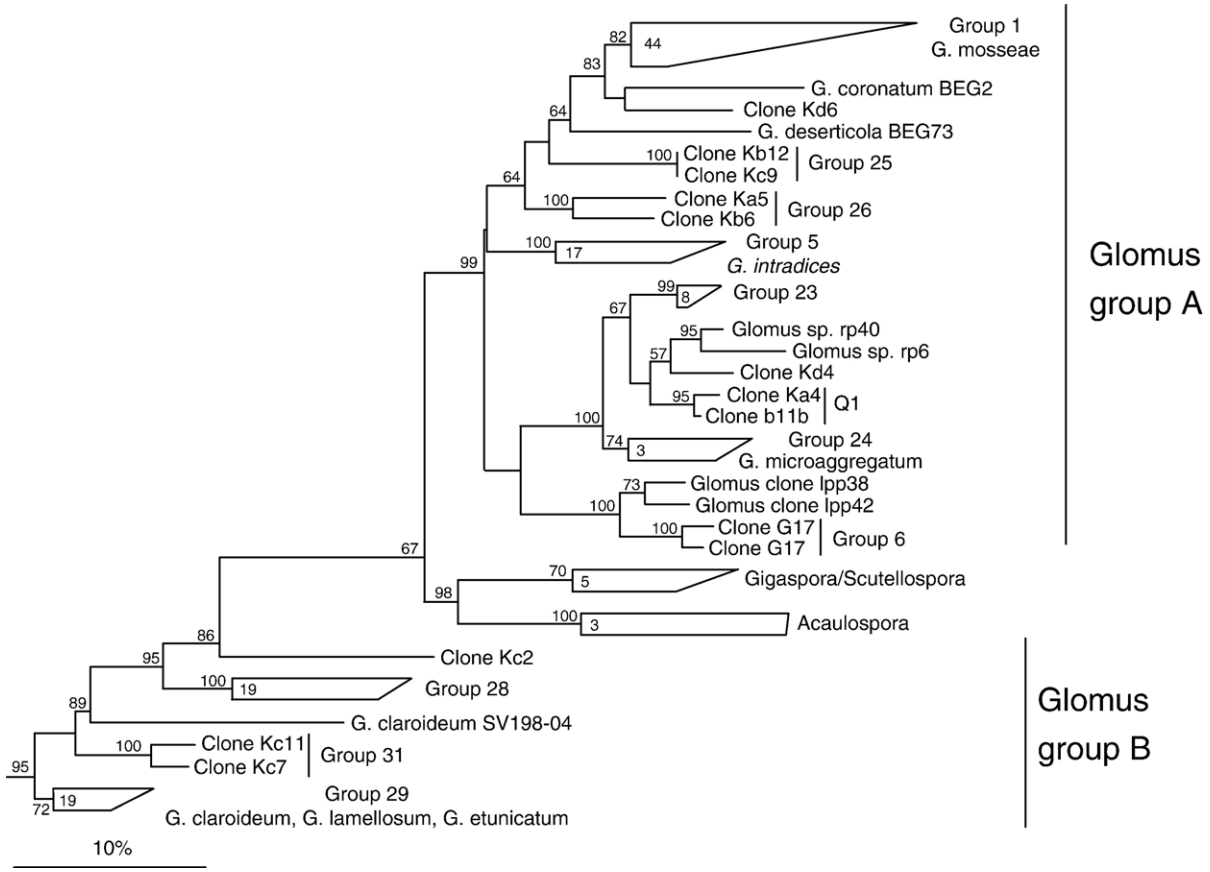

Fig. 1. Neighbor joining tree (Kimura 2-paramenter) constructed using the computer program PAUP* (Swofford, 1998) representing relationships between sequences cloned from glasshouse-grown plant roots in this study and sequences derived from public databases. Numbers within wedges indicate the number of sequences within each group. Numbers at nodes indicate percent bootstrap support (1000 replications). *Glomus*-groups A and B are as defined by Schüssler et al. (2001).

