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Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora* sp.

J. P. CLAPP,* A. H. FITTER and J. P. W. YOUNG
Department of Biology, University of York, PO Box 373, York, YO10 5YW, UK

Abstract

Roots of bluebell (*Hyacinthoides nonscripta*) were sampled from a woodland in Yorkshire, UK and spores of an arbuscular mycorrhizal fungus *Scutellospora* sp., were obtained from the surrounding soil. Partial small subunit (SSU) ribosomal RNA sequences were amplified from both roots and spores using either the universal forward primer SS38 or the Glomales-specific primer VANS1, with the reverse Gigasporaceae-specific primer VAGIGA. Amplified products were cloned and sequenced. Both spores and roots yielded sequences related to those known from fungi within the Glomales, with up to four distinct SSU sequences obtained from individual spores. The VANS1 primer-binding site varied considerably in sequence and only a subset of *Scutellospora* sequences were amplified when the VANS1 primer was used. In addition to glomalean sequences, a number of different sequences, apparently from ascomycetes, were obtained from both root and spore samples.

Keywords: 18S, arbuscular mycorrhiza, diversity, Glomales, rRNA, *Scutellospora*, SSU, VANS1

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Introduction

A principal requirement for the ecological investigation of community structure in natural ecosystems is the correct and reproducible identification of the organisms present. This most basic task is hard to achieve in many ecologically important groups of organisms, especially obligate symbionts such as arbuscular mycorrhizal fungi (AMF). Methods for *in situ* identification and characterization of species composition within roots are critical to the effective study of AMF ecology because morphologically similar isolates may have very different effects on their hosts (Streitwolf-Engel *et al*. 1997; Van der Heijden *et al*. 1998). The taxonomy of AMF is based upon the morphological description of spores and their related structures. However, because the number of available taxonomic characters is limited and many features are highly variable in expression, the use of spore morphology for identification in the study of the ecology of AMF may lead to a reduced estimate of diversity (Sanders *et al*. 1996). The crucial information necessary for field-based investigations of AMF ecology is the species composition and relative abundance of AMF present in colonized roots and associated rhizosphere. To date, however, ecological investigations have relied heavily upon spore surveys extrapolated to the situation within the root. Such investigations yield only circumstantial information (Klironomos *et al*. 1993; Roldan-Fajardo 1994). Clapp *et al*. (1995) reported the widespread presence of *Glomus* spp. in roots of the bluebell (*Hyacinthoides nonscripta* L. Chouard ex. Rothm.) using family-specific primers and PCR, but found very few *Glomus* spores in the associated rhizosphere. This demonstrated the potential error of using spore surveys to extrapolate to the situation within the root. Therefore the identification of AMF within the plant root seems most accurately achieved by the use of molecular markers.

As is the case with many organisms, the majority of nucleic acid information derived from the Glomales has been from the small subunit (SSU) ribosomal RNA gene (Simon *et al*. 1992b; 1992a; 1993a; 1993b; Clapp *et al*. 1995; Simon 1996; Helgason *et al*. 1998). These are high-copy-number genes existing in tandem arrays, and in the majority of organisms little sequence variation between copies has been detected. The ribosomal RNA genes have been useful sources of phylogenetic information

Correspondence: J. P. Clapp. E-mail: J.P.Clapp@ukc.ac.uk
*Present address: Department of Biosciences, The University of Kent, Canterbury, Kent, CT2 7NZ, UK.

