Manuscript type: Original article

Title: A simple model system for identifying arbuscular mycorrhizal fungal taxa that actively colonize rice roots grown in field soil.

Running title: DNA analysis on AM fungal infection unit

Authors: Yoshihiro Kobae[1,*], Ryo Ohtomo[1], Norikuni Oka[1], Sho Morimoto[1]

[1] National Agriculture and Food Research Organization (NARO) Hokkaido Agricultural Research Center, Agro-environmental Research Division, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido, 062-8555 Japan

Yoshihiro Kobae, Phone: +81-11-857-9241; Fax: +81-11-859-2178; E-mail: kobae@affrc.go.jp

Ryo Ohtomo, Phone: +81-11-857-9241; Fax: +81-11-859-2178; E-mail: rotm@affrc.go.jp

Norikuni Oka, Phone: +81-11-857-9243; Fax: +81-11-859-2178; E-mail: okan@affrc.go.jp

Sho Morimoto, Phone: +81-11-857-9243; Fax: +81-11-859-2178; E-mail: shomo@affrc.go.jp

*** Corresponding Author:** Yoshihiro Kobae

National Agriculture and Food Research Organization (NARO) Hokkaido Agricultural

Research Center, Agro-environmental Research Division, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido, 062-0045 Japan

Phone: +81-11-857-9241; Fax: +81-11-897-2178; E-mail: kobae@affrc.go.jp

Abstract

Even a few centimetres of roots in field soils can be colonized by genetically diverse arbuscular mycorrhizal (AM) fungi. The DNA sequences of AM fungi in roots suggest the fungal identities; however, it is difficult to determine which AM fungal taxa is physiologically active. In this study, we took advantage of the characteristics of rice (*Oryza sativa*) mycorrhizal roots, in which active colonization in roots is easily detected via histochemical staining of fungal succinate dehydrogenase activity (vital staining) and individual active colonization regions (infection units) in roots rarely coalesce. Root segments (<3 mm) containing an active infection unit were dissected and squashed, LSU ribosomal RNA genes were amplified using fungal universal primers and the sequences were directly determined by Sanger sequencing. All obtained sequences of colonization regions were of glomeromycotan origin. Phylogenetic analysis revealed that the levels of genotypic heterogeneity within an active colonization site were different among different clades. The methodology presented in this study offers researchers a novel tool for investigating the DNA information of physiologically active AM fungi in roots, whereas the factors that affect genetic diversity among active colonization remain to be clarified.

Key words

active colonization; arbuscular mycorrhizas; fungal taxa; infection unit; *Oryza sativa*; vital staining

1 Introduction

Most terrestrial plant species are colonized by multiple species of a fungal lineage belonging to *Glomeromycota*, namely arbuscular mycorrhizal (AM) fungi. These fungi play a crucial role in a number of plant physiological processes, such as nutrient uptake (Javot *et al*. 2007; Leigh *et al*. 2009), water uptake (Khalvati *et al*. 2005), disease resistance (Song *et al*. 2010), fruit ripening (Chialva *et al*. 2016) and the production of secondary metabolites (Toussaint *et al*. 2007). Strict host specificity has not been recognized in this symbiosis (Smith and Read 2008) and a few centimetres of root fragments can be colonized by genetically diverse multiple fungal species (van Tuinen *et al*. 1998). Laboratory experiments with inoculations of single AM fungi illustrated that the outcome of colonization strongly depends on fungal taxa (Munkvold *et al*. 2004; Avio *et al*. 2006; Mensah *et al*. 2015). Characterization of the taxa (DNA information) of individual AM fungi in roots grown in field soils thus appears to be of crucial importance for understanding the molecular cues that regulate the outcome of colonization.

Determining the taxa of AM fungi in roots grown in field soils has generally been performed using clone library analysis or next-generation amplicon sequencing against whole root systems with AM fungus-specific oligonucleotide primers (Lee *et al*. 2008; Krüger *et al*. 2009). In this method, the taxa of individual colonizing fungi are not distinguished and the abundance of DNA is not necessarily correlated with their physiological activity (Vandenkoornhuyse *et al*. 2007; Berruti *et al.* 2013; Janoušková *et al*. 2015). Accordingly, the individual taxa of AM fungi co-colonizing roots have not been linked to the outcome of colonization.

Physiologically active colonization can be detected on the basis of AM fungal

enzymatic activities (Tisserant *et al*. 1993). Vital staining, which histochemically visualizes the activity of succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme in AM fungi, using the reduction of nitroblue tetrazolium (NBT) into insoluble formazan, detects metabolically active colonization (MacDonald and Lewis 1978). Using this method, all metabolically active AM fungal structures such as hyphae, spores, vesicles and arbuscules are stained (Vierheilig *et al*. 2005). All colonizing fungi revealed by fungal cell wall (CW) staining are metabolically active in young plants, but this may not be the case in older plants (Smith and Gianinazzi-Pearson 1990; Abdel-Fattah 2001). Our previous study also illustrated that the numbers of colonization site were comparable between vital staining and CW staining at least up to 15 days post-plantation (dpp) in rice roots colonized by the model AM fungus *Rhizophagus irregularis* DAOM197198 (Kobae *et al*. 2016). However, it should be noted that the observations of similar colonization levels between vital staining and CW staining have been, to the best of our knowledge, obtained from the inoculation of single fungal strains (Hamel *et al*. 1990; Smith and Gianinazzi-Pearson 1990; Abdel-Fattah 2001) opposed to multiple fungal species in roots grown in field soils. Given that physiological characteristics differ among AM fungal taxa (Boddington and Dodd 1998; Vivas *et al*. 2003), it is possible that only some root-colonizing AM fungi are physiologically active.

To identify the AM fungal taxa that actively colonize roots grown in field soil with high spatial resolution, it is important to determine the taxon of individual colonization. The spread of colonization is achieved by the successive formations of small colonization sites, namely infection units (IUs), each of which comprises an internal mycelium arising from one entry point (Cox and Sanders 1974; Walker and Smith 1984; Javot *et al*. 2007). Hyphae penetrate epidermal cells and longitudinally spread from the

97 entry points intercellularly (*Arum*-type) or intracellularly (*Paris*-type) through the root
98 cortex (Smith and Read 2008). Arbuscules, highly branched structures of AM fungi that
99 are regarded as major sites for nutrient exchange (Harrison 2005), are formed in cortical
100 cells in parallel with the spreads of intraradical hyphae. The spread of intraradical hyphae
101 from one entry point is limited to approximately 5 mm (Cox and Sanders 1974; Smith
102 and Read 2008). The subsequent spread of colonization occurs through the growth of
103 external hyphae, both along and between roots, originating from established IUs and by
104 the formation of new IUs (Sanders and Sheikh 1983; Kobae and Fujiwara 2014). It is
105 notoriously difficult to dissect a single IU from well-developed mycorrhizal roots because
106 numerous IUs coalesce to form a larger colonized region, at which the border of each IU
107 is not identified at least within a few days from the beginning of colonization (Sanders
108 and Sheikh 1983). In addition, fungal DNA isolated from mycorrhizal roots that are
109 treated using conventional CW staining methods (e.g. trypan blue staining) is hardly
110 amplified by PCR, probably due to DNA damage during the root clearing process
111 involving high concentrations of potassium hydroxide (Pitet *et al*. 2009). To overcome
112 these difficulties in isolating and determining the taxa of individual IUs, in this study, rice
113 (*Oryza sativa*) was used as the host plant because (i) the morphology of the development
114 of IUs is well understood (Kobae and Hata, 2010; Kobae and Fujiwara 2014), (ii) active
115 IUs rarely coalesce in roots (Kobae and Fujiwara 2014), probably due to the small number
116 of cortical cell layers (Fiorilli *et al*. 2015; Gutjahr *et al*. 2015), and (iii) the vital staining
117 is convenient for detecting a single IU (Kobae and Hata 2010; Kobae *et al*. 2014; Kobae
118 *et al*. 2016). In PCR amplification of IU fungal DNA, we used the universal fungal
119 primers LR1/FLR2 (van Tuinen *et al*. 1998; Trouvelot *et al*. 1999) to detect a wide range
120 of LSU ribosomal RNA genes (LSU-rDNAs) of diverse AM fungal species.

In this study, we demonstrated that not all IUs of indigenous AM fungi are metabolically active in roots and successfully determined the DNA sequences of individual metabolically active IUs via Sanger sequencing. All obtained sequences of IUs were of glomeromycotan origin. The presented methodology will permit the investigation of the DNA information of individual colonizing fungi in rice roots grown in field soils.

2. Materials and Methods

2.1. Plant materials and sample preparation

Unhulled rice seeds (*Oryza sativa* L. cv Nipponbare) were surface sterilized with bleach (2.5% available chloride) for 5 min, rinsed with excess deionized water five times and immersed in deionized water for 2 days at 28°C. The germinated seeds were grown in 100-mL pots (D-100; Teraoka, Osaka, Japan), which had two holes of 5 mm in diameter at the bottom. The soil consists of 20 g (bottom layer) of Akadama soil (tuff loam) (Setogahara Kaen, Gunma, Japan) and 90 g (upper layer) of 2 mm-sieved soil obtained from a non-cultivated area of a farm field (43°00′ 30″ N, 141°24′ 25″ E) at NARO Hokkaido Agricultural Research Center, Japan. The main vegetation was turf. The soil type of the field was Thapto-upland Wet Andosol (Typic Endoaquands [USDA Soil Taxonomy]) (Obara *et al*. 2011). The area has not experienced agricultural management (e.g. tillage, fertilization) for at least 20 years and it is located more than 5 m from the cultivated area. Pots were placed on a flat bottom tray under 16-h light/8-h dark photoperiods (26°C/23°C). No nutrients were added and water was supplied from the bottom.

2.2. Fungal CW staining

To detect AM fungal colonization, the CWs of AM fungi were detected using 3,3′ -diaminobenzidine (DAB) staining (Kobae and Ohtomo 2016). Roots were cleared using 10% (weight/volume [w/v]) potassium hydroxide by boiling for 15 min and then rinsed three times with water and once with phosphate-buffered saline (PBS; pH 7.5). Roots were then immersed in PBS containing 1% (w/v) bovine serum albumin (Wako, Osaka, Japan) and 0.4 μg mL^{-1} wheat germ agglutinin-conjugated horseradish peroxidase (HRP) (Vector, Burlingame, CA, USA). Roots were maintained in this solution for more than 16 h at room temperature before being rinsed twice with PBS and then immersed in PBS containing 0.2 mg mL^{-1} DAB tetrahydrochloride (Nakarai Tesque, Kyoto, Japan) and 0.1 μL mL^{-1} 30% H_2O_2. The roots were incubated in the DAB solution for at least 1 h at room temperature and then soaked in Tris-ethylene diamine tetraacetic acid (EDTA) buffer (TE buffer; 10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to stop the HRP reaction. Images were obtained using a stereomicroscope (SMZ800; Nikon, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera.

2.3 Fungal vital staining

To detect metabolically active AM fungal colonization in roots, the viability was assessed by vital staining, which detects *in situ* SDH activity using NBT (MacDonald and Lewis 1978). Washed roots were incubated at room temperature for 30 min in the dark in NBT solution containing 50 mM Tris-HCl buffer (pH 7.4), 1 mg mL^{-1} NBT, 0.5 mM $MgCl_2$ and 250 mM sodium succinate and then rinsed with water. Images were obtained using a stereomicroscope equipped with a CCD camera.

2.4 PCR and sequencing of IUs

To identify the AM fungal taxa of IUs, a novel method has been developed. A single root segment (<3 mm) containing IUs (<2 mm) detected via vital staining (**Supplementary Figure 1A, 1B**) was dissected with micro-scissors under a stereomicroscope and placed in 12 μL of TE buffer on a rectangular (24 × 50 mm^2) coverslip (**Figure 1**). The sample was covered with a smaller coverslip (18 × 18 mm^2) with caution to avoid the formation of air bubbles between the coverslips. The samples were squashed via pressing with the eraser of a PILOT FRIXION erasable pen (PILOT, Tokyo, Japan) (**Supplementary Figure 1C**). Approximately 5 μL of sample solution that leaked from the side of the upper coverslip were recovered, 1 μL of which was used as the PCR template. KOD-Plus-Neo High-Fidelity DNA polymerase (TOYOBO, Osaka, Japan) was used for PCR with the fungal universal primers LR1/FLR2 (van Tuinen *et al*. 1998; Trouvelot *et al*. 1999). The reaction mix was prepared according to the manufacturer's instructions. Thermal cycling was performed in a thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, Foster City, CA, USA) with the following conditions: 5 min of initial denaturation at 94°C; 40 cycles of 15 s of denaturation at 98°C, 30 s of annealing at 58°C and 45 s elongation at 68°C; and a final extension phase at 68°C for 10 min. PCR products were separated by gel electrophoresis on a 1.0% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2 and 1 μg/mL ethidium bromide) and DNA was visualized under UV light. PCR products with the expected size (680–780 bp, Kawahara and Ezawa 2013) were excised, extracted using a gel extraction kit (QIAEX II; QIAGEN, Hilden, Germany) and then sequenced with the LR1 primer using a capillary sequencer (ABI 3130; Applied Biosystems) with the BigDye v3.1 sequencing chemistry (Applied Biosystems).

193 ***2.5. DNA cloning and sequencing***

194 Mixed DNAs of two IUs (IU4 and IU23) were cloned in *Escherichia coli* and
195 then sequenced. Since blunt-ended PCR products produced by KOD-Plus-Neo High-
196 Fidelity DNA polymerase have poor cloning efficiency and are not suitable for ensuring
197 more clones, purified IU PCR products were re-amplified by PCR using Ex-Taq DNA
198 polymerase (TaKaRa Bio, Shiga, Japan) with the primers LR1 and FLR2 and ligated into
199 a pMG20 TA-cloning vector (TaKaRa Bio) following the manufacturer's instructions.
200 Cloned DNA in *E. coli* (HST02; TaKaRa Bio) was amplified using DNA polymerase
201 (PrimeSTAR HS; TaKaRa Bio) with M13 forward and reverse primers, treated with
202 ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) to remove unconsumed dNTPs and
203 primers and sequenced using the LR1 primer. The sequences of seven and nine clones
204 were determined for IU4 (*Gigasporaceae* clade) and IU23 (*Claroideoglomeraceae* clade),
205 respectively.

206

207 ***2.6. Sequence analysis and classification***

208 The sequences of the obtained PCR products were submitted to National Center
209 for Biotechnology Information BLAST searches to verify the taxon of the sequences.
210 Sequences including isolated IUs sequences and AM fungal SSU-ITS-LSU reference data
211 (http://www.amf-phylogeny.com, Krüger *et al*. 2012) were aligned using the MUSCLE
212 program (Edger 2004) on MEGA v7.0.14 (Kumar *et al*. 2016). After the aligned
213 sequences were shortened to the length corresponding to the nucleotide region 2433–2935
214 of *Rhizophagus irregularis* MUCL43195 (consensus 28) (Krüger *et al*. 2012) covering
215 the variable D1 and D2 regions of the LSU ribosomal RNA gene, the sample scores were
216 analysed using AB1 Peak Reporter (Applied Biosystems,

https://apps.thermofisher.com/ab1peakreporter/). The quality values of each nucleotide position called by ABI Prism DNA Sequencing Analysis (v5.1) (Applied Biosystems) were determined according to the manufacturer's instructions of AB1 Peak Reporter. The mean values were regarded as the sample scores. A phylogenetic tree was generated with MEGA v7.0.14 using the maximum likelihood method with the Kimura 2 parameter model plus gamma. *Paraglomus* was used as an outgroup, as it represents the most basal glomeromycotan branch. The reliability of the clades of the phylogenetic tree was assessed using the bootstrap method with 500 replications. Electropherograms were drawn using DNADynamo v1.452 (Blue Tractor Software Ltd.)

3. Results

3.1 Not all IUs revealed by CW staining are metabolically active

To investigate whether all colonizing AM fungi (IUs) in roots are metabolically active, rice seedlings were inoculated with native AM fungi using field soils and the numbers of colonizing fungi detected by fungal CW staining and vital staining were compared. Intraradical mycelia detected by CW staining revealed that most IUs contained intact arbuscules at 10 and 12 dpp. The numbers of IUs evaluated by the two methods were comparable at 7 dpp, but the number of IUs evaluated by CW staining was significantly larger than that detected by vital staining at 12 dpp (**Figure 2**), suggesting that not all IUs in roots grown in field soils are metabolically active.

