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Wild *Solanum cajanumensis* species as a potential reservoir of arbuscular mycorrhizal fungi diversity compatible with cultivated *Solanum betaceum* species

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Abstract

The community structure of arbuscular mycorrhizal fungi (AMF) is wide in terms of composition and distribution and can be influenced by the host plant. The aim of this research was to compare the diversity of AMF communities associated with cultivated (*Solanum betaceum*) and wild tree tomato (*Solanum cajanumensis*) species. Roots of both species were collected from two sampling sites in Southern Ecuador. The microscopic analysis revealed a heavy colonization by AMF in the roots of both species. An 18S rDNA barcoding analysis was conducted on DNA samples isolated from root tissue to determine the AMF community composition. Sequences from the partial 18S rDNA region were used to reconstruct operational taxonomic units (OTUs) using the UPARSE algorithm with a similarity cut-off of 97%. In total, seven OTUs were retrieved from both species. A higher number of Glomeromycota OTUs were associated with the wild *Solanum* host and two out of seven OTUs were shared between both *Solanum* species. Based on the phylogenetic relationships observed among family-specific OTUs, it was speculated that the wild individuals of *S. cajanumensis* could constitute a natural reservoir of AMF, potentially transferable to the cultivated tree tomato species as part of a crop management strategy.

Introduction

The ecological importance and physiological plant and soil benefits of arbuscular mycorrhizal fungi (AMF) are recognized, particularly they are an important component of natural and agricultural ecosystems (Smith and Read, 2008). AMF are obligated symbionts that belong to the phylum Glomeromycota (Schüßler et al., 2001). The mycorrhizal symbiosis can benefit the host plants in several ways, for example, enhancing uptake of mineral nutrients (especially phosphate), improving water status, protecting against microbial pathogens (Smith and Read, 2008; Wehner et al., 2010) and facilitating the formation and maintenance of soil structure and dynamics (Van Der Heijden et al., 2006).

In addition, the differences in community composition of AMF may have different effects in plants, and play a potential role in determining plant diversity, ecosystem variability and productivity (Lee et al., 2013) that has been evidenced in experimental greenhouse studies (Van Der Heijden et al., 1998; Grandillo et al., 2011) and in natural ecosystems (Barness, 1975; Newsham et al., 1995).

In particular, wild plants may differ from cultivated plants in their response to AMF and pathogens, as they were not selected to maximize yield under optimal conditions. The species richness and diversity of AMF differ among ecosystems, which usually are greater in natural ecosystems compared to agricultural ecosystems (Wang et al., 2013). In fact, natural selection may favor individuals with

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different responses to AMF and pathogens than agriculturally selected plants. For example, one of the few wild plants investigated, *Vulpia ciliata*, was protected by AMF from fungal pathogens in the field (Newsham et al., 1994; 1995).

Particularly in Ecuador, the cultivated species called "tree tomato" or "tamarillo" (*Solanum betaceum* Cav.) (Bohs, 1995), is a small tree native to the Andean region in South America (Bohs, 1991; Bohs and Nelson 1997; Sinton et al., 2002) that produces fruits of a high commercial value. In fact, New Zealand is the main producer of this fruit (Prohens and Nuez, 2001) but its production is also locally important for farmers in Ecuador, Colombia and Peru (Acosta-Quezada et al., 2015). Due to an increase in the demand of the fruit by local consumers, cultivation of tree tomato has increased in Ecuador since the 1980's (Mertz et al., 2010). One wild relative to tree tomato is *Solanum cajanumensis* Kunth, also known as "casana" and can be found in Colombia, Ecuador and Peru from 1,500 to 3,000 meters above sea level (m asl) (Maguire, 1966). Putative wild subpopulations are small, occurring in restricted areas in Ecuador. Wild representatives display a rich genetic variation and are instrumental for the genetic improvement of many economically important traits (Rick, 1987). On the other hand, the cultivated tree tomato has reduced genetic variation (Grandillo et al., 2011).