for numerous organisms including fungi, where comparisons have been made between morphologically based phylogenies and sequence data (Bruns *et al*. 1992), although there are instances where sequence and morphology suggest different relationships (Waters *et al*. 1992). However, differences and similarities in the base sequence of rRNA genes are generally accepted as likely to reflect the true phylogenetic relationships between groups. There is a large amount of DNA sequence information available with which to compare newly obtained sequences. Several complete SSU, internal transcribed spacer (ITS) and partial large subunit (LSU) region sequences have now been determined for AMF. Sequence variation has been reported in the ITS regions within single spores (Sanders *et al*. 1995; Lloyd-MacGilp *et al*. 1996) complementing reports in other fungi (O'Donnell *et al*. 1998). SSU and LSU (Van Tuinen *et al*. 1998) sequences have been used to design several primers for the identification of AMF at the level of order (VANS1) and family (VAGLO [*Glomus*], VAACAU [*Acaulospora*] and VAGIGA [*Gigaspora* and *Scutellospora*], used in conjunction with VANS1 (Simon *et al*. 1992a; 1993b). Clapp *et al*. (1995) addressed the detection of AMF diversity in a natural ecosystem using such primers, demonstrating the presence of multiple sequences from each of three genera (*Glomus*, *Scutellospora* and *Acaulospora*) in the roots of bluebells. This was initially interpreted as indicating the presence of more than one species of each genus within a single root. The order-specific and family-specific primers gave no results from field material, however, without resorting to a more complex procedure known as selective enrichment of amplified DNA (SEAD). This technique offered a reproducible method of overcoming the difficulties encountered in obtaining amplification from field material with the Glomales-specific primer VANS1, but was less straightforward than a single-step amplification of field roots. The investigation presented in this study was undertaken to determine the source of the difficulties found with the use of VANS1, and to use the partial rRNA sequences obtained from AMF to investigate sequence diversity in a natural community. Previous work has shown sequences corresponding to the genus *Scutellospora* to be common in roots and because this genus was also represented by many spores in the soil at the study site (Clapp *et al*. 1995), it was selected for detailed examination by sequence analysis.

Materials and methods

Rhizosphere soil and bluebell roots were sampled at Pretty Wood, Castle Howard, North Yorkshire, UK in March and May 1995 (Grid SE 732 687). Samples were collected from a site where the upper storey vegetation was dominated by oak (*Quercus petraea* (Mattuschka) Liebl.). The site has been described in detail by Merryweather & Fitter (1995).

Extraction of DNA from spores

AMF spores that appeared to be intact and unparasitized in field soil were isolated by wet-sieving (Walker 1991) and selected for DNA isolation (single-spore isolations SA, SB, SC and SD; five-spore isolations SE and SF). The spores were vigorously washed by vortexing, picked from sterile water and examined for obvious external fungal contamination such as hyphae. The spore(s) were placed in 0.5-mL Eppendorf tubes, centrifuged to the base and crushed with a sterile microhomogeniser in 10 µL of Instagene matrix (Bio-Rad) followed by the addition of a further 10 µL of matrix. The extraction mixture was incubated at 56 °C for 30 min, vortexed and incubated again for 10 min at 100 °C. The mixture was vortexed once more for 10 s and centrifuged at 12 100 *g* (14 000 rpm) for 3 min to pellet cellular debris and the Instagene matrix. The subsequent PCR amplifications used 10 µL of supernatant as template.

Extraction of DNA from roots

Five plants (RA, RB, RC, RD and RE), randomly chosen from sites ≈ 20 m apart, were sampled at the same time as soil samples were taken. Roots (≈ 5 cm in length) were selected from each plant only if they were completely intact. The roots were brushed to remove contaminating debris and surface sterilized in 0.1% household bleach (0.05% NaOCl) for 30 s. DNA was extracted using a CTAB buffer and AMF colonization determined by SEAD (Clapp *et al*. 1995).

PCR

Amplified products were obtained from roots after SEAD using *Taq* polymerase and the universal forward primer SS38 (White *et al*. 1990) or the VANS1 primer (Simon *et al*. 1992a) in conjunction with the family-specific primer VAGIGA (Simon *et al*. 1993b). Amplification parameters were as described by Clapp *et al*. (1995). DNA extracted from spores was amplified using the same primers and amplification conditions.

Sequencing

Amplified products were purified using Wizard DNA purification system columns (Promega) and ligated into the vector pGEM-T (Promega). Recombinant plasmids were extracted from bacterial clones using Wizard Minipreps columns (Promega) and sequenced using a T7 DNA polymerase kit (Pharmacia) according to the manufacturers'

Table 1 Sequences cloned from *Scutellospora* spores or bluebell roots after amplification with the small subunit ribosomal RNA primers SS38 and VAGIGA

Source	Sample	Cloned sequences*			
		Putative *Scutellospora*	Other Glomales	*'Thelobolus–Verpa'* cluster	Others
Spores	SA	4	0	1	1
	SB	2	0	0	0
	SC	5	1	0	0
	SD	2	0	0	0
	SE (multi)	2	2	0	0
	SF (multi)	2	0	0	1
Roots	RA	4 (4)†	0	10	2
	RB	1 (2)	0	13	0
	RC	1 (3)	1	5	0
	RD	0 (2)	0	5	1
	RE	0 (1)	0	1	0