3.2 Identifying the taxa of metabolically active IUs

To detect active IUs in rice roots grown in soil, vital staining was performed at 8, 12 and 21 dpp. Most root regions outside (<1 cm) IUs had no colonization up to 10

241 dpp, suggesting that IUs at 8 dpp might be derived from independent propagules in soils.
242 To determine the taxa of IUs, 16 IUs were isolated from roots at both 8 and 12 dpp. DNA
243 sequences were not obtained from one IU, probably due to inefficient PCR amplification.
244 BLAST analysis of the obtained IU sequences indicated that all sequences were of
245 glomeromycotan origin. To assess whether this technique is also capable of determining
246 the taxa of IUs in later colonization stages, vital staining and IU sequencing was also
247 performed at 21 dpp. At this stage, in contrast to the findings at 8 dpp, colonization may
248 be derived from both independent propagules in soils and secondary infection within the
249 root system. The purpose of this experiment is to investigate whether the type of active
250 AM fungi of IUs is complicated (intermingled) at this stage, due to the presence of another
251 inactive hypha in the roots that are not detected by vital staining. We dissected 24 root
252 segments (3 mm) containing a metabolically active IUs and obtained the LSU-rDNA
253 sequences. BLAST analysis indicated that all sequences obtained were of
254 glomeromycotan origin
255
256 ***3.3 Levels of genotypic heterogeneity within IUs are different among fungal clades***
257 Some IU sequences produced a mixed electropherogram (**Supplementary**
258 **Figure 2A**), resulting in a lower quality value for sequence calling. This result is
259 consistent with previous studies demonstrating that rDNAs exist within multi-copy
260 repeated arrays in the genome of AM fungi (Corradi *et al*. 2007) and highly nucleotide
261 polymorphisms including insertions/deletions even within a single nucleus (Tisserant *et*
262 *al*. 2013; Lin *et al*. 2014). The best strategy for deciphering the taxa of IUs is to determine
263 'all' of the varieties of LSU-rDNA sequences without any experimental biases; however,
264 comprehensive sequencing of LSU-rDNA genes within individual IUs is extremely

265 laborious and costly. Thus, a simple method enabling amplification and determination of
266 the DNA sequences of IUs is warranted to explore numerous genetically diverse IUs. To
267 this end, the mixed LSU-rDNA sequences of individual IUs were handled in this study.
268 Despite the mixed electropherogram, ABI Prism DNA sequencing analysis produced
269 more than 600 bp of LSU-rDNA sequences, reflecting the predominant LSU-rDNA gene
270 within IUs. Because the obtained IU sequences derived from mixed electropherograms
271 are not actual sequences but only illustrate the representative 'meta'-sequences, we called
272 these sequences 'IU meta-sequences (IMSs)'. The heterogeneity of the IMSs was
273 expressed as the mean of the quality value for base calls (sample score). Phylogenetic
274 analysis with the alignment of 55 IMSs and AM fungal rDNA reference sequences
275 (Krüger *et al*. 2012) suggested that they could be classified into four major family clades,
276 namely clades 1 (11 IMSs), 2 (10 IMSs), 3 (19 IMSs) and 4 (8 IMSs), in addition to some
277 minor clades (**Figure 3**). The average sample scores of IMSs were significantly different
278 among the four major clades (**Supplementary Figure 2B**); i.e. the total average sample
279 score of clade 3 (20.0) was significantly lower than those of the other three major clades
280 (clade 1, 54.5; clade 2, 33.8; clade 4, 42.9), suggesting a different level of LSU-rDNA
281 sequence variations among fungal types. BLAST analysis supported that all IMSs with
282 middle–high scores (≥20) were correctly classified into the same family (i.e. clade 1,
283 *Gigasporaceae*; clade 2, *Glomeraceae*; clade 4, *Paraglomeraceae*); however, the group
284 of low-score IMSs (<20) was hardly determined by BLAST analysis. To investigate the
285 reason behind the lower sample scores, the cloned sequences of IMSs were analysed. We
286 examined the PCR products of one IU each as a representative from clade 1 with a high
287 average sample score and clade 3 with a lower average sample score. Multiple alignment
288 of nine cloned sequences of IU23 from clade 3 revealed that the number of single-

nucleotide base substitution and single-nucleotide base insertions/deletions that occurred in more than two cloned sequences were 45 and 5, respectively (**Supplementary Figure 3B**). By contrast, in a multiple alignment of seven cloned sequences of IU4 from clade 1, neither single-nucleotide substitution nor single-nucleotide insertion/deletion occurred in greater than two cloned sequences (**Supplementary Figure 3A**), suggesting that high nucleotide polymorphism within rDNAs might lower the sample scores. The total average sample scores of IMSs at 8, 12 and 21 dpp were 25.6, 36.2 and 38.1, respectively. AM fungi of clade 3 (low sample scores) tended to rapidly colonize roots; accordingly, the total average sample scores of IMS at 8 dpp might be reduced. It is noteworthy that the total average sample scores were not decreased in IMSs at 21 dpp, at which point multiple AM fungi possibly colonize root samples, suggesting that fungal genotypes in IUs are not intermingled during this period in rice mycorrhizal roots.

4. Discussion

In this report, we developed a novel model system for determining the taxa of active IUs in rice roots. The fungal rDNAs of IUs were certainly amplified using the LR1/FLR2 fungal universal primers and all of them represented the sequences of AM fungi, suggesting that this model system is feasible for the analysis of a wide range of AM fungal taxa that actively colonize rice roots grown in field soils.

Double detection of IUs by vital staining and CW staining revealed that not all IUs of native AM fungi are metabolically active. The number of IUs determined by fungal CW staining is much increased from 10 dpp to 12 dpp, whereas the number of metabolically active IUs is not increased. There are two possible explanations for this observation. First, IUs with little metabolic activity may not be allowed further

313 development by plants. Since (i) growing colonized regions have high metabolic activity
314 in either infection fronts or internal regions in which new IUs develop (Amijee *et al*.
315 1989; Saito *et al*. 1993; Kobae *et al*. 2016); (ii) arbuscules lower their metabolic activity
316 according to their development (Dickson *et al*. 2003; Kobae *et al*. 2014), it is not
317 surprising that IUs with low activity are often observed at 12 dpp, and at the same time
318 this observation implies that many IUs are not forming new arbuscules and contributing
319 to the development of mycorrhizal roots. Given that the majority of IUs detected by CW
320 staining contained arbuscules at 12 dpp, it is likely that they are functional. As arbuscules
321 are ephemeral structures with a lifetime at maturity of approximately 2–3 days (Kobae
322 and Hata 2010), subsequent growth and function of colonization may be determined by
323 the taxa of metabolically active IUs. The AM fungal taxa of active IUs in roots may be
324 determined by host developmental stages or nutritional status or by environmental factors
325 through the regulation of successive development of IUs by unknown mechanism.
326 Another possibility is that the majority of IUs by native AM fungi can occur without high
327 metabolic activity. In that case, IUs with abnormally high metabolic activity may
328 consume much more carbon than other normal IUs. These extremely active IUs may
329 either have a physiologically important contribution to host plant or act as a parasite in
330 an environment-dependent manner (e.g. Johnson *et al*. 1997). Although this hypothesis is
331 speculative, in any case, the taxa of metabolically active colonizing fungi may possibly
332 have great effects on the current and subsequent characteristics of mycorrhizal roots.
333 The method presented in this study determined the LSU-rDNA sequences of IUs
334 for the first time. Most IU sequences of clade 3 produced mixed electropherograms, which
335 are probably due to the presence of several single-nucleotide substitutions and
336 insertions/deletions, suggesting a high level of heterogeneity of rDNA within IUs.

337 Intriguingly, the heterogeneity of rDNA sequences within IUs varies in a phylogenetic
338 clade-dependent manner. The IU sequences of clade 1 produced significantly
339 homogeneous electropherograms compared with those of clades 2 and 3. Three possible
340 scenarios for the difference can be postulated. First, the level of polymorphism of AM
341 fungal rDNA is not uniform throughout the Glomeromycota phylum (Sanders and
342 Rodriguez 2016), and may be at least partly conserved within phylogenetic clade in the
343 same soil. Second, soil management has decreased the nucleotide variation of rDNAs.
344 There is a positive correlation between plant diversity and AM fungal diversity in
345 agricultural fields (Helgason *et al*. 1998; Oehl *et al*. 2003). Although the soils of the non-
346 cultivated area used in this study have not experienced agricultural management (e.g.
347 tillage, fertilization) for at least 20 years and they are located more than 5 m from the
348 cultivated area, the soils were occasionally affected by the use of agricultural machineries
349 and weed management was constantly conducted, leading to decreased diversity of host
350 plant species. The sensitivity of intramycelium genetic diversity to agricultural
351 management may differ among fungal types; accordingly, the IU sequences of clade 1
352 might display homogeneous electropherograms. Third, despite the latent genetic diversity
353 in AM fungal propagules in soil, a limited number of genotypes actively populate certain
354 host plant species. A recent study suggested that the genotype and phenotype of an AM
355 fungus can be changed depending on the host plant species (Angelard *et al*. 2014).
356 Limpens and Geurts (2014) theoretically proposed that the selection of nucleus may occur
357 through molecular dialogue between plants and cooperative AM fungi in arbuscules. It is
358 thus possible that a certain genotype of clade 1 might be selected in the roots of rice
359 seedlings. It has also been suggested that the types of infecting AM fungi are affected by
360 previously infected types (priority effect) (Werner and Kiers 2015). In future research,

IU-based spatiotemporal analysis of interaction among the IUs as well as its genotypes will give new information on how mycorrhizal roots including various AM fungi are formed.

The underlying reason for the variable genetic diversity among fungal taxa is currently speculative, but it provides a framework for future experiments. More studies using different soil types, different field management strategies and different environmental conditions are needed to understand the mechanism for biased IU genotypes. More importantly, recent molecular studies have provided several fluorescence (e.g. GFP)-labelled protein markers that illustrate a tight association between colonization and function (e.g. endomembrane system, phosphate, ammonium transport) (Kobae and Hata 2010; Kobae *et al*. 2010; Genre *et al*. 2012; Ivanov *et al*. 2012; Lota *et al*. 2013; Breuillin-Sessoms *et al*. 2015; Zhang *et al*. 2015). These markers will be useful in characterizing the AM fungal DNA information of non-functional and functional colonization regions. In addition, transcriptome analysis of IUs will be an important challenge for investigating the molecular mechanism of the outcome of colonization at the IU level.

Acknowledgments

This work was supported partly by ACCEL from the Japan Science and Technology Agency. We thank Shingo Hata for critical reading of the manuscript. We thank Taro Maeda for recommending the use of AB1 peak reporter and Tatsuhiro Ezawa for providing LR1/FLR2 primers.

Disclosure statement

Conflicts of interest: No conflicts of interest declared.

Funding

This work was partly supported by ACCEL from the Japan Science and Technology Agency.

References

Abdel-Fattah GM 2001: Measurement of the viability of arbuscular-mycorrhizal fungi using three different stains; relation to growth and metabolic activities of soybean plants. *Microbiol. Res.*, **156**, 359–367.

Amijee F, Tinker PB, Stribley DP 1989: The development of endomycorrhizal root systems. VII. A detailed study of effects of soil phosphorus on colonization. *New Phytol.*, **111**, 435–446.

Angelard C, Tanner CJ, Fontanillas P, Niculita-Hirzel H, Masclaux F, Sanders IR 2014: Rapid genotypic change and plasticity in arbuscular mycorrhizal fungi is caused by a host shift and enhanced by segregation. *ISME J.*, **8**, 284–294.

Avio L, Pellegrino E, Bonari E, Giovannetti M 2006: Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. *New Phytol.*, **172**, 347–357.

Berruti A, Borriello R, Lumini E, Scariot V, Bianciotto V, Balestrini R 2013: Application

409 of laser microdissection to identify the mycorrhizal fungi that establish arbuscules inside
410 root cells. *Front. Plant Sci.*, **4**, 135.
411
412 Boddington CL, Dodd JC 1998: A comparison of the development and metabolic activity
413 of mycorrhizas formed by arbuscular mycorrhizal fungi from different genera on two
414 tropical legumes. *Mycorrhiza*, **8**, 149–157.
415
416 Breuillin-Sessomsa F, Floss DS, Gomez K, Pumplin N, Ding Y, Levesque-Tremblay V,
417 Noar RD, Daniels DA, Bravo A, Eagleshama JB, Beneditob VA, Udvardib MK, Harrison
418 MJ 2015: Suppression of arbuscule degeneration in *Medicago truncatula* phosphate
419 transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3.
420 *Plant Cell*, **27**, 1352–1366.
421
422 Chialva M, Zouari I, Salvioli A, Novero M, Vrebalov J, Giovannoni JJ, Bonfante P 2016:
423 *Gr* and *hp-1* tomato mutants unveil unprecedented interactions between arbuscular
424 mycorrhizal symbiosis and fruit ripening. *Planta*, **244**, 155–65.
425
426 Corradi N, Croll D, Colard, A, Kuhn G, Ehinger M, Sanders IR 2007: Gene copy number
427 polymorphisms in an arbuscular mycorrhizal fungal population. *Appl. Environ.*
428 *Microbiol*., **73**, 366–369.
429
430 Cox G, Sanders F 1974: Ultrastructure of the host–fungus interface in a vesicular-
431 arbuscular mycorrhiza. *New Phytol*., **73**, 901–912.
432

433 Dickson S, Schweiger P, Smith FA, Söderström B, Smith S 2003: Paired arbuscules in
434 the *Arum*-type arbuscular mycorrhizal symbiosis with *Linum usitatissimum*. *Can. J. Bot.*,
435 **81**, 457–463.
436
437 Edgar RC 2004: MUSCLE: multiple sequence alignment with high accuracy and high
438 throughput. *Nucleic Acids Res*., **32**, 1792–1797.
439
440 Fiorilli V, Vallino M, Biselli C, Faccio A, Bagnaresi P, Bonfante P 2015: Host and non-
441 host roots in rice: cellular and molecular approaches reveal differential responses to
442 arbuscular mycorrhizal fungi. *Front. Plant Sci*., **6**, 636.
443
444 Genre A, Ivanov S, Fendrych M, Faccio A, Žárský V, Bisseling T, Bonfante P 2012:
445 Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis
446 in arbuscular mycorrhizas. *Plant Cell Physiol*., **53**, 244–255.
447
448 Gutjahr C, Sawers RJH, Marti G, Andres-Hernandez L, Yang SY, Casieri L, Angliker H,
449 Oakeley EJ, Wolfender JL, Abreu-Goodger C, Paszkowski U 2015: Transcriptome
450 diversity among rice root-types during asymbiosis and interaction with arbuscular
451 mycorrhizal fungi. *Proc. Natl. Acad. Sci. U.S.A*., **112**, 6754–6759.
452
453 Hamel C, Fyles H, Smith DL 1990: Measurement of development of endomycorrhizal
454 mycelium using three different vital stains. New Phytol., **115**, 297–302.
455
456 Harrison MJ 2005: Signaling in the arbuscular mycorrhizal symbiosis. *Annu. Rev.*

457 *Microbiol*., **59**, 19–42.

458

459 Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW 1998: Ploughing up the
460 wood-wide web? *Nature*, **394**, 431.

461

462 Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T 2012:
463 Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule
464 formation. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 8316–8321.

465

466 Janoušková M, Püschel D, Hujslová M, Slavíková R, Jansa J 2015: Quantification of
467 arbuscular mycorrhizal fungal DNA in roots: how important is material preservation?
468 *Mycorrhiza*, **25**, 205–214.

469

470 Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ 2007: A *Medicago truncatula*
471 phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc. Natl.*
472 *Acad. Sci. U.S.A.*, **104**, 1720–1725.

473

474 Johnson NC, Graham JH, Smith FA 1997: Functioning of mycorrhizal associations along
475 the mutualism-parasitism continuum. *New Phytol.*, **135**, 575–586

476

477 Kawahara A, Ezawa T 2013: Characterization of arbuscular mycorrhizal fungal
478 communities with respect to zonal vegetation in a coastal dune
479 ecosystem. *Oecologia*, **173**, 533–543.

480

Khalvati MA, Hu Y, Mozafar A, Schmidhalter U 2005: Quantification of water uptake by arbuscular mycorrhizal hyphae and its significance for leaf growth, water relations, and gas exchange of barley subjected to drought stress. *Plant Biol.* (Stuttg), 7, 706–712.

Kobae Y, Fujiwara T 2014: Earliest colonization events of *Rhizophagus irregularis* in rice roots occur preferentially in previously uncolonized cells. *Plant Cell Physiol*., **55**, 1497–1510.

Kobae Y, Gutjahr C, Paszkowski U, Kojima T, Fujiwara T, Hata S 2014: Lipid droplets of arbuscular mycorrhizal fungi emerge in concert with arbuscule collapse. *Plant Cell Physiol*., **55**, 1945–1953.