Given that there are few reports of AMF association in the rhizosphere of *Solanum* (Diop et al., 2003; Dennett et al., 2011) as wild species, it is becoming increasingly important to gain a better understanding of mycorrhizal diversity in *S. cajanumensis* and *S. betaceum*. For example, AMF association in *S. khasianum*, *S. sisymbriifolium* and *S. torvum* were investigated for the first time by Songachan and Kayang (2012), with the numbers of AMF species isolated and identified from the rhizosphere soil being 12, 11 and 11, respectively.

Furthermore, identifying and exploiting diversity of the AMF present in wild tomato species could be crucial for the improvement of production traits in cultivated tree tomato, (Grandillo et al., 2011) considering the potential transfer of these AMF and the desired benefits of improved pathogen resistance and tolerance to abiotic stress (Jacott et al., 2017).

The aim of the present study was to investigate the molecular diversity of AMF associated with cultivated and wild relative tree tomato plants. Therefore, DNA from plant roots was isolated and amplified using specific primer combinations known to detect families of the Glomeromycota phylum. Used primers target the nuclear rDNA coding for the small subunit rRNA gen (18S rDNA). To the authors' knowledge, this is the first report showing the community composition of the AMF associated with *S. cajanumensis* and *S. betaceum* plants near the center of the Ecuadorian Andes.

Material and Methods

Study site and root sampling

Root samples were collected from *Solanum betaceum* individuals planted at the research station of Universidad Técnica Particular de Loja located in Zamora-Huayco, Loja Province, Ecuador (4°0' S and 79°10' W; 2160 m asl) corresponding to the Southern Andes montane humid shrub forest formation (Sierra, 1999) with an average annual temperature of 15°C and an annual rainfall of 780 mm (Acosta-Quezada et al., 2011). Sampling of *Solanum cajanumensis* was carried out from standing individuals in a protected area in Santiago, Loja Province, Ecuador (3°47' S and 79°16' W; 2735 m asl) corresponding to a secondary forest with a mean annual temperature of 13°C and an annual rainfall of 850 mm. The study was carried out at two sites in the south of Ecuador during June 2007.

At both study sites, 10 sampling plots (10 m × 10 m) were selected, from which five single standing plants in each were randomly selected and their fine roots were sampled. From each plant individual, five parts of the roots were selected for the analysis. Overall, 25 samples were analyzed from each study site.

Root preparation and microscopy

Roots were washed under running tap water to remove attached soil and kept at -80°C for subsequent microscopic and molecular studies. Five randomly selected root fragments of approximately 5 cm were selected per plant, providing a total of 50 root fragments analyzed. The 50 fragments were later split in two parts for the analyses: one half (2.5 cm lengths) was used to determine the percentage of AMF colonization and the other half was kept at -80°C for subsequent molecular analysis.

To determine the frequency of AMF colonization, roots were cleaned and stained with methyl blue following the method proposed by Grace and Stribley (1991). Stained root samples were analyzed under a light microscope with magnification of 400× and the colonization percentage was calculated according to Giovannetti and Mosse (1980) using Equation 1:

$$\text{Root colonization \%} = \frac{\text{Number of colonized segments}}{\text{Total number of examined segments}} \times 100 \qquad (1)$$

Statistical analysis

Data analysis was performed using the statistical software R version 1.1.447 (R Core Team, 2019). An independent two sample t test analysis was carried out to test statistical significances in mycorrhizal root colonization between *S. cajanumensis* and *S. betaceum*. The data were expressed as mean ± SD.

Molecular characterization

Five root pieces of 2.5 cm per each plant individual were used for DNA extraction. The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen; Hilden, Germany) following the manufacturer's instructions. A fragment of 800 bp of 18S rDNA of the AMF genome was amplified using polymerase chain reaction (PCR) using different primer combinations in the nested PCRs: *Glomus* Group A: first SSU128 / SSU1536IH, second SSU300 / GLOM1310rc; *Glomus* Group B: first SSU 817 / NS8, second SSU817 / LETC1670rc;