*Four distinct sequence classes are defined on the basis of their phylogenetic positions (see Fig. 1 and text).
†In parentheses are shown the number of additional sequences obtained using the VANS1/VAGIGA primer pair.

instructions. Twenty-three spore clones were sequenced in both strands from mixed (SE and SF) and single (SA, SB, SC and SD) spores. Fifty-six sequences were obtained from root samples in total. The sequence data are available in the EMBL/GenBank database (Accession nos AF062656–AF062707).

Sequence analysis

Sequences, excluding the primer sites, were aligned with published sequences using CLUSTALX (Thompson *et al*. 1997) with some manual adjustment. The published sequences were taken from the EMBL/GenBank database: *Ajellomyces dermatitidis* (M63096), *Aspergillus fumigatus* (M55626), *Auricularia auricula* (L22254), *Chytridium confervae* (M59758), *Emericella nidulans* (U77377), *Glomus intraradices* (X58725), *Gigaspora margarita* (X58726), *Neurospora crassa* (X04971), *Pleurotus ostreatus* (U23544), *Sclerotinia sclerotiniorum* (X69850), *Thelobolus crustaceus* (U53394), *Thelomma mammosum* (U86697), *Verpa bohemica* (U42645) and *Verticillium dahliae* (U33637). The alignment was 199 bases in length. A distance matrix with Kimura's 2-parameter correction for multiple substitutions (Kimura 1980) was used to construct a neighbour-joining tree (Saitou & Nei 1987) using CLUSTALX (Thompson *et al*. 1997). The tree was displayed using TREEVIEW (Page 1996).

Amplification of cloned products

The specificity of the VANS1 primer was tested by using cloned SS38/VAGIGA products of known sequence as PCR templates, with VAGIGA as the reverse primer. The amplification conditions were the same as those described for PCR amplification of spores.

Results

PCR amplification products of approximately 240 bp corresponding to part of the SSU rRNA gene were amplified using the SS38/VAGIGA primer combination from both bluebell roots and *Scutellospora* sp. spores. The products were cloned and a total of 23 spore-derived and 44 root-derived clones were sequenced (Table 1). Apart from nucleotide substitutions, the sequences differ in a number of single-base insertions or deletions, usually not more than one in a sequence but up to five in one case. While it is possible that some of these reflect errors introduced by the DNA polymerase, the expected error rate is not high enough to affect interpretation of the results (see Lloyd-MacGilp *et al*. 1996).

A phylogenetic tree, based on these sequences plus published homologues, is shown in Fig. 1. The resolution of this tree is limited by the low level of variation seen at the 5′ end of the SSU, but all sequences appear to be of fungal origin and cluster with published sequences of the Glomales or of Ascomycetes. The sequences can be divided into four classes: (1) sequences close to that of *Gigaspora margarita* Becker & Hall DAOM 194757 (now re-classified as *G. rosea*, Bago *et al*. (1998)), which are therefore putatively from the *Scutellospora* sp. found at the site (in the family Gigasporaceae); (2) other species in the Glomales branch; (3) a large cluster of very closely related sequences that are unidentified but within the Ascomycetes branch; (4) diverse other sequences associated with the Ascomycetes or Glomales but mostly of uncertain placement. All four classes were obtained from spores, but the great majority of spore-derived sequences were, as expected, in the first class (Table 1). Only six putative *Scutellospora* sp. sequences were cloned from