Table 1
Terminal restriction fragment sizes derived from all clones sequenced in this study and database sequences after simulated digestion with each of three different restriction enzymes

TaqI		*MboI*		*AluI*	
F	R	F	R	F	R
Acaulosporaceae					
101	48	**70**	26	**243**	70
239	76	168	**154**	**244**	71
252	77	169	**155**	**245**	72
254	**213**	182	334	258	73
255	**315**	224		260	
256	334	225		261	
257		300		262	
264		**302**		263	
315		**303**		**270**	
334		**305**			
		334			
Glomus group B					
47	43	59	26	45	71
53	76	151	51	56	72
54	137	152	138	69	73
55	215	187	213	258	370
66	216	299	305	259	
105	217	329	306	260	
150	218	330	329	261	
151	302	331	330	295	
152	329	332	331	296	
190	330	333	332	297	
251	331	366	333	299	
329	332	367	366	370	
330	333	368	367		
331	366	369	368		
332	370	370	369		
333	373	372	370		
366		373	372		
370			373		
373					
Gigasporaceae					
89	**46**	**110**	**55**	**126**	**184**
90	**89**	169	96	**127**	**185**
91	136	170	97	**128**	**188**
111	137	191		**148**	
Glomus group A					
F	R	F	R	F	R
49	48	**39**	**45**	44	**52**
50	49	**75**	**46**	45	70
139	54	**106**	**89**	**54**	71
143	55	**107**	**90**	68	72
144	73	**108**	**91**	**76**	**190**
145	75	150	96	**77**	**191**
146	76	151	97	**78**	**192**
149	136	152	98	**174**	**193**
150	137	153	**99**	**175**	**194**
152	**139**	**155**	**101**	**179**	**196**
153	**143**	**158**	**135**	**180**	**294**
182	**176**	**164**	137	**181**	**296**
183	**178**	**165**	138	**182**	**297**
184	**186**	**166**	**140**	**183**	**298**
185	**187**	167	**144**	**185**	**368**
186	**188**	168	**184**	300	371
228	**189**	169	**203**	**368**	**374**
229	**220**	**178**	**204**	371	
233	**221**	183	**217**	**374**	

Table 1 (*continued*)

TaqI		*MboI*		*AluI*	
F	R	F	R	F	R
Glomus group A					
369	**320**	**184**	**262**		
370	369	**185**	366		
371	370	186	370		
	371	187	371		
		188			
		189			
		191			
		224			
		269			
		270			
		273			
		277			
		366			
		370			
		371			

Values are fragment lengths (bp). Values in bold indicate T-RF sizes that are unique (a difference of at least 2 bp) to each of the four major Glomeromycota groups indicated on the right. F and R indicate forward and reverse sequence directions, respectively. Accession numbers for public database sequences included in the analysis: AF304894, AF304977, AF304990, AF378435, AF378445, AF378505, AF389004, AF389017, AF396782, AF396797, AJ271927, AJ459326, AJ459327, AJ459341, AJ459352, AJ459373, AJ459376, AJ510229, AJ510232, AJ549321, AJ746249, AM040404, AM040420, AM040426, AY541822, AY541859, AY541860, AY541861, AY541863, AY541865, AY541866, AY541879, AY541880, AY541905, AY639180, AY639334, DQ273790, DQ273828, X99640, Y07656, Y12075, AJ459338, AJ459329, AJ459330, AJ459334, AJ459332, AJ459337, AJ459345, AJ459331, AJ459324, AJ459323, AJ459364, AJ459333, AJ459328, AJ459342, AJ459346, AJ459347.

Van Tuinen et al., 1998) and the second using primers FLR3 and FLR4. The 25 μl reaction mixtures included 1 μl soil or root extracted template DNA, 100 pmol of each deoxynucleoside triphosphate, 10 pmol of each primer and 2 U HotMaster™ Taq DNA polymerase (Eppendorf, Hamburg, Germany). Thermal cycling for all reactions included an initial denaturing step of 95 °C for 5 min, 25 cycles (primer pair LR1 and FLR2) or 30 cycles (primer pair FLR3 and FLR4) consisting of 1 min at 95 °C, 1 min at 58 °C and 1 min at 65 °C, followed by a final extension step of 65 °C for 10 min. The resulting PCR products were purified using the GenCatch™ PCR cleanup kit (Epoch Biolabs, Inc., Sugar Land, TX) and cloned into the pCR®4-TOPO® vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Based on blue/white screening, randomly selected colonies were used to inoculate 500 μl LB broth containing kanamycin (50 μg ml^{-1}) and incubated at 37 °C (4 h). Each culture (1 μl) was used as template for PCR (25 μl; primers M13F and M13R; 25 cycles). Reactions yielding amplicons of correct size were used as templates in cycle sequencing reactions employing BigDye terminators (Applied Biosystems Inc., Fremont, CA). Products of these reactions were analyzed using a 3100 automated DNA sequencer (Applied Biosystems Inc.) and the resulting sequences were deposited in GenBank under accession numbers DQ468685–DQ468824, DQ677384–DQ677482 and EF066393–EF066479.