Acaulosporaceae: first SSU 817 / NS8, second SSU817 / ACAU1660rc; and Archaeosporales: first SSU817 / SSU1536IH, second SSU 817 / ARCH1375rc. Each primer combination had been optimized to target a different group of Glomeromycota (Table 1). The PCR conditions were: initial denaturation at 94°C for 3 min; 35 cycles, each cycle consisting of one step of denaturation at 94°C for 30 s; annealing between 40°C and 60°C for 45 s depending on the primer; and extension at 72°C for 1 min. A final extension at 72°C for 7 min was performed to guarantee the synthesis of unfinished PCR products. The reaction mixture consisted of 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 pmol/µL of each primer, 10% bovine serum albumin (BSA-SIGMA; Hamburg, Germany) with a final concentration of 60 pmol/µL (Iotti and Zambonelli, 2006) 1 U GoTaq® DNA polymerase (Promega; Mannheim, Germany), and 2 µL of DNA in a final volume of 20 µL. The success of each PCR was tested in 0.7% agarose stained in a solution of 0.5 mg mL^{-1} ethidium bromide.

The PCR products were cloned using the PCR® 2.1-TOPO® vector system (Invitrogen; Carlsbad, USA). Up to 30 clones per plant were selected for colony-PCR assay with the GoTaq® DNA Polymerase (5U Rl-1; Promega; Mannheim, Germany) and modified M13F and M13R primers (Stockinger et al., 2010) The DNA fragments amplified from selected colonies were then subjected to restriction fragment length polymorphism (RFLP) analysis to differentiate between clones. RFLP was performed as described by Krüger et al. (2009). Clones showing distinctive restriction patterns were afterward purified following the S.N.A.P.™ miniprep kit (Invitrogen; Carlsbad, USA) protocol and finally were sequenced by Macrogen (Seoul, Korea) using the universal primers M13F and M13R. The sequence chromatograms, editing and generating of consensus sequences from matching forward and reverse sequencing reads per each clone were performed using the Sequencer software version 4.6 (Gene Codes; Ann Arbor, MI, USA).

Operational taxonomic units delimitation and phylogenetic analysis

Sequenced data were analyzed with the UPARSE algorithm implemented in USEARCH (Edgar, 2013). Briefly, quality filtered per clone consensus sequences (see above) were pooled in a per sample multifasta file (*Solanum betaceum* and *Solanum cajanumensis*) and treated as sequence reads. Reads were then sorted by decreasing abundance using the 'sort by size' command with the –minsize 2 option to discard singletons. OTUs were defined for each sample at a 97% similarity cut-off with the 'cluster_otus' command with abundance-sorted reads as input. Chimera OTUs were automatically discarded by the 'cluster_otus' command. Finally, taxonomy assignment for each OTU was performed using the Glomeromycota phylogeny database (http://www.amf-phylogeny.com; Redecker et al., 2013). To ensure the identity of mycorrhizal OTUs, these OTUs were compared against the NCBI nucleotide database (www.ncbi.nlm.nih.gov) using BLAST (Altschul et al., 1997). A consensus sequence of each OTU was submitted to GenBank (accession numbers MF784401–MF784407).

Identical OTUs in both species were found by performing an all-versus-all global alignment of the OTU representative sequences with the 'needleall' command which is an implementation of the Needleman-Wunsch global alignment algorithm distributed as part of the EMBOSS toolkit (Rice et al., 2000).

OTU sequences were aligned using the 'L-INS-I' strategy as implemented in MAFFT v6.620b (Katoh and Toh, 2008) including the closest sequence matches obtained from the NCBI database (National Center for Biotechnology Information, GenBank) using BLAST (Kozakov et al., 2006) and consensus sequences from Krüger et al. (2012). A second alignment was produced using the first alignment, which was subsequently corrected manually by excluding ambiguous regions. The final multiple alignment matrix comprised 30 sequences and a total length of 694 bp.

The phylogenetic reconstruction was performed using a Bayesian approach based on Markov chain Monte Carlo (B/MCMC) and a maximum parsimony (MP) analysis. The B/MCMC analyses were conducted for each alignment using the 'MrBayes program (Huelsenbeck and Rannala 2004). The procedure used the most complex substitution model available (GTR+I+G), including two runs each involving four incrementally heated Markov chains over 4,000,000 generations and using random starting trees (Whelan et al., 2001; Douady et al., 2003; Huelsenbeck and Rannala, 2004). Trees were sampled every 100 generations resulting in 40,000 trees from which the last 24,000 were used to compute a 50% majority-rule consensus tree, enabling the use of Bayesian posterior probabilities as node support. Stationarity of the process and effective sample size values were checked visually using the software Tracer 1.5 (Drummond and Rambaut, 2007).