Kobae Y, Hata S 2010: Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. *Plant Cell Physiol*., **51**, 341–353.

Kobae Y, Ohmori Y, Saito C, Yano K, Ohtomo R, Fujiwara T 2016: Phosphate treatment strongly inhibits new arbuscule development but not the maintenance of arbuscule in mycorrhizal rice roots. *Plant Physiol*., **171**, 566–579.

Kobae Y, Ohtomo R 2016: An improved method for bright-field imaging of arbuscular mycorrhizal fungi in plant roots. *Soil Sci. Plant. Nutr*., **62**, 27–30.

Kobae Y, Tamura Y, Takai S, Banba M, Hata S 2010: Localized expression of arbuscular

505 mycorrhiza-inducible ammonium transporters in soybean. *Plant cell physiol*., **51**, 1411–
506 1415.
507
508 Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A 2012: Phylogenetic reference
509 data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to
510 species level. *New Phytol*., **193**, 970–984.
511
512 Krüger M, Stockinger H, Krüger C, Schüßler A 2009: DNA-based species level detection
513 of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytol*.,
514 **183**, 212–223.
515
516 Kumar S, Stecher G, Tamura K 2016: MEGA7: Molecular Evolutionary Genetics
517 Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol*., **33**, 1870–1874.
518
519 Lee J, Lee S, Young JPW 2008: Improved PCR primers for the detection and
520 identification of arbuscular mycorrhizal fungi. *FEMS Microbiol. Ecol*., **65**, 339–349
521
522 Leigh J, Hodge A, Fitter AH 2009: Arbuscular mycorrhizal fungi can transfer substantial
523 amounts of nitrogen to their host plant from organic material. *New Phytol*., **181**, 199–207.
524
525 Limpens E, Geurts R 2014: Plant-driven genome selection of arbuscular mycorrhizal
526 fungi. *Mol. Plant Pathol*., **15**, 531–534.
527
528 Lin K, Limpens E, Zhang Z, Ivanov S, Saunders DG, Mu D, Pang E, Cao H, Cha H, Lin

529 T, Zhou Q, Shang Y, Li Y, Sharma T, van Velzen R, de Ruijter N, Aanen DK, Win J,
530 Kamoun S, Bisseling T, Geurts R, Huang S 2014: Single nucleus genome sequencing
531 reveals high similarity among nuclei of an endomycorrhizal fungus. *PLoS Genet*., **10**,
532 e1004078.
533
534 Lota F, Wegmuller S, Buer B, Sato S, Brautigam A, Hanf B, Bucher M 2013: The cis-
535 acting CTTC-P1BS module is indicative for gene function of LjVTI12, a Qb-SNARE
536 protein gene that is required for arbuscule formation in *Lotus japonicus*. *Plant J*., **74**, 280–
537 293.
538
539 MacDonald RM, Lewis M 1978: The occurrence of some acid phosphatases and
540 dehydrogenases in the vesicular–arbuscular mycorrhizal fungus *Glomus mosseae*. *New*
541 *Phytol*., **80**, 135–141.
542
543 Mensah JA, Koch AM, Antunes PM, Hart MM, Kiers ET, Bücking H 2015: High
544 functional diversity within arbuscular mycorrhizal fungal species is associated with
545 differences in phosphate and nitrogen uptake and fungal phosphate metabolism.
546 *Mycorrhiza*, **25**, 533–546.
547
548 Munkvold L, Kjoller R, Vestberg M, Rosendahl S, Jakobsen I 2004: High functional
549 diversity within species of arbuscular mycorrhizal fungi. *New Phytol*., **164**, 357–364
550
551 Obara H, Ohkura T, Takata Y, Kohyama K, Maejima Y, Hamazaki T 2011:
552 Comprehensive soil classification system of Japan First approximation. *Bull, Natl, Inst.*

Agro-Environ. Sci., **29**, 1–73.

Oehl F, Sieverding E, Ineichen K, Mader P, Boller T, Wiemken A 2003: Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. *Appl. Environ. Microbiol*., **69**, 2816–2824.

Pitet M, Camprubì A, Calvet, Estaùn V 2009: A modifiedstaining technique for arbuscular mycorrhiza compatible with molecular probes. *Mycorrhiza*, **19**, 125–131.

Saito M, Stribley DP, Hepper CM 1993: Succinate dehydrogenase activity of external and internal hyphae of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae* (Nicol.&Gerd.) Gerdmann and Trappe, during mycorrhizal colonization of roots of leek (*Allium porrum* L.), as revealed by in situ histochemical staining. *Mycorrhiza*, **4**, 59–62.

Sanders IR, Rodriguez A 2016: Aligning molecular studies of mycorrhizal fungal diversity with ecologically important levels of diversity in ecosystems. *ISME J*., doi: 10.1038/ismej.2016.73.

Sanders FE, Sheikh NA 1983: The development of vesicular-arbuscular mycorrhizal infection in plant root systems. *Plant Soil*, **71**, 223–246.

Smith SE, Gianinazzi-Pearson V 1990: Phosphate uptake and arbuscular activity in mycorrhizal Allium cepa L: Effects of photon irradiance and phosphate nutrition. *Aust. J. Plant Physiol*., **17**, 177–188.

Smith SE, Read DJ 2008: Mycorrhizal symbiosis, 2nd edn. London, UK: Academic Press Ltd.

Song YY, Zeng RS, Xu JF, Li J, Shen X, Yihdego WG 2010: Interplant communication of tomato plants through underground common mycorrhizal networks. *PLoS ONE*, **5**, e13324.

Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A 1993: *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. *Mycol. Res*., **97**, 245–250.

Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Frei dit Frey N, Gianinazzi-Pearson V, Gilbert LB, Handa Y, Herr JR, Hijri M, Koul R, Kawaguchi M, Krajinski F, Lammers PJ, Masclaux FG, Murat C, Morin E, Ndikumana S, Pagni M, Petitpierre D, Requena N, Rosikiewicz P, Riley R, Saito K, San Clemente H, Shapiro H, van Tuinen D, Becard G, Bonfante P, Paszkowski U, Shachar-Hill Y, Tuskan GA, Young PW, Sanders IR, Henrissat B, Rensing SA, Grigoriev IV, Corradi N, Roux C, Martin F. 2013. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. 2013: Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, 562–563.

600 Toussaint JP, Smith FA, Smith SE 2007: Arbuscular mycorrhizal fungi can induce the
601 production of phytochemicals in sweet basil irrespective of phosphorus nutrition.
602 *Mycorrhiza*, **17**, 291–297.
603
604 Trouvelot S, van Tuinen D, Hijri M, Gianinazzi-Pearson V 1999: Visualization of
605 ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence *in situ*
606 hybridization. *Mycorrhiza*, **8**, 203–206.
607
608 Vandenkoornhuyse P, Mahé S, Ineson P, Staddon P, Ostle N, Cliquet JB, Francez AJ, Fitter
609 AH, Young PW 2007: Active root-inhabiting microbes identified by rapid incorporation
610 of plant-derived carbon into RNA. *Proc. Natl. Acad. Sci. U.S.A*., **104**, 16970–16975.
611
612 van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V 1998:
613 Characterization of root colonization profiles by a microcosm community of arbuscular
614 mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Mol. Ecol*., **7**, 879–887.
615
616 Vierheilig H, Schweigerb P, Brundrett M 2005: An overview of methods for the detection
617 and observation of arbuscular mycorrhizal fungi in roots. *Physiol. Plant.*, **125**, 393–404.
618
619 Vivas A, Marulanda A, Gomez M, Barea JM, Azcon R 2003: Physiological characteristics
620 (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus*
621 *thuringiensis* inoculation under two phosphorus levels. *Soil Biol. Biochem*., **35**, 987–996.
622
623 Walker NA, Smith SE 1984: The quantitative study of mycorrhizal infection. II. The

relation of rate of infection and speed of fungal growth to propagule density, the mean length of the infection unit and the limiting value of the fraction of the root infected. *New Phytol*., **96**, 55–69.

Werner GD, Kiers ET 2015: Order of arrival structures arbuscular mycorrhizal colonization of plants. *New Phytol*., **205**, 1515–1524.

Zhang X, Pumplin N, Ivanov S, Harrison MJ 2015: EXO70I is essential for development of a sub-domain of the periarbuscular membrane during arbuscular mycorrhizal symbiosis. *Curr. Biol*., **25**, 2189–2195.

