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TECHNICAL DEVELOPMENT FOR THE ISOLATION OF SINGLE NUCLEI FROM MULTINUCLEATE SPORES AND HYPHAE USING *GLOMUS IRREGULARE* ISOLATE DAOM 181602

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Abstract

Isolation of single nuclei from multinucleate spores and hyphae of arbuscular mycorrhizal fungi (Glomeromycota) is technically challenging but provides important information about genetic diversity and genome organization. This study aimed to isolate single nuclei from hyphae and spores of *Glomus irregulare* isolate DAOM 181602, amplify marker genes and get an idea about presence of homokaryons and heterokaryons in arbuscular mycorrhizal fungi. Arbuscular mycorrhizal fungi grown on Laser Capture Microdissection (LCM) slides in bicompartmental Petri plates produced hyphae which showed reduced autofluorescence, enabling excellent visualization and successful catapulting of nuclei. Single nucleus was also successfully isolated from spores by gentle crushing and staining on LCM slides. Isolated nuclei were subjected to multiple-strand displacement amplification (MDA) followed by multiplex PCR targeting nine *Glomus irregulare* genes. The Binding protein (*BiP*) gene amplified successfully from approximately 50% of single nuclei, while most other genes showed inconsistent amplification. Cloning and sequencing of *BiP* from six single nuclei revealed 1-7 allelic variants per nucleus. Direct sequencing traces from additional nuclei confirmed that some nuclei contain single *BiP* sequences while others harbor multiple variants, providing insights into the genetic organization of this arbuscular mycorrhizal fungus.

Key words: Single-nucleus isolation; Laser capture microdissection; *Glomus irregular*; Arbuscular mycorrhizal fungi (AMF); Multiple displacement amplification (MDA); *BiP* gene

Introduction

Arbuscular mycorrhiza (AM) is a mutualistic symbiosis between plant roots and fungi belonging to the phylum Glomeromycota. In this symbiosis, plants provide the fungus with carbohydrates and get phosphorus in return. Colonization occurs after a relationship is established between the young hyphae and a transformed hairy root. The fungi produce intra-radical and extra-radical hyphae, arbuscules and spores. The number of nuclei in a single spore varies greatly from species to species. These nuclei are firmly packed inside the spore and share a common cytoplasm (Bécard & Pfeffer, 1993; Pawlowska & Taylor, 2004). The amount of DNA in a single nucleus is very low (Cavalier-Smith, 1985). Isolation of single nuclei is challenging due to the presence of hundreds of nuclei in a single spore and strong autofluorescence (Dreyer *et al.,* 2006; Gange *et al*., 1999; Helber & Requena, 2008; Manjarrez *et al*., 2009; Sejalon-Delmas *et al*., 1998; Vierheilig *et al*., 1999; Vierheilig *et al*., 2001), presumably from intraradical and extraradical structures which complicates the identification of nuclei from debris. AM fungi can be cultured axenically with transformed roots in bicompartmental petri plates, where fungal hyphae extend into a root-free compartment, providing pure fungal material for molecular studies.

Very little is known about the genetics of AM fungi (Pawlowska, 2005) due to problems in identification, inability to cultivate all AM fungal species axenically, and obtain large amounts of pure fungal DNA for various molecular studies (Corradi *et al.*, 2004; Redecker *et al*., 1999). AM fungi reproduce asexually and produce multinucleate spores and hyphae (Burggraaf & Beringer, 1987; Cooke *et al*., 1987; Mosse, 1973; Viera & Glenn, 1990). There are two hypotheses proposing the possible organization of AM fungal genome, homokaryosis, heterokaryosis (Fig. 1). Polyploid genome (referring to first hypothesis) suggests all sequence variants are present in each nucleus in multiple copies, and nuclei are genetically identical. This hypothesis is supported by the findings when all three variants of the Internal Transcribed Spacer were found in each of six isolated single nuclei (Pawlowska & Taylor, 2004; Stukenbrock & Rosendahl, 2005a; Stukenbrock & Rosendahl, 2005b). Authors defined the observed variation as variation that is not lost during segregation. Haploid genome (referring to second hypothesis) propose sequence variants are distributed between nuclei, where each nucleus has one copy of each gene (Hijri & Sanders, 2005; Kuhn *et al*., 2001). The present study presents a method for the isolation of single nuclei from *Glomus irregulare* spores and hyphae via laser capture microdissection, overcoming the limitation of autofluorescence. Genetic

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analysis and sequencing of marker genes from the isolated single nuclei was performed to get an insights in genome organization of AM fungi. This study aimed to isolate single nuclei from hyphae and spores of *Glomus irregulare* isolate DAOM 181602, amplify marker genes and get an idea about presence of homokaryons (presence of genetically similar nuclei) or heterokaryons (presence of different nuclei) in AMF spores.