Table 1 Glomeromycota group, primer names, sequences and references

Fungal group	Primer	Sequence (5´- 3´)	Reference
Glomus Group A	SSU128	GGA TAA CCG TGG TAA TTC TAG	Haug et al., 2010
	SSU1536IH	RTT GYA ATG CYC TAT CCC CA	Borneman and Hartin, 2000
	SSU300	CAT TCA AAT TTC TGC CCT ATC A	Haug et al., 2010
	GLOM1310 rc	TAA CAA TGT TAG RCC TAG CT	after Redecker, 2000
Glomus Group B	SSU817	TTA GCA TGG AAT AAT RRA ATA GGA	Borneman and Hartin, 2000
	NS8	TCC GCA GGT TCA CCT ACG GA	White et al., 1990
	LETC1670rc	ACT CAC CGA TCG CCG ATC	after Redecker, 2000
Acaulosporaceae	ACAU1660rc	CCG ATC CGA GAG TCT CA	after Redecker, 2000
Archaeosporales	ARCH1375R	TCA AAC TTC CGT TGG CTA RTC GCR C	Russell et al., 2002

MP analysis for each alignment was calculated using the MEGA software, V. 7.0.21 (Kumar et al., 2016). Heuristic searches with 1,000 random taxon addition replicates were conducted using the tree-bisection-reconnection method with search level 1 (Molina et al., 2017). Clade support was inferred from bootstrapping (Felsenstein, 1985) performed based on 1,000 pseudoreplicates with the same settings as for the heuristic search. Only clades that received bootstrap support of greater than or equal to 70% in the MP or posterior probabilities of greater than or equal to 0.90 in the MrBayes analysis were considered to be well supported (Molina et al., 2017). Phylogenetic trees were drawn using the FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) and statistically well supported clades containing the calculated OTUs were marked in the final tree.

Richness estimation of arbuscular mycorrhizal fungi operational taxonomic units

Potential richness and inventory completeness of *Solanum cajanumensis* and *Solanum betaceum* AMF OTUs were evaluated using the program EstimateS version 8.2.0 (Colwell et al., 2012). Individual-based species accumulation curves were calculated for each *Solanum* species. The obtained curves describing their 95% confidence intervals were fitted to a Clench curve (Soberón and Llorente, 1993) using Statistica version 7.0.61.0 (StatSoft; Tulsa, OK, USA) following Jimenez and Hortal (2003). The asymptote of each curve was calculated as the fraction a / b, where a and b are the two parameters of the Clench curve. The sampling effort needed to record 95% of the estimated proportion of OTUs for each *Solanum* species was calculated according to Jimenez and Hortal (2003).

Results

Frequency of mycorrhizal colonization

Microscopic analysis of the root samples of *S. cajanumensis* showed notably higher root colonization than for *S. betaceum,* with 88.2% and 68.8%, respectively.

The results of the independent two sample t test analysis showed statistically significant differences among mycorrhizal root colonization of both plant species (t = -10.44; df = 48; $p < 0.001$). The mean for mycorrhizal root colonization of *Solanum betaceum* was 68.8±7.21 and for *Solanum cajanumensis* was 88.2±5.84.

Molecular identification and phylogenetic analysis

In total, 82 DNA fragments were amplified solely for the primer sets *Glomus* Group B and *Acaulospora* between both *Solanum* species. The PCR products were cloned and for each amplified fragment up to six clones were analyzed. One or two clones for each restriction pattern were sequenced. Overall, 121 sequences (694 bp in length) were obtained from both plants. RFLP was performed to detect sequence variability in the clones; 91 sequences were removed after redundancy filtering based on RFLP and sequence quality analysis and finally 30 sequences were obtained of AMF isolated from the two plant species (Table 2). These sequences were then used as input for the UPARSE software to delineate OTUs. The USEARCH algorithm found seven OTUs that were later taxonomically annotated as members of the Glomeromycota phylum.