Figure legends

Figure 1 Schematic drawing of the procedure of identifying AM taxa of infection units. Infection units (IUs) were detected via vital staining. Root pieces (<3mm in length) containing IU were dissected with scissors under stereomicroscope. A single root piece was placed in 12 µl of TE buffer on a rectangular (24 × 50 mm^2) coverslip. The sample was covered with a smaller coverslip (18 × 18 mm^2), and then squashed. Sample solution leaked from the side of upper coverslip was recovered and applied to PCR with the fungal universal primers (LR1/FLR2). Fungal DNA of expected size (approx. 680–780 bp) was separated by electrophoresis, extracted and applied to Sanger sequencing

Figure 2 Comparison of numbers of infection units (IUs) determined via fungal cell wall (CW) staining and vital staining. Rice roots colonized with native AM fungi in

field soils were subjected to CW staining and vital staining at 7, 10 and 12 days post-plantation (dpp) to detect the intraradical mycelium. The numbers of IUs per plant (n = 3, biologically independent) were compared. Data are presented as means ± SD. *P <0.05, Welch's t test (vital staining versus CW staining); ns, no significant difference

Figure 3 Phylogenetic placement of LSU-rDNA partial sequences of infection units (IUs) inferred by maximum likelihood phylogenetic analysis. For tree construction, 55 IU sequences (IU meta-sequences, IMSs) and 39 AM fungi rDNA consensus sequences (Krüger *et al*. 2012) were used. Numbers above branches denote bootstrap values from 500 replications. *Paraglomus occultum* cons.39 sequence was used as the outgroup

Supplementary Figure 1 Vital staining of infection units (IUs). (A and B) Images of IU visualized with vital staining (bidirectional arrow). (C) Image of squashed root fragment (root is same as B).

Supplementary Figure 2 Genotypic heterogeneity of infection units (IUs). (a) Electropherogram of the partial sequence of LSU-rDNA obtained from an IU of clade 1 (IU4), clade 3 (IU23), and a clone of IU23. Numbers beside electropherogram indicate sample scores. (b) Comparison of sample scores among IU meta-sequences (IMSs) of clade 1 (n=11), clade 2 (n=10), clade 3 (n=19), clade 4 (n=8), and cloned sequences (n=9) of an IU (IU23 from clade 3). Multiple sequence alignment of IMSs and AM fungi consensus sequences were generated by MUSCLE program (Edger 2004) on MEGA v7.0.14 software (Kumar *et al*. 2016). Sequences outside nucleotide region 2433-2935 of

Rhizophagus irregularis MUCL43195 (consensus 28) (Krüger *et al*. 2012) were trimmed. The sample scores were calculated by AB1 Peak Reporter (Applied Biosystems, https://apps.thermofisher.com/ab1peakreporter/). Data are presented as means ± SD. Different letters indicate significant differences as assessed by Tukey's HSD test ($P < 0.05$)

Supplementary Figure 3 Multiple alignment of cloned sequences of infection unit (IU) sequences. (a) Multiple sequence alignment of IU4 (clade 1) cloned sequences with its IU meta-sequences (IMSs). (b) Multiple sequence alignment of IU23 (clade 3) cloned sequences with its IMSs. Multiple sequence alignments were generated by MUSCLE program (Edger 2004) on MEGA v7.0.14 software (Kumar *et al*. 2016). IMSs were shown at the top of the alignment. Sequences corresponding to nucleotide region 2433-2935 of *Rhizophagus irregularis* MUCL43195 (consensus 28) (Krüger *et al*. 2012) were shown

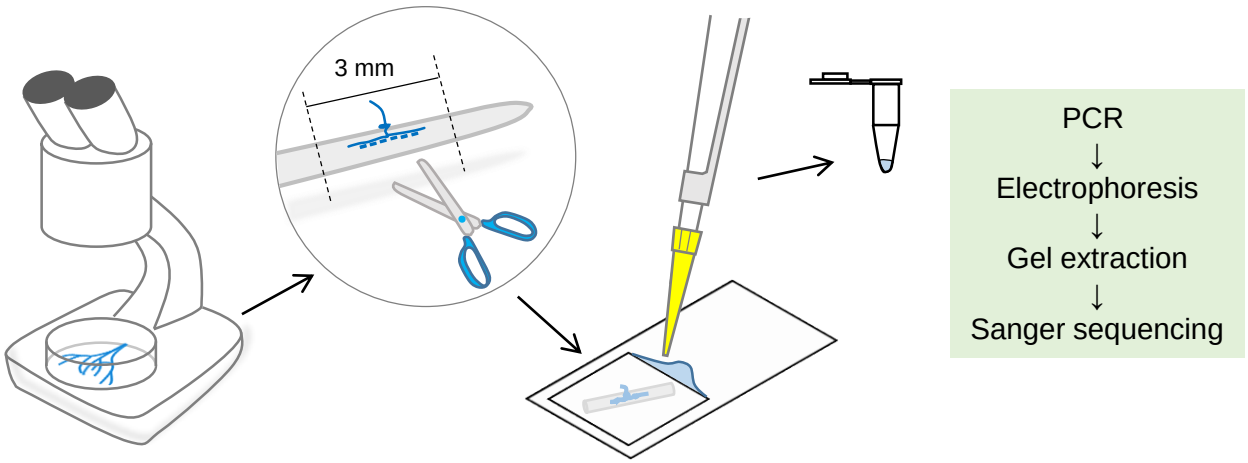


Figure 1
Genotyping of infection units
Schematic drawing of the procedure of infection unit (IU) genotyping. IUs were detected via vital staining and root pieces (<3mm in length) containing IU were dissected with scissors under stereomicroscope. The root piece was placed in 12 µl of TE buffer on a rectangular (24 × 50 mm^2) coverslip. The sample was covered with a smaller coverslip (18 × 18 mm^2), and then squashed. Sample solution leaked from the side of upper coverslip was recovered and applied to PCR with the fungal universal primers (LR1/FLR2). Fungal DNA of expected size (approx. 680–780 bp) was separated by electrophoresis, extracted and applied to Sanger sequencing.

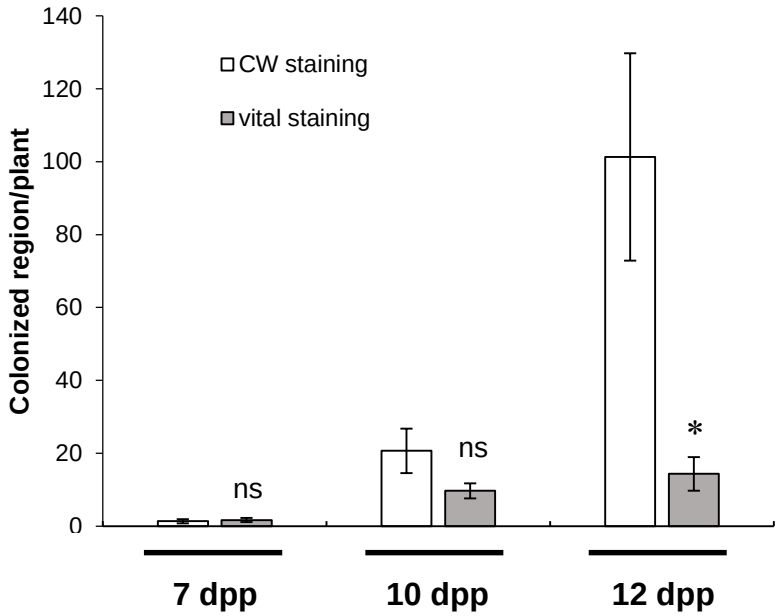


Figure 2

Comparison of numbers of infection units (IUs) determined via fungal cell wall (CW) staining and vital staining

Rice roots colonized with native AM fungi in field soils were subjected to CW staining and vital staining at 7, 10 and 12 days post-plantation (dpp) to detect the intraradical mycelium, and the numbers of colonized regions per plant (n = 3, biologically independent) were compared. Data are presented as means ± SD. **P* <0.05, Welch's t test (vital staining versus CW staining); ns, no significant difference.