A database was constructed containing these sequences and reference sequences obtained from Genbank selected to broadly

represent the Glomeromycota. These sequences were aligned using Clustal W and the alignment used to generate tree topologies (Fig. 1).

Simulated digestion of these sequences with a range of common tetrameric restriction enzymes (*AluI*, *BstUI*, *HhaI*, *HinfI*, *MboI*, *MspI*, *NlaIII*, *RsaI* and *TaqI*) was conducted using the computer program TRFSEQ (Mahaffey et al., 2002; available upon request) to determine restriction enzymes allowing for optimal discrimination of the AMF groups identified as above on the basis of differences in T-RF sizes. Restriction enzyme selection was based on the following criteria: (1) ability to discriminate between AMF groups, (2) obtaining unique T-RF sizes for each group that were as distinct as possible (in this case at least 2 bp), and (3) that the resulting T-RF sizes were greater than 35 bp to allow for size calling.

Two datasets were examined. The first consisted of all (n=323) of our sequenced clones derived from soils and roots and 93 public database sequences broadly representing the diversity within the Glomeromycota. We examined this dataset to determine the overall efficacy of different restriction enzymes for T-RFLP discrimination of major AMF lineages *Glomus* groups A and B (as defined by Schüssler et al., 2001), the Acaulosporaceae and the Gigasporaceae. The second dataset, consisting of only sequences derived from our glasshouse-grown root samples (n=184), was examined to evaluate the efficacy of study-specific analyses for discrimination of all AMF groups identified via phylogenetic analyses.

Based on the criteria specified above, all restriction enzymes except *AluI*, *MboI* and *TaqI* were found to have relatively poor ability to discriminate between AMF groups in either dataset and were excluded from further analysis.

Simulated digestion of dataset 1 indicated that *MboI* and *TaqI* yielded a greater number of T-RF sizes (57 and 56 forward and 43 and 44 reverse T-RF sizes, respectively) than *AluI* (42 forward and 26 reverse T-RF sizes) (Table 1). However, T-RF sizes yielded by each enzyme unique to each of the four primary AMF groups (*Glomus* A and B, the Acaulosporaceae and the Gigasporaceae), and therefore useful for group discrimination, were greatest for *TaqI* (69%), followed by *AluI* (62%) and least for *MboI* (59%).

Simulated digestion of dataset 2 showed that *MboI* resulted in the greatest number of T-RF sizes (19 and 16 in forward and reverse directions, respectively) compared with *TaqI* (13 and 15) and *AluI* (12 and 8) (Table 2). Moreover, numbers of T-RF sizes unique to each AMF group identified from phylogenetic analyses were greatest after simulated digestion with *MboI* (68% overall, 80 and 55% for forward and reverse directions, respectively) than for *AluI* (40% overall, 78 and 3% for forward and reverse directions, respectively) or *TaqI* (42% overall, 43 and 41% for forward and reverse directions, respectively).

Simulated digestion of dataset 2 with *MboI* resulted in unique T-RF sizes in either forward or reverse directions for all AMF groups identified through sequence analysis with the exception of the two clones affiliated with g26 and one of the g29 clones (Fig. 1). *TaqI* yielded unique T-RF sizes for all clones affiliated with groups g23 and g28, and 96, 24, 88 and 2% of clones affiliated with groups g29, g31, g5 and g1, respectively, and none of the clones affiliated with groups g24, g25, g6 or g26.

Table 2
Specific analysis of cloned sequences (n=184) derived from plant roots in our glasshouse studies