OTU 1 included seven sequences belonging to the *Glomus* genera in *S. cajanumensis* being the OTU with the highest number of sequences in wild *Solanum* species. From the seven OTUs, only OTU 5 and OUT 7 (*Scutellospora* sp.1 and *Acaulospora* sp.2 respectively) were found in both plant species. Three OTUs (1, 2 and 4) were found in the wild tomato species *S. cajanumensis* while only two OTUs (3 and 6) were unique to *S. betaceum* (Table 2). The phylogenetic analysis of the seven OTU sequences showed that the OTUs were not segregated at the family level based on their origin (wild or cultivated tomato) but rather mixed with each other (Fig. 1). The two OTUs shared by both *Solanum* species (OTU 5 and OTU 7) were clustered closely to Gigasporaceae and *Acaulospora* sp., respectively. Three singleton sequences were removed from the analysis due to low sequence similarity to any other sequence in the dataset.

Table 2 Information on number of clones of each operational taxonomic unit (OTU; 17 and 13 in *S. cajanumensis* and *S. betaceum,* respectively), where sizes of OTUs shared between species are highlighted in bold and the genera assigned to each OTU are based on comparison with the closest sequence in NCBI

			Host Plant		
OTUs ID	Genus	Accession number	*Solanum cajanumensis*	*Solanum betaceum*	Identity (%)
OTU 1	*Glomus*	MF784401	7	0	99.85
OTU 2	*Acaulospora* sp.1	MF784404	2	0	97.32
OTU 3	*Rhizophagus*	MF784405	0	2	99.79
OTU 4	*Claroideoglomus*	MF784403	2	0	100
OTU 5	*Scutellospora* sp.1	MF784402	**2**	**7**	100
OTU 6	*Scutellospora* sp.2	MF784406	0	2	99.67
OTU 7	*Acaulospora* sp.2	MF784407	**4**	**2**	100
	Total		17	13	