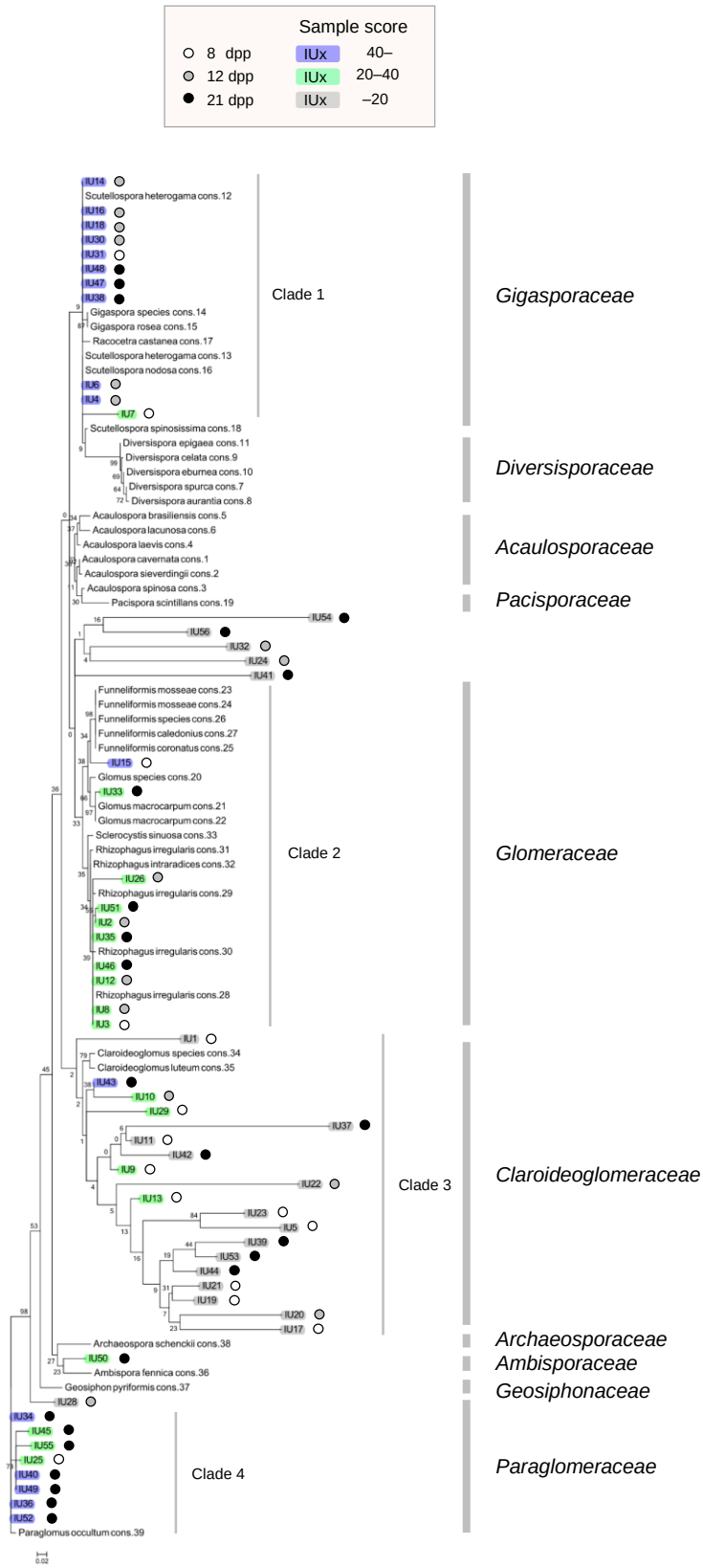
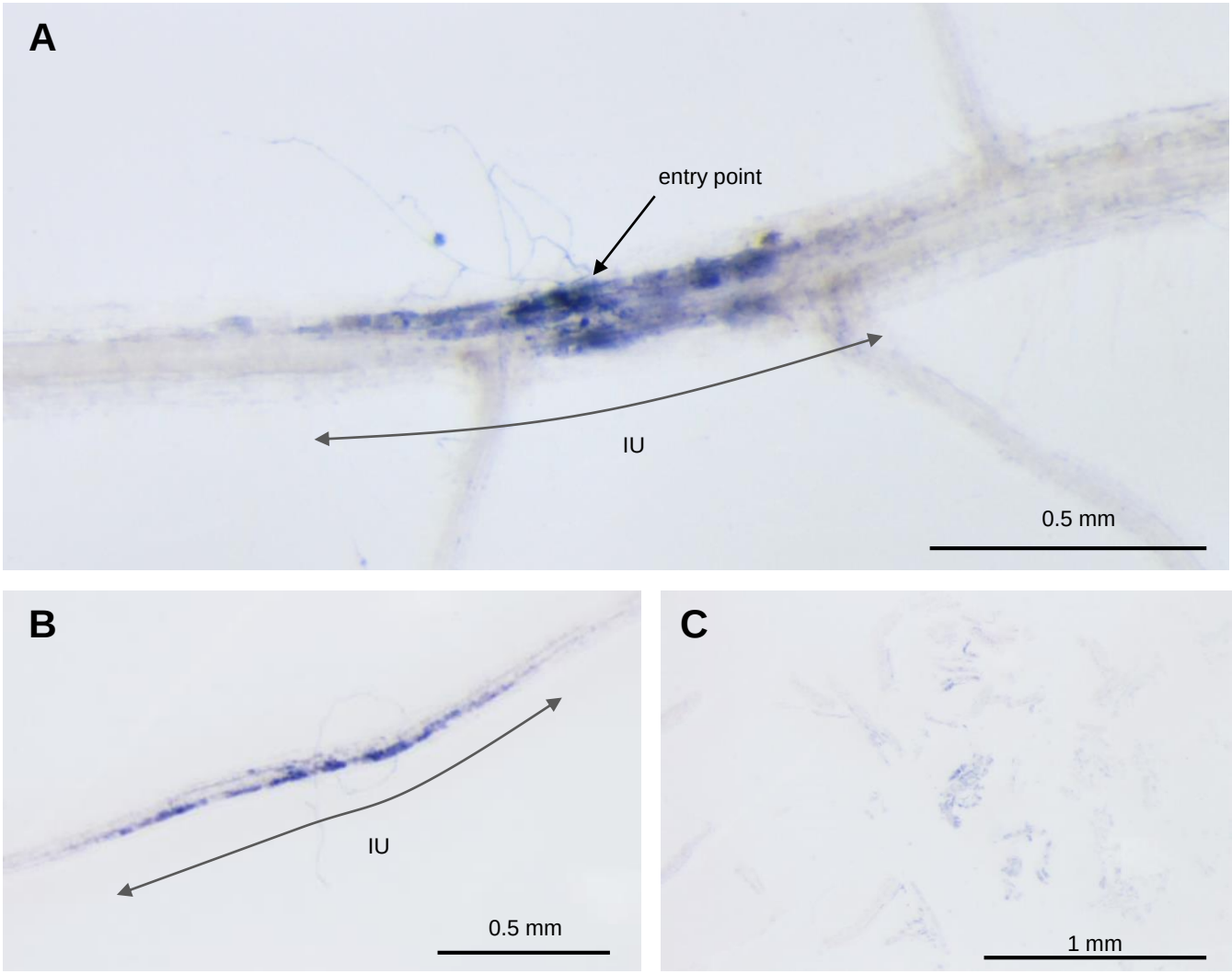


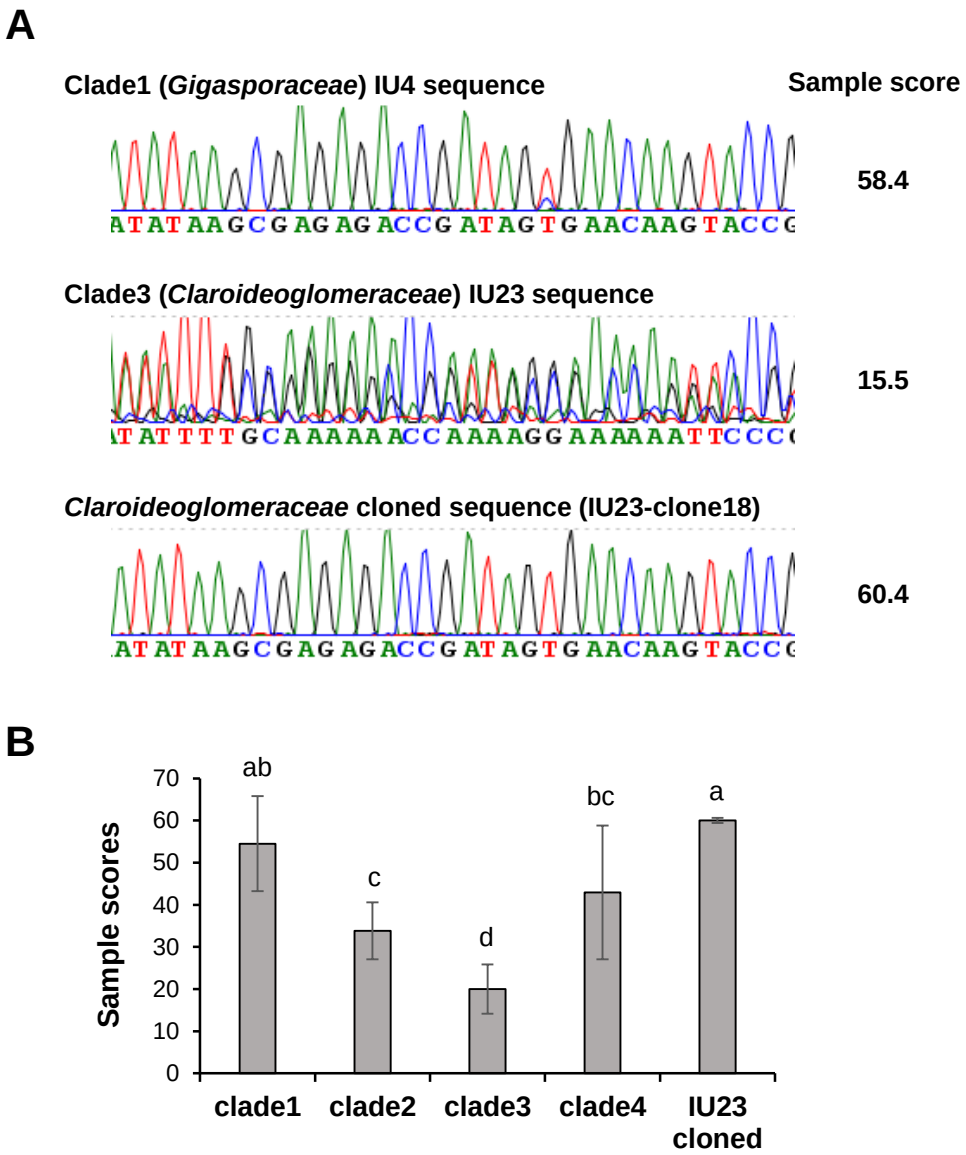
Figure 3

Phylogenetic placement of LSU-rDNA partial sequences of infection units (IUs) inferred by maximum likelihood phylogenetic analysis

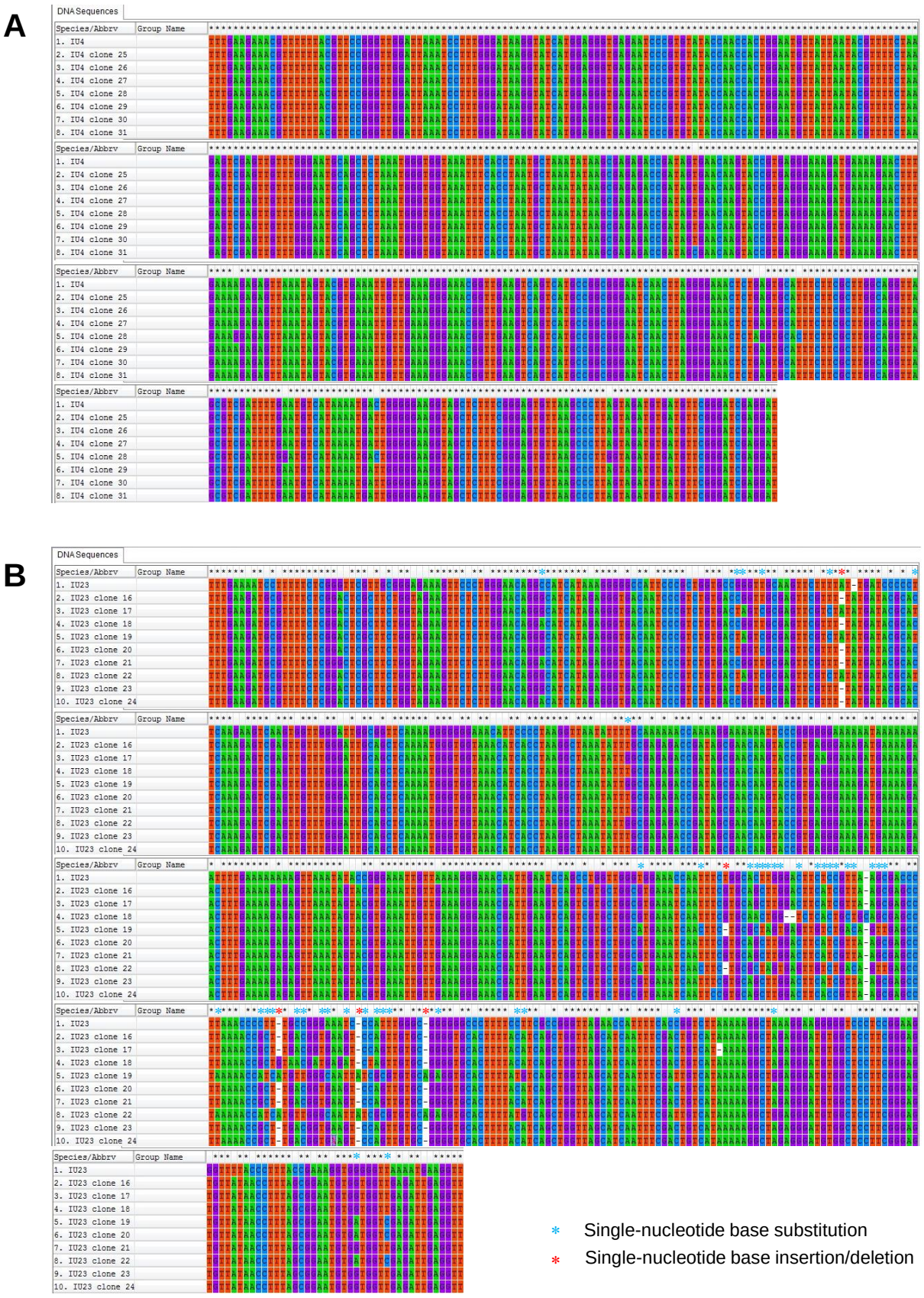
For tree construction, 55 IU sequences (IU meta-sequences, IMSs) and 39 AM fungi rDNA consensus sequences (Krüger et al., 2012) were used. Numbers above branches denote bootstrap values from 500 replications. *Paraglomus occultum* cons.39 sequence was used as the outgroup.



Supplementary Figure 1
Vital staining of infection unit (IU)
(A and B) Images of IU visualized with vital staining (bidirectional arrow). (C) Image of squashed root fragment (root is same as B).



Supplementary Figure 2
Genotypic heterogeneity of infection units (IUs)
(A) Electropherogram of the partial sequence of LSU-rDNA obtained from an IU of clade 1 (IU4), clade 3 (IU23), and a clone of IU23. Numbers beside electropherogram indicate sample scores. (B) Comparison of sample scores among IU meta-sequences (IMSs) of clade 1 (n=11), clade 2 (n=10), clade 3 (n=19), clade 4 (n=8), and cloned sequences (n=9) of an IU (IU23 from clade 3). Multiple sequence alignment of IMSs and AM fungi consensus sequences were generated by MUSCLE program (Edger, 2004) on MEGA v7.0.14 software (Kumar et al., 2016). Sequences outside nucleotide region 2433-2935 of *Rhizophagus irregularis* MUCL43195 (consensus 28) (Krüger et al., 2012) were trimmed. The sample scores were calculated by AB1 Peak Reporter (Applied Biosystems, https://apps.thermofisher.com/ab1peakreporter/). Data are presented as means ± SD. Bars topped by the same letter do not differ significantly at P <0.05 by Tukey's HSD test.



Supplementary Figure 3

Multiple alignment of cloned sequences of infection unit (IU) sequences

(A) Multiple sequence alignment of IU4 (clade 1) cloned sequences with its IU meta-sequences (IMSs). (B) Multiple sequence alignment of IU23 (clade 3) cloned sequences with its IMS. Multiple sequence alignments were generated by MUSCLE program (Edger, 2004) on MEGA v7.0.14 software (Kumar et al., 2016). IMSs were shown at the top of the alignment. Sequences corresponding to nucleotide region 2433-2935 of *Rhizophagus irregularis* MUCL43195 (consensus 28) (Krüger et al., 2012) were shown.