Clones	*TaqI*		*MboI*		*AluI*		Amplicon length	AMF group
	F	R	F	R	F	R		
1	185	189	**107**	99	**54**	72	376	g1
1	186	188	**108**	98	**54**	71	376	g1
1	187	185	**107**	184	**54**	71	374	g1
1	185	188	**57**	98	**54**	71	374	g1
1	185	188	**101**	98	**54**	71	376	g1
1	184	188	**107**	98	**54**	71	376	g1
5	185	189	**107**	99	**54**	72	376	g1
11	185	188	**107**	98	**54**	71	375	g1
34	185	188	**107**	98	**54**	71	375	g1
1	183	48	184	**90**	180	192	372	g2/g5
8	**149**	**76**	**371**	**371**	180	191	371	g23
2	50	187	**155**	184	45	192	377	g24
1	188	50	**189**	98	**185**	71	378	g25
1	188	188	**189**	98	**185**	71	378	g25
1	184	189	185	99	78	72	375	g26
1	184	188	185	98	181	71	374	g26
1	**331**	**331**	**331**	**331**	259	72	**332**	g28
1	**333**	**333**	**333**	**333**	260	73	**333**	g28
1	**333**	**333**	**333**	**333**	261	72	**333**	g28
4	**332**	**332**	**332**	**332**	261	71	**332**	g28
9	**332**	**332**	**332**	**332**	260	72	**332**	g28
10	**332**	**332**	**332**	**332**	260	71	**332**	g28
14	**331**	**331**	**331**	**331**	260	71	**331**	g28
1	**54**	43	**369**	**369**	297	72	369	g29b
1	**54**	215	**369**	**369**	71	71	369	g29b
1	151	216	152	213	**297**	72	369	g29b
2	**54**	215	**369**	**369**	297	71	369	g29b
14	**54**	216	**369**	**369**	297	72	369	g29b
7	**54**	217	**369**	**369**	297	73	369	g29b
3	151	216	**59**	**306**	297	72	369	g29b
1	**47**	216	**59**	213	45	72	369	g31
1	**47**	43	**59**	**51**	45	72	369	g31
1	**47**	43	**59**	**51**	45	**49**	370	g31
1	151	44	**59**	**52**	45	73	370	g31
3	**47**	43	**59**	**51**	45	72	370	g31
4	151	43	**59**	**51**	45	72	370	g31
7	151	43	**59**	**51**	45	**49**	370	g31
7	151	44	**59**	**52**	45	**50**	370	g31
1	184	**48**	**191**	**90**	78	191	372	g5
1	185	49	186	**91**	78	72	375	g5
7	184	**48**	185	**90**	78	192	373	g5
2	50	50	151	**137**	370	370	370	g6

Presented are the number of sequenced clones having the associated T-RF size pattern, T-RF sizes after simulated digestion with each of three different restriction enzymes, total amplicon lengths, and taxonomic affiliation as in Fig. 1. Values in bold indicate T-RF sizes unique (at least a two bp difference) to each group.

AluI resulted in unique T-RF sizes for all clones within groups g1, g25, g28, g31 and g6, 98% of g29 clones and 65% of g31 clones, but none of the clones affiliated with groups g23, g5 g24 and g26.

While there is no a priori way of selecting restriction enzymes optimal for revealing the diversity in a given community short of extensive sequence analyses, our analyses indicated three restriction enzymes (*MboI*, *TaqI* and *AluI*) having overall greater ability to discriminate between AMF phylogenetic groups. While *TaqI* yielded greater numbers of T-RF sizes unique to the four primary AMF groups when public database sequences were

included in the analysis (Table 1), *MboI* would be the better choice for our site-specific studies. This highlights the importance of collecting site- or study-specific sequence data to optimize restriction enzyme selection and to determine the overall resolution of the method.

To reveal the diversity present in a sample it is imperative that terminal restriction fragments be both unique to each phylogenetic group of interest and that these differences in fragment sizes be as pronounced as possible. This is especially important if a goal is tentative discrimination or identification of specific components of the AMF community since predicted T-RF sizes (anticipated from sequence analysis) and observed T-RF sizes (indicated by T-RFLP analysis) can vary (Marsh, 2005). Such problems can be largely eliminated by determining observed T-RF size for each cloned sequence of interest after PCR amplification and restriction enzyme digestion. In some cases, such as shown here for our glasshouse studies, optimization of restriction enzyme selection may allow for relatively large differences in T-RF sizes for most AMF groups; relatively large size "bins" can potentially accommodate discrepancies between predicted and observed T-RF sizes.

In summary, PCR primers FLR3 and FLR4 are highly specific to Glomeromycota in our study systems. Moreover, the information content in the amplicon is relatively high and, with judicious restriction enzyme selection, T-RFLP analysis based on these primers is a potentially valuable tool for analysis of AMF communities. However, collection of site-specific sequence data is necessary before conducting T-RFLP in order to optimize restriction enzyme selection, determine reaction specificity and for evaluation of overall analysis efficacy.

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