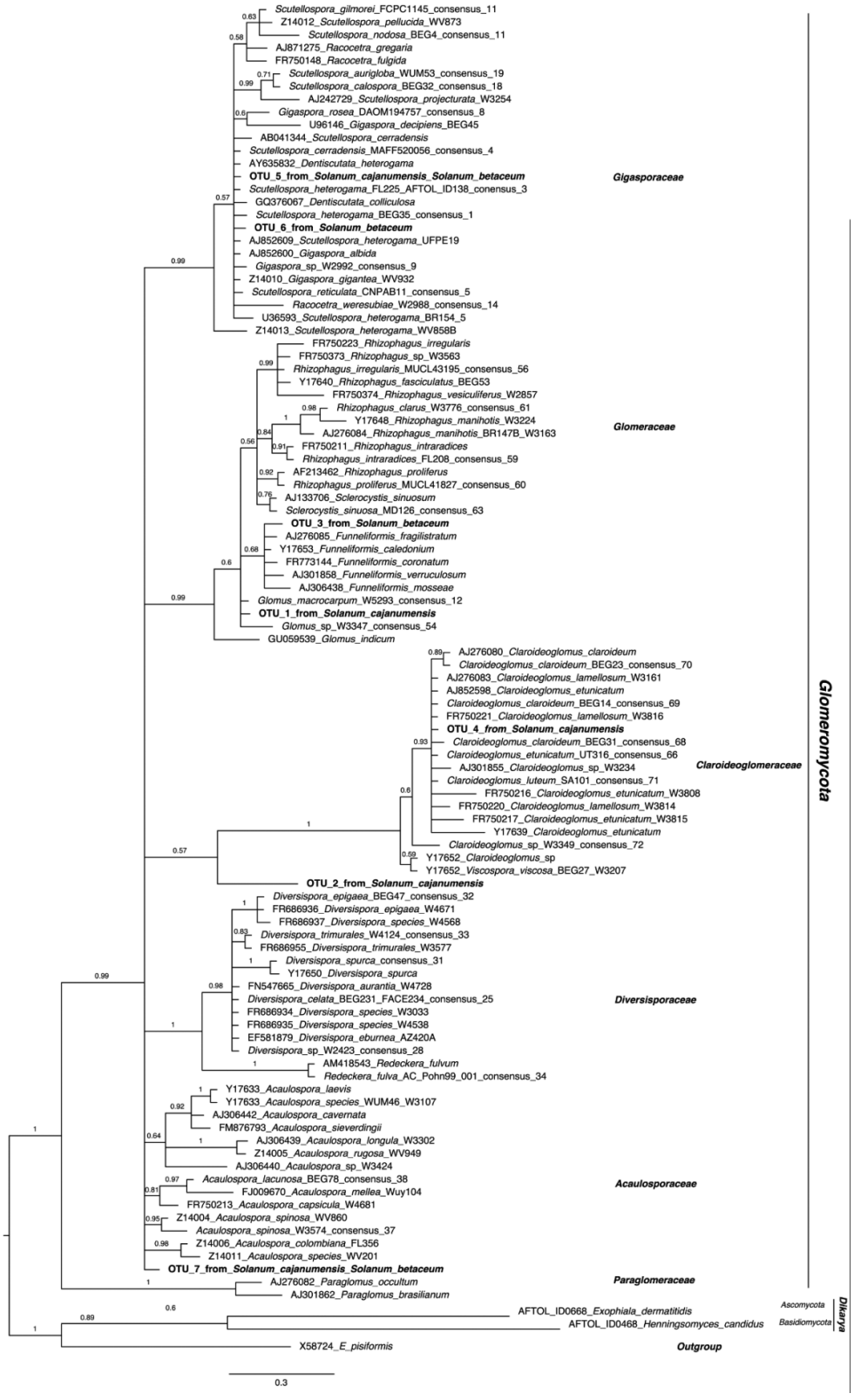

Fig. 1 Phylogenetic relationships among identified operational taxonomic units defined by the UPARSE software corresponding to a partial region of 694 bp of 18S rDNA gene from *S. cajanumensis* and *S. betaceum* including closest sequence matches obtained from the NCBI database (≥ 97% percent identity) and consensus sequences from Krüger et al. (2012). The phylogenetic tree was calculated using a Bayesian approach. Each OTU id is followed by plant species *S. betaceum*, *S. cajanumensis* or both and the corresponding accession number from NCBI. A sequence of *Endogone pisiformis* X58724 was used to root the tree. Numbers above nodes show the Bayesian posterior probabilities.

Richness and similarity of arbuscular mycorrhizal fungi operational taxonomic units

The accumulation curves of the AMF OTUs indicated an inventory completeness of 42.7% on *S. cajanumensis* and 34.5% on *S. betaceum* (Fig. 2). In total, 7 Glomeromycota OTUs were obtained and the non-saturated accumulation of fungal OTUs suggested that more locally distributed rare species may be present at the sites sampled in the current study. Richness between OTUs of *S. cajanumensis* and *S. betaceum* was considered significantly different when the 95% confidence intervals of the corresponding accumulation curves did not overlap.

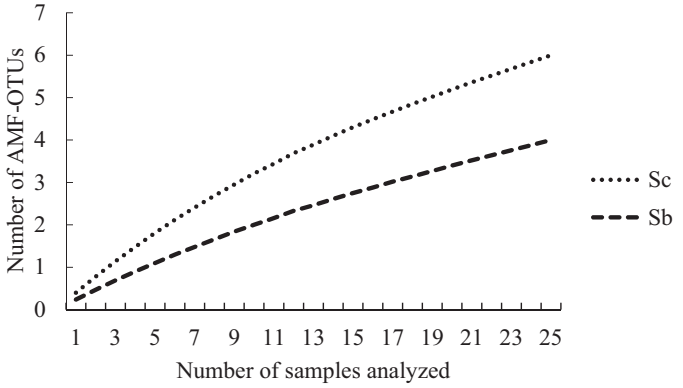

Fig. 2 Rarefaction curves of arbuscular mycorrhizal fungi (AMF) operational taxonomic unit (OTU) richness in *Solanum cajanumensis* (Sc) and *Solanum betaceum* (Sb)

Discussion

The roots of the investigated *Solanum* species were frequently colonized by AMF (68.8% and 88.2% for *S. betaceum* and *S. cajanumensis*, respectively). Information on the occurrence of associations of AMF in members of the Solanaceae is very limited. Only 25 solanaceous plant species have been assessed for their mycorrhizal status (Wang and Qiu, 2006; Reddy et al., 2007; Thangavelu and Tamiselvi, 2010). The extent of fungal colonization by AMF and the percentage of root length with different structures of AMF varied significantly between species (Muthukumar and Sathya, 2017).

The current results were consistent with others indicating a medium-to-high AMF colonization in members of the *Solanum* genera: *Capsicum frutescens*, *Lycopersicon esculetum* and *Solanum tuberosum* having colonization percentages of 42%, 50% and 44%, respectively (Tawaraya, 2003). Songachan and Kayang (2012) determined the colonization levels for *Solanum khasianum*, *Solanum sisymbriifolium* and *Solanum torvum* as 39%, 42% and 36%, respectively. In addition, Muthukumar and Sathya (2017) determined the total colonization percentage, ranging between 34% for *Solanum trilobatum* and 81.15% for *Solanum viarum* among 20 root samples. These values might suggest that lower colonization of *S. betaceum*, compared to *S. cajanumensis* is a consequence of cropping practices or a lower dependence of *S. betaceum* on AMF; however more information is needed to establish an explanation for such differences.

The highest number of sequences in the reconstructed OTUs corresponded to the genus *Glomus*, a frequent Glomeraceae group found in various habitats (Stürmer and Siqueira 2011; Gai et al., 2012; Chaiyasen et al., 2014) and plant species (Shi et al., 2006; Kramadibrata et al., 2007; Wang et al., 2013).

In the current investigation, *Glomus* from the family Glomeraceae and *Acaulospora* from the family Acaulosporaceae were the most frequent AMF identified in *S. cajanumensis* species. For *S. betaceum, Scutellospora* from the family Gigasporaceae was the most frequent AMF. Studies in the same genus *Solanum*, for example *Solanum tuberosum*, have reported *Acaulospora* as the dominant genus in AMF communities associated with potato plantations in the Andes of Ecuador, Peru and Bolivia (Senés-Guerrero and Schüßler, 2016) and Songachan and Kayang (2012) reported that *Glomus* and *Acaulospora* were the most frequent AMF in *Solanum khasianum*, *Solanum sisymbriifolium* and *Solanum torvum*. The current study used the partial 18S rDNA region because it is the only gene with broad taxon sampling in Glomeromycota (Redecker and Raab 2006). However, considering the available primers when the current study was carried out, several Glomeromycota groups may be underrepresented compared with actual studies (Krüger et al., 2012).

The molecular characterization of AMF associated with both *S. betaceum* and *S. cajanumensis* revealed a higher diversity of fungal species associated with the wild species compared to the cultivated one. A higher number of AMF species was associated to wild plants rather than field crops, with 35 and 22 AMF species isolated in each plant type, respectively (Gai et al., 2000; 2004). Ohsowski et al. (2014) showed that natural, undisturbed systems and wild plants are associated with more uncultured AMF than disturbed systems and cultivars.

The interpretation of OTU diversity must be done with caution, especially due to the limited number of recovered sequences. However, it is speculated based on the current study that the OTUs found in the wild species could colonize the cultivated species as well, based on their phylogenetical proximity (Fig. 1). This opens an interesting opportunity for using the roots or rhizosphere soil of *S. cajanumensis* as natural reservoirs of AMF that could improve agronomic parameters in *S. betaceum* in highly disturbed agroecosystems as has been observed to benefit other species (Marx et al., 2002). Sangabriel-Conde et al. (2015) suggested similar management practices in maize based on using nested PCR and observing that regarding AMF rDNA, there was a higher number of Glomeromycota OTUs in four landrace species compared to a domesticated hybrid.

The accumulation curves of AMF OTUs in the current study indicated a low inventory completeness in both species, as well the non-saturated accumulation of fungal OTUs, suggesting that more locally-distributed species may be present at sites in the current study. In order to assess this richness, comparison with other sites are necessary in conjunction with intensifying the sampling effort. Further studies will confirm whether the AMF diversity detected on *S. cajanumensis* is compatible with *S. betaceum,* as well the benefits of such associations to improve desired agronomic traits.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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