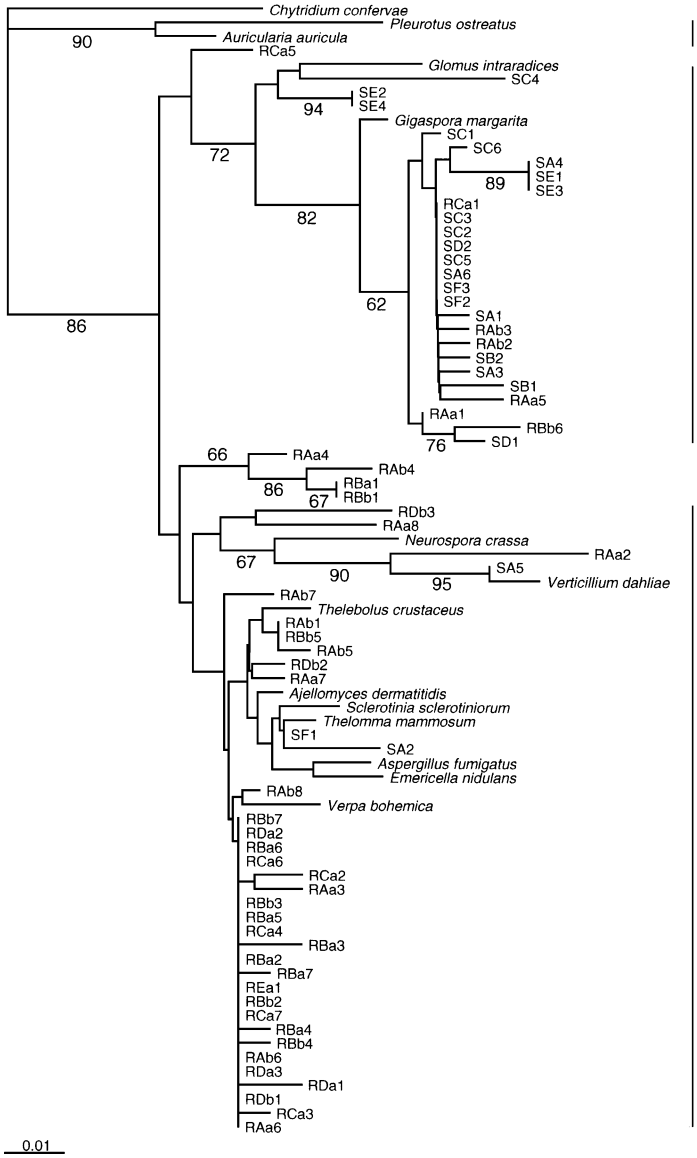

Fig. 1 Phylogeny of the partial SSU sequences obtained from spores (S) and roots (R) using the primer combination SS38 and VAGIGA. The primer sequences are not included in the alignment (length 199 bp), nor are sequences obtained using VANS1. Sequences of other fungi were obtained from the EMBL sequence database. The tree was constructed by the neighbour-joining method (see text) and rooted with *Chytridium confervae* as outgroup. Percentage bootstrap support (1000 replicates) is indicated where this exceeds 60%. The scale bar represents 1% sequence divergence.

roots and one of these was also amplified from a spore (RCa1). Most of the root sequences are in class (3). Two spore-derived sequences (SA2 and SF1) are also related to this group, although not identical to any of the sequences obtained from roots. The distinction between classes (3) and (4) is rather arbitrary, as there is a group of 15 identical sequences that form the core of class (3) but many other sequences only differ from these by one or two substitutions. Many of the sequences in classes (3) and (4) are similar to sequences from named Ascomycete species, but none is identical and the phylogenetic information in these short sequences is, in any case, not sufficient to allow confident identification.

Clones derived from a single spore sometimes differed in sequence. For example, the sequences SA4 and SD1 differed substantially from other SA and SD sequences; these differences are too great to ascribe to polymerase error and must reflect heterogeneity within the spores. Among the Glomales-like sequences, the most variable region is the part originally proposed by Simon *et al*. (1992a) as the target site for a primer, VANS1, that was intended to be universal for the Glomales. In fact, only two of the sequences in our study match the VANS1 primer exactly. These two sequences (SE2 and SE4) are most similar to the published sequences from *Glomus intraradices* DAOM 197198, Accession no. X58725. Most of the putative *Scutellospora* sequences fail to match VANS1 by three single-base insertions and one base substitution (Table 2). In view of the poor conservation of the VANS1-binding site, we tested representative clones as templates for PCR using VANS1 as the forward primer rather than SS38. While all AMF templates could be amplified when

Table 2 Polymorphism of the VANS1 primer site in putative *Scutellospora* sequences obtained from field-collected spores and roots

		Number of times sequence encountered	
	VANS1 site sequence*	Spores	Roots
1.	GTCT.AGTAT.AATCGTT.ATACAGG	2	0
2.	GTCT**T**AGTAT.AATCGTT.ATACAGG	1	0
3.	GTCT**A**AGTAT**A**AATC**A**TT**T**ATACAGG	11	5
4.	GTCT**A**AGTAT**A**AATCGTT**T**ATACAGG	1	1
5.	GTCT**A**AGTAT.AATCGTT.AT**G**CAGG	3	0
6.	GTCT**A**AGTAT**A**AATC**A**TT**C**ATACAGG	1	0
7.	GTCT**A**AGTAT**A**AA**CCA**TT**T**ATACAGG	1	0

*The VANS1 primer matches sequence 1.

Table 3 Differing efficiency of amplification from clones using VANS1/VAGIGA primers at two annealing temperatures. Sequences SA2 and SA5 do not group with AMF sequences

		Anneal temperature	
Sequence	VANS1 site†	50 °C	55 °C
SE4	1	***	***
SC4	2	***	***
SA1	3	**	*
SA4	5	**	*
SC6	6	**	*
SC1	7	**	*
SA2	—	—	—
SA5	—	—	—

***strong amplification; *poor amplification; — no amplification.
†Defined in Table 2.

the annealing temperature was 50 °C, most amplifications were less effective when 55 °C was used. Templates derived from putative Ascomycete sequences (SA2 and SA5) did not amplify under either conditions (Table 3).

As the SS38/VAGIGA primer combination was apparently not specific to the Glomales, it was difficult to obtain a high proportion of Gigasporaceae sequences from roots. We therefore also amplified SSU sequences from root samples using VANS1/VAGIGA after SEAD (Clapp *et al*. 1995). Twelve clones were sequenced (Accession nos AF062700–AF062707), all of which cluster with the Gigasporaceae. These sequences are omitted from the phylogenetic tree (Fig. 1) because they are shorter than the other sequences and lack the informative region of the VANS1 site itself. Apart from a few differences that were each confined to a single clone, *Scutellospora* sequences show almost complete identity over their lengths when the VANS1 site is excluded from the comparison (results not shown). There is, however, a single transition (either C or T) at position 154 of the alignment, where a C occurred three times in the 23 *Scutellospora* sequences obtained with the SS38/VAGIGA primers. It was significantly more frequent ($\chi^2 = 5.64$, 1 d.f., $P < 0.025$) in sequences obtained using the VANS1/VAGIGA primer combination, occurring six times out of 12.

Discussion

The sequences obtained during this study were categorized into four groups on the basis of the phylogenetic analyis shown in Fig. 1: (1) putative *Scutellospora* sp. sequences (most probably *S. calospora*, J. C. Dodd, personal communication); (2) sequences allied with other Glomales; (3) a large group of related sequences associated with Ascomycetes; and (4) diverse sequences allied with the Ascomycetes or Glomales. A single putative *Scutellospora* sequence was obtained from both spores (RCa1) and roots and provides an unambiguous link between the spores isolated in the rhizosphere and the colonists of the roots themselves. These sequences were represented in the greatest proportion when amplified from spores, as might be expected. In the second group, three sequences (SC4, SE2, SE4) appear to be more closely related to *Glomus* than to *Scutellospora*, despite the fact that the spores were identified by morphology as *Scutellospora* and other sequences from the same DNA extraction cluster with the Gigasporaceae. Two of the sequences were derived from a DNA extraction from five spores but it seems unlikely that any *Glomus* spores were included by mistake as it is relatively straightforward to separate Glomaceae from Gigasporaceae spores on the basis of the bulbous morphology of the hyphal attachment (sporogenous cell). The third group represents the largest group of sequences obtained from roots. They were isolated from all the root samples and therefore may represent a widespread endophytic fungus. Some similar sequences were also isolated from spores, suggesting either internal parasitism or external contamination. The closest published sequences include *Ajellomyces* (*Blastomyces*) *dermatitidis*, *Thelobolus crustaceus* and *Verpa bohemica*, but there are many other fungi with very similar sequences and there is too little information in these relatively short sequences to allow any firm identification.

These results indicate that the sequence polymorphism previously reported within single spores (Sanders *et al*. 1995; Lloyd-MacGilp *et al*. 1996) is not restricted to the ITS region but is also frequent in the SSU gene. The origin of this diversity is still undetermined but it may arise from either distinct rRNA gene clusters within a genome or from variation between nuclei. Rosendahl & Taylor (1997) demonstrated that single spores from a pot culture each had a unique amplified fragment length polymorphism

(AFLP) profile. This suggests that each spore is composed of a unique combination of nuclei with differing genomic compositions. In the present study, up to four sequences were identified from single spores (e.g. SC). The presence of multiple sequences in the SSU indicates that the population genetics of these fungi could be unusual and merits detailed study, and also has implications for other research on these fungi, specifically in the areas of phylogenetic reconstruction and primer design.

The ribosomal SSU genes have long been the preferred target for phylogenetic reconstruction and databases of considerable size are available for comparison. The presence of multiple SSU sequences within single spores suggests that phylogenies within the Glomales may be ambiguous. For example, currently available SSU sequences suggest that the genus *Acaulospora* is more closely allied to the Gigasporaceae than to the Glomaceae (Simon 1996). This relationship is contrary to that indicated by developmental and morphological characters (Morton & Benny 1990). It is possible that the use of single unrepresentative sequences could be responsible for this contradiction. To overcome this, intra-isolate phylogenies could be created using sequences from several spores to identify the ancestral or common sequence for each. Phylogenies based on these sequences could be compared with the taxonomic relationships indicated by other criteria. Of course, this approach will only be possible if intra-isolate phylogenies are shallow in comparison with those relating to different taxa. The sequence data presented here are from field material; it is possible that biases arising from single-spore inoculations during the initiation of pot cultures could mask sequence diversity encountered in natural communities. Until the frequencies, diversity and significance of different sequences occurring within single taxa have been assessed, molecular phylogenies based on the SSU should be interpreted with caution.

On the basis of our data, primers targeting variable regions of the SSU are unlikely to amplify DNA from all homologous genes present within a single spore. This has implications for the use of molecular methods for *in situ* identification and determination of species composition within roots. Such methods are critical to the effective study of AMF ecology because, for example, morphologically similar isolates may have very different effects on their hosts (Streitwolf-Engel *et al*. 1997; Van der Heijden *et al*. 1998). It is possible that unique sequences or sequence combinations could be identified within each isolate and exploited to allow identification of each taxon. However, it is unfortunate that the number of unique AMF SSU sequences found within a root does not necessarily reflect the number of independent colonization events or the number of taxa present. Thus sequence diversity may not reflect species diversity.

The region sequenced in this study represents one of the more conserved areas of the SSU. The VANS1 primer (Simon *et al*. 1992a) was initially designed from sequences of *Gigaspora margarita* Becker and Hall DAOM 194757 (*G. rosea*, Bago *et al*. 1998) and *Glomus intraradices* Schenck and Smith DAOM 197198 and its Glomales-specific status inferred from its ability to amplify other cultured fungi in the Glomales. The use of the universal primer SS38 to obtain sequence data covering the VANS1 annealing site has allowed us to investigate the specificity of VANS1. The degree of variation we report in the VANS1 priming site (Table 2) may explain the difficulties previously experienced in using the VANS1 primer on field material (Clapp *et al*. 1995). The sequence information obtained in this study indicates that the VANS1 primer is specific in its ability to amplify DNA from Glomales, but that the amplified products represent a biased sample of the sequences present. This is demonstrated by the differences seen in the sequences obtained using SS38 or VANS1 as forward primers. Thus VANS1 has limitations for studies of natural populations. Although only sequences from one species of *Scutellospora* have been investigated in this study, the difficulties in amplifying other AMF (Clapp *et al*. 1995) suggest that a similar situation may occur in other genera. The presence of multiple SSU ribosomal RNA gene sequences has been reported for *Glomus mosseae* (Vandenkoornhuyse & Leyval 1998).

The conserved morphology of the Glomales has led to the description of relatively few genera and species considering the evolutionary history and wide adaptability of the group. It is becoming increasingly apparent that the conservative nature of spore morphology belies considerable genetic variation and functional diversity (Sanders *et al*. 1996). It is certain that considerably more fundamental research is required into this group of fungi before their full importance can be adequately recognized and investigated in natural communities.

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