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Comparison of different PCR primers on detecting arbuscular mycorrhizal communities inside plant roots

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Abstract: [**Objective**] Communities of arbuscular mycorrhizal fungi (AMF) colonizing roots have been increasingly investigated by molecular approaches with AMF-specific PCR primers. However, it is difficult to compare the species diversity and species compositions of AMF communities across various studies due to the PCR primers used differently, and also little is known if significant difference of community compositions is characterized by different primers. We aim to compare the difference of efficiency of four primers for AMF. [**Methods**] We chose four commonly used AMF-specific primer combinations (NS31-AM1, AML1-AML2, NS31-AML2 and SSUmCf-LSUmBr), and used 18S rDNA clone libraries to describe the AMF diversity and community. [**Results**] Our results showed that the specificity and coverage varied among the tested primers, different primer combinations would yield distinct patterns of species diversity and composition of AMF community. SSUmCf-LSUmBr had the best specificity and coverage in amplifying AMF sequences, followed by NS31-AML2 and NS31-AM1, and AML1-AML2 showed the lowest specificity towards AMF sequences. [**Conclusion**] SSUmCf-LSUmBr is not the optimal primer pair for AMF community study in current stage due to limited reference sequences and large DNA size. As an alternative, NS31-AML2 is more suitable in AMF community study, because its target rDNA region could well match the increasingly used virtual taxonomy database (http://maarjam.botany.ut.ee) and also its suitable DNA size could be efficiently used in high-throughput sequencing.

Keywords: Mycorrhiza, Glomeromycota, community ecology, rDNA, DNA barcoding, primer

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Arbuscular mycorrhizal fungi (AMF) in the phylum Glomeromycota are one of the most important root-associated microorganisms on the earth[1]. About 70% – 90% land plant species form mutualistic associations with diverse AMF communities in natural conditions[2], and through the mycorrhizal symbionts AMF provide phosphorus and nitrogen to their hosts in exchange for plant photosynthates[3]. In addition, increasing evidence shows that AMF could regulate plant communities[4-5] and biogeochemical cycles[6-7], and also influence many ecosystem processes via direct and indirect ways[8]. Although the importance of AMF has been well accepted, the diversity and composition of AMF communities in most ecosystems are not well understood, and which is partly due to the difficulty in identifying AMF communities in fields.

The study of AMF community is traditionally dependent on the morphological identification of asexual spores collected from soil[9]. However, it is obvious that the communities of dormant spores could not reflect the active AMF communities[10]. In recent years, PCR-based molecular approaches have been increasingly used in the study of AMF community[11-15], because these methods can rapidly and accurately identify the diversity and composition of AMF communities in both root and soil samples. Using molecular methods to identify AMF communities are highly dependent on the specific PCR primers, which should amplify all members of AMF, but meanwhile exclude sequences from other organisms[16]. In 1998, Helgason et al[17] designed an AMF-specific primer AM1 from small subunit (SSU) rDNA sequences, and successfully used it with a general eukaryotic primer NS31 in detecting AMF communities in arable and forest fields. The NS31-AM1 has been used to identify AMF communities in many ecosystems[15, 18-21], but increasing evidence shows that AM1 could not amplify the AMF members within Paraglomerales and Archaeosporales[16], and it would also amplify non-AMF sequences under some circumstances[19, 22]. To improve the specificity and coverage of AMF PCR primers, several new primers and primer combinations that target SSU rDNA region have been developed in recent years. For instance, Lee et al.[16] designed a new primer pair (AML1-AML2) with higher specificity and coverage than NS31-AM1; Liu et al.[23] paired the NS31 with AML2 and successfully used this primer combination in detecting AMF communities from root samples. However, some researchers proposed that inclusion of the internal transcribed spacers (ITS) and the large subunit (LSU) rDNA regions could largely improve the species resolution of AMF than on basis of SSU region[24]. On this basis, Krüger et al.[24] design four primer mixtures (SSUmAf, SSUmCf, LSUmAr and LSUmBr) that cover the partial SSU, whole ITS and partial LSU regions, and they found that these primers could be efficiently used in detection and identification of AMF. All the PCR primers mentioned above have been frequently used in detecting AMF communities in fields, but which is the best primer pair and whether different primers would yield distinct AMF communities are poorly understood.

Several recent studies have evaluated the abilities of different AMF PCR primers in detecting AMF communities. For example, Kohout et al.[25] compared five AMF-specific primer systems using six plant species with a PCR-cloning-sequencing approach, and found that the primers covering the partial SSU-ITS-partial LSU region tended to yield the highest AMF diversity than the SSU primers. Geel et al.[26] evaluated six AMF PCR primer pairs using 454 pyrosequencing techniques and showed that the SSU primers could work better than the LSU primers in characterizing AMF communities colonizing apple roots. The above two studies clearly show that different primer pairs would lead to different results, indicating that the choice of target rDNA marker region was crucial in analyzing AMF communities. However, inconsistent PCR primers recommended in both studies make it is difficult to choose the suitable primers in

future AMF community study, moreover, single plant species tested in both cases may not reflect the difference of the primers accurately, because AMF often show host preference in natural conditions[21,23]. To fully address whether there have significant differences of community compositions characterized by different PCR primers, we compared four commonly used AMF-specific primer combinations using mixed root samples collected from undisturbed grassland. Our results will greatly facilitate the choice of primers in the community study of AMF.

1 Materials and Methods

1.1 Selection of tested primers and root samples

We selected the NS31-AM1[17], AML1-AML2[16], NS31-AML2[23] and SSUmCf-LSUmBr[24] as our tested primer combinations, because they were frequently employed in AMF community researches. The former three primer sets target SSU rDNA and have an overlapped region of *c.* 550 base pairs (bp), and the SSUmCf-LSUmBr cover partial SSU, whole ITS and partial LSU rDNA (*c.* 1500 bp; Figure 1).

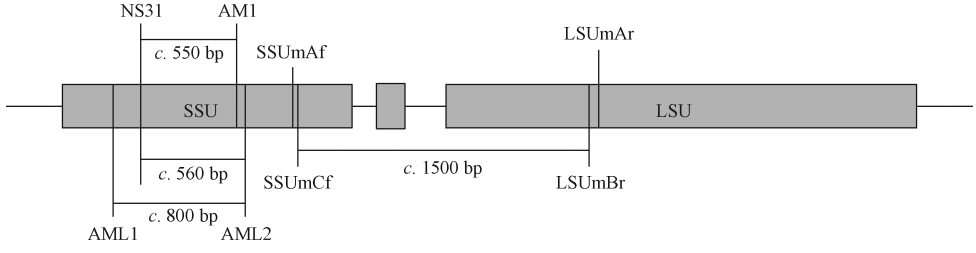

Figure 1. The location of each primer combination on target nuclear ribosomal DNA regions. The amplified DNA length is about 550, 560, 800 and 1500 bp with primer pairs NS31-AM1, NS31-AML2, AML1-AML2 and SSUmCf-LSUmBr, respectively.

Our root samples were collected from the Walaka experimental site of the Research Station of Alpine Meadow and Wetland Ecosystems of Lanzhou University, which is located in the eastern Qinghai-Tibet Plateau of China (33°59′ N, 102°00′W, 3500 m a. s. l.) and belongs to a typical alpine meadow ecosystem. The major reason we chose the tested root samples from this experimental site is that the species diversity of both plants and AMF are relatively high[13]. On August 2011, five tested root samples were collected from five (2 × 2) m^2 plots with similar vegetation and soil conditions, and in each plot, five soil cores were taken randomly and mixed as one sample. All active fine roots were isolated carefully from each soil sample and washed clearly for DNA extraction.

1.2 Molecular analysis of AMF community

DNA was extracted from 50 mg fresh roots of each sample using a Plant DNA Extraction Kit (Tiangen Biotech, Beijing, China) and subjected to nested PCR with four primer systems respectively. For the former three SSU primers, the first PCR was performed using the universal fungal primers of GeoA2-Geo11[27]. For the SSUmCf-LSUmBr primer pair, we used SSUmAf-LSUmAr primer mixtures as the primers of first PCR. The first PCR was carried out in a final volume of 25 μL including 2.5 μL 10 × PCR buffer, 2 μL extracted DNA dilution, 0.2 mmol/L dNTPs, 0.2 μmol/L of each primer and 1.5U *Taq* polymerase (New England Biolabs, MA, USA). The first PCR product was diluted with ddH_2O (1∶100) and 2 μL of this dilution was used as template for the second PCR. The second PCR conditions were the same as the first PCR except that the final volume and reaction reagents were double.

We constructed 20 clone libraries using the purified DNA fragments of the second PCR products (5 samples × 4 primer sets). For each clone library, 48 putative positive clones were screened using PCR and restriction fragment length polymorphism (RFLP) analysis with the restriction enzymes *Hin*f I and *Hin*1II (Fermentas, Vilnius, Lithuania). RFLP patterns were only compared within the five samples in the same primer system. One representative clone of each RFLP

type was sequenced (138 in total), and the remaining clones were classified by RFLP typing. All sequences were checked for chimeras using Chimera-Check program[28] and compared with GenBank database using BLAST. All obtained AMF sequences (113 in total; these sequences were submitted to GenBank database under accession numbers KF939879-KF939919 and KF939921-KF939992) were used in phylogenetic analysis and phylotype delimitation. Considering that the SSUmCf-LSUmBr sequences have no overlap with the sequences obtained by the three SSU primer sets, their phylogenetic analysis and the phylotype delimitation were separately preformed. Sequences from the SSU fragment were blasted against the MaarjAM database (http://www.maarjam.botany.ut.ee/) and grouped into the corresponding molecular virtual taxa (VT; similar with named AMF phylotypes) with the sequence identity ≥ 97%. For the SSUmCf-LSUmBr sequences, we used the monophyletic clade approach to combine sequence groups into phylotypes that formed monophyletic clades manually[29-30]. Representative sequences of each AMF phylotype and some reference sequences from GenBank were aligned using ClustalW 2.0, and the maximum-likelihood tree was constructed using MEGA 5.0 with Kimura two-parameter model and 1000 bootstrap replications[31].

1.3 Statistical analysis

The community compositions of AMF were calculated on the basis of the relative abundance of each phylotype in each clone library. Rarefaction curves were generated with EstimateS 9.1.0 for each primer combination[32], and the Shannon's diversity and Evenness of each detected AMF community were calculated using PAST version 1.61[33]. The effects of PCR primer on amplification efficiency and phylotype abundance were tested by one-way analysis of variance (ANOVA). To determine whether different primer combinations would yield distinct AMF communities, the communities obtained by the three SSU primer sets were compared using a principle component analysis (PCA) and an Adonis analysis with R package 'Vegan'[34].

2 Results and Discussion

The proportion of detected AMF clone/sequence varied among primer combinations: the primer pair SSUmCf-LSUmBr had the highest specificity in amplifying AMF sequences from our samples, followed by NS31-AM1 and NS31-AML2, and AML1-AML2 was the lowest (Table 1). About 26% sequences amplified by AML1-AML2 were related to plant sequences, corroborating a community study in semiarid Mediterranean using the same primer pair[35], in which 34% of sequences were plant origins. It is possible that AML1-AML2 could excellently exclude non-AMF fungi and some plant sequences[16], but our findings indicate that more cautions need to be paid when using this primer combination to analyze AMF community colonizing roots.

Table 1. Proportion of AMF clones and AMF diversity indexes identified by different primer combinations

Primer combinations	Proportion of AMF clones (%)	Total number of detected phylotype (family)	Phylotype richness	Shannon's diversity	Evenness
NS31-AM1	98.5 ± 0.6 a	9 (Glomeraceae)	7.0 ± 0.6 ab	1.55 ± 0.06 a	0.71 ± 0.06 a
NS31-AML2	94.8 ± 1.9 a	10 (Glomeraceae)	8.2 ± 0.6 a	1.65 ± 0.09 a	0.64 ± 0.02 ab
AML1-AML2	74.1 ± 4.4 b	11 (Glomeraceae, Acaulosporaceae)	7.8 ± 0.5 a	1.66 ± 0.07 a	0.68 ± 0.04 a
SSUmCf-LSUmBr	100.0 ± 0.0 a	12 (Glomeraceae, Acaulosporaceae, Ambisporaceae)	5.8 ± 0.6 b	1.15 ± 0.11 b	0.56 ± 0.02 b

All data are means ± SE ($n=5$), with exception of the total number of detected phylotype. Significant differences across different primer combinations within each variable were determined using Least Significant Difference at the 5% level and indicated by dissimilar letters.

Even though the majority of AMF diversity was captured by each primer combination, the total numbers of AMF phylotypes and families as well as the mean phylotype richness, Shannon's diversity index and evenness were highly dependent on the used primer combination. The SSUmCf-LSUmBr detected

higher numbers of phylotype and family, and followed by AML1-AML2 (Table 1). Surprisingly, the lowest phylotype richness, diversity index and evenness were synchronously detected by SSUmCf-LSUmBr. This might be attributed to few common phylotypes were detected from our root samples, and emphasizes that more clones need to be analyzed when using the SSUmCf-LSUmBr in community study. Nonetheless, higher AMF lineage coverage of SSUmCf-LSUmBr observed in our case supports the idea proposed by Stockinger et al.[36], who recommoned the SSUmCf-LSUmBr rDNA region as a barcode region for AMF species identification. All sequences identified by NS31-AML2 and NS31-AM1 were belonging to

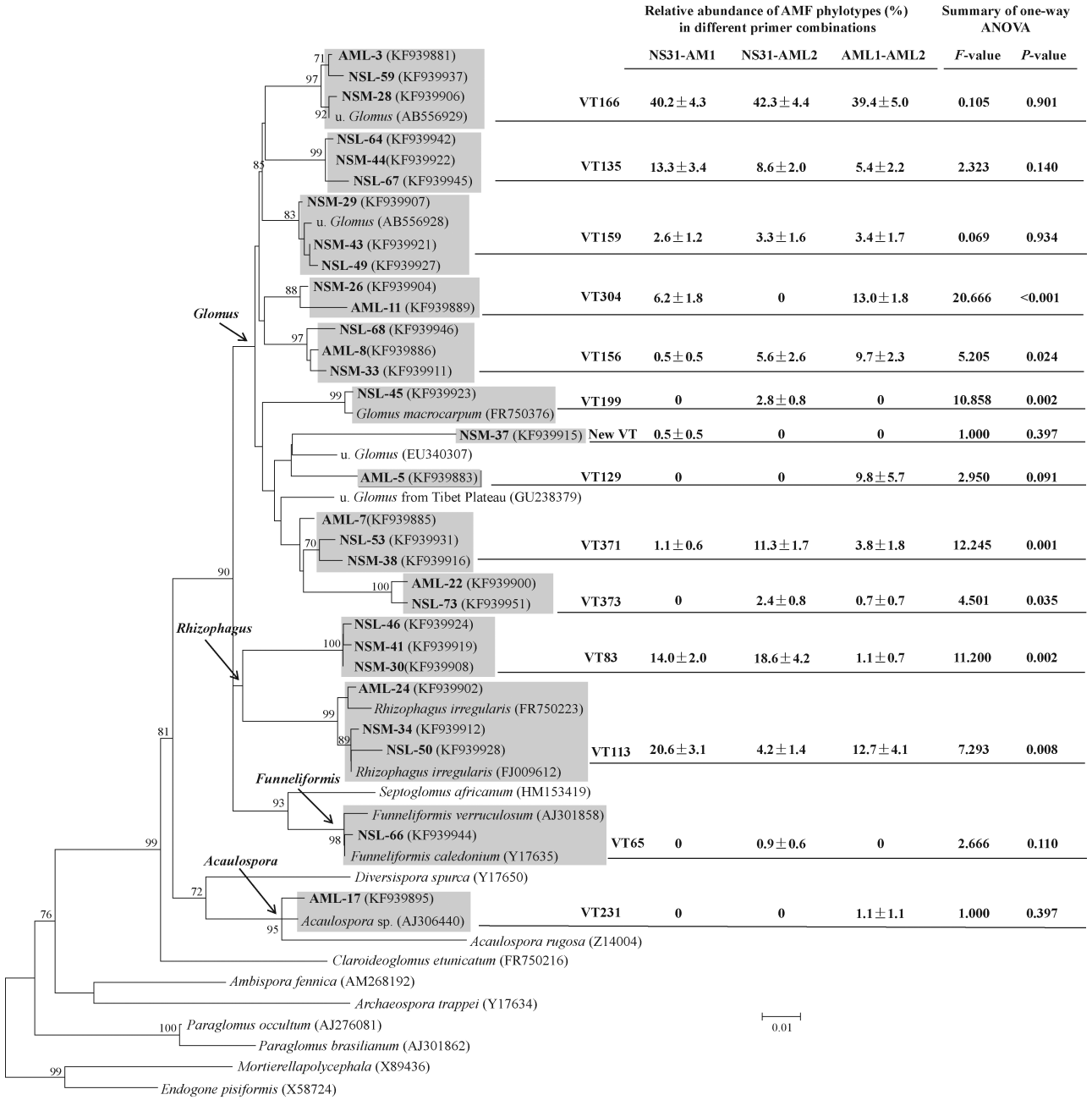

Figure 2. Maximum-likelihood tree inferred from the SSU representative sequences of each AMF phylotype obtained in this study and reference sequences from GenBank. Bootstrap values above 70% are shown. The relative abundances of each AMF phylotype in different primer combinations are means ± SE ($n=5$). The results of one-way ANOVA for each phylotype were joined onto this figure.

Glomeraceae, indicating that the sequences of Ambisporaceae and Acaulosporaceae that were successfully detected by SSUmCf-LSUmBr could not be amplified by these two primer sets. In fact, the coverage limitation of NS31-AM1 has been well reported[16], but rich evidence shows that NS31-AML2 could detect near all AMF families[12-14, 23, 37-40].

A total of 14 AMF phylotypes were detected by the SSU primers, with 3 – 5 phylotypes missed by each primer combination (Figure 2). Relative abundance of half phylotypes was significantly affected by primer combination (Figure 2), and the AMF communities detected by the three SSU primer combinations were different in their phylotype compositions (Figure 3; Adonis analysis: all $P < 0.01$).

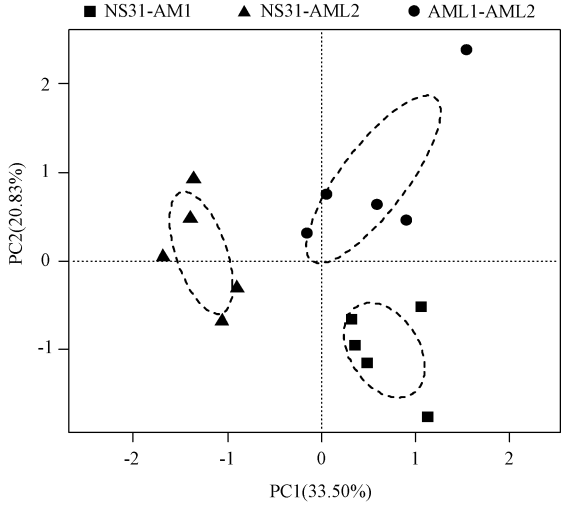

Figure 3. Principal component analysis showing distinct community compositions of AMF detected by three SSU primer combinations. Ellipses with different colors indicate 95% confidence ellipses for each primer combination. Adonis analysis also indicates significant difference among different primer combinations (all $P < 0.01$).

Such distinct communities detected in this study should be related with the primer sensitivity to less-represented DNA populations in the mixture; furthermore, as the NS31-AM1 preferentially amplifies Glomeraceae[16], it is possible that other primer combinations would also have a certain degree of taxon or sequence preference. It is impossible to compare the AMF communities identified by SSUmCf-LSUmBr and SSU primers, but we can expect that the community detected by SSUmCf-LSUmBr might be largely different with that by other three primer pairs. For example, the SSU primes hosted a high percentage of the *Rhizophagus* (13% – 34%) in the AMF communities, but none was detected by the SSUmCf-LSUmBr (Figure 4).

In conclusion, our results showed that different primer combinations would yield distinct patterns of species diversity and species composition of AMF community. Among the four tested primer combinations, the SSUmCf-LSUmBr should be the first choice in identification of AMF species, because it has relatively higher coverage, specificity and taxonomic resolution[36]. However, the major problem of using the SSUmCf-LSUmBr in community researches is that limited reference sequences have been published; furthermore, long DNA region covered by SSUmCf-LSUmBr would also double the experimental expense compared with other primers. More works on identification of AMF taxa and environmental sequences using the SSUmCf-LSUmBr are encouraged in future, and only this can SSUmCf-LSUmBr be used as an efficiently AMF barcoding primer set. As an alternative choice in current stage, the NS31-AML2 might be more suitable in the studies of AMF community. The sequences obtained by NS31-AML2 can be well grouped into the virtual taxonomy of AMF SSU sequences, which was established by Dr. Maarja Öpik and is gradually improved and increasingly used[41]. In addition, suitable DNA size amplified by NS31-AML2 will also facilitate the application of next-generation sequencing techniques in AMF community studies, and indeed this primer pair has been widely used in 454 pyrosequencing[14, 38, 42-45]. Nonetheless, as the primer design is highly dependent on published sequences, we can expect that with more environmental DNA sequenced, new primers with reliable and robust resolution in AMF identification will be designed in near future.

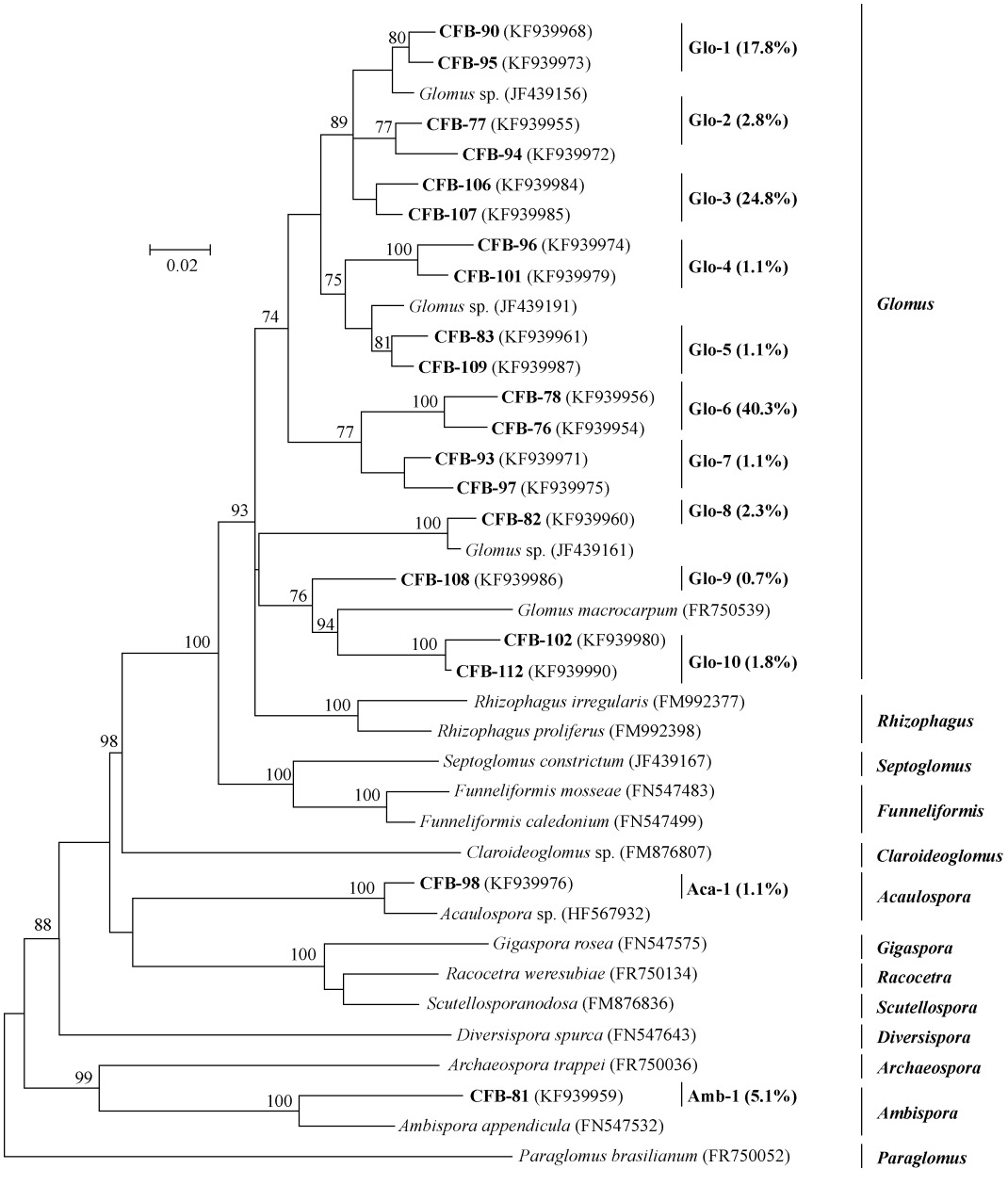

Figure 4. Maximum-likelihood tree of our representative sequences (in bold) identified by the SSUmCf-LSUmBr primer pair and the referenced sequences.

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不同 PCR 引物在根系丛枝菌根真菌群落研究中的应用比较

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摘要:【目的】基础 PCR 的各种分子技术已广泛地应用于丛枝菌根真菌(AMF)的群落研究。为探讨不同 PCR 引物对丛枝菌根真菌群落的特异性差异扩增。【方法】本研究选取 4 对 AMF 特异性引物(NS31-AM1, AML1-AML2, NS31-AML2 和 SSUmCf-LSUmBr),通过 PCR、克隆及测序技术对 AMF 的多样性及群落结构进行了分析比较。【结果】不同引物对 AMF 的扩增特异性及覆盖度均有显著性差异,且不同引物得到的 AMF 群落结构也存在显著性差异。SSUmCf-LSUmBr 的扩增特异性及覆盖度最高,NS31-AML2 和 NS31-AM1 次之,而 AML1-AML2 则相对较差。【结论】NS31-AML2 的扩增区段能很好地与越来越被认可的 AMF VT 分类数据库(http://maarjam.botany.ut.ee)相匹配,且扩增片段长度也适合于目前的高通量测序技术。基于此,本文推荐 NS31-AML2 为 AMF 群落研究中的首选引物。

关键词:菌根,球囊菌门,群落生态学,rDNA,DNA 条形码,PCR 引物

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