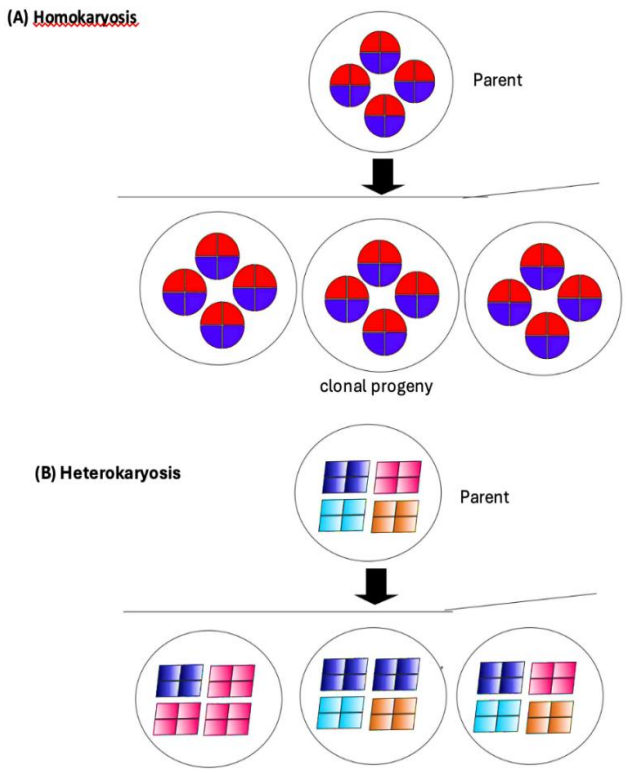

Fig. 1. Two possible genome organizations of AM fungi (Homokaryosis, Heterokaryosis). Large size circles in figure above indicate AMF spores, small four-compartment circles and rectangles indicate genetically identical and genetically different single nuclei within each spore respectively.

Materials and Methods

Germplasm: *Glomus irregulare* isolate DAOM 181602 was grown axenically on transformed carrot roots in bicompartmental Petri plate (St-Arnaud *et al*., 1996) system using modified MSR medium (Declerck *et al*., 1998). In this system, roots and fungi are grown in one compartment, and the fungal hyphae extend into the other compartment. Spores and extraradical hyphae obtained from these cultures were the two sources used for the isolation of single nuclei using LCM. The isolated single nuclei were subjected to MDA based whole genome amplification and sequences of known *G. irregulare* genes amplified using Multiplex PCR.

Isolation of single nuclei from spores: Approximately 200 spores isolated from axenically propagated AM cultures were introduced onto the membrane slide of LCM after being thoroughly washed in sterile water to remove any contaminants. The spores were then squashed by gently pressing a cover slip against the LCM slide to release nuclei. The released nuclei were fixed with 100% ethanol for 20 minutes and then stained with Propidium Iodide (PI, 5 µg/ml) for 1 hour on the slide. The nuclei were viewed under fluorescence microscope to confirm the staining and used for LCM.

Isolation of single nuclei from hyphae: Nuclei were isolated (using Zeiss PALM Laser Microdissection) from AM hyphae grown on LCM membrane slides in the fungal compartment of bicompartmental Petri plates.

LCM used for catapulting single nuclei: Using LCM, single nuclei from either spores or hyphae were isolated in 10-20 µl mineral oil using 0.5 ml Eppendorf tube. Stained slides were viewed through different filters under fluorescence illumination, and well-spaced single nuclei were targeted.

MDA based whole genome amplification: The isolated single nuclei from either hyphae or spores were subjected to MDA, where 2 µl reaction volume was employed, and three different reaction volumes (i.e. 5 µl, 10 µl and 15 µl) were employed for hyphal nuclei and spore nuclei, respectively (Table S1). The idea behind using different reaction volumes was to identify the optimum condition for getting better results. For MDA, Repli-g Mini kit was used according to the manufacturer's instructions.

Multiplex PCR: In case of spore nuclei, multiplex PCR was performed with 8 *G. irregulare* specific primer pairs notably BiP, ITS, Hsp, type IIc etc. but in case of hyphal nuclei**,** two primer pairs (*BiP* and *ITS*) were used because frequency of amplification from the said two markers (*BiP*, *ITS*) was higher than other primers in most of the test conducted.

Cloning, sequencing, restriction digestions and phylogeny of the catapulted nuclei: Sequences of *BiP* gene were cloned from 6 single nuclei (Table S2, batch-1 supplementary) using pGem-T Easy Vector system according to the manufacturer's instructions. *E. coli* strain JM109 was used as a cloning strain. The 48 clone sequences obtained from each of the 6 single nuclei during the study were restricted digested with two restriction enzymes *Alu*1 and *Rsa*1, according to the supplier's instructions (New England Biolabs). Out of 48 clones, only 30 clones were sequenced based on the pattern of RFLP using Sanger sequencing (Macrogen, South Korea). These clone sequences, along with BiP alleles reported by Kuhn, (2001), were used to construct Neighbour-joining (NJ) tree. NJ tree was also constructed for *ITS* gene sequences derived from the hyphal 6 nuclei. The nucleotide sequences of *ITS* gene retrieved from GenBank were used as reference sequences in the phylogeny.

Results

Isolation of single nuclei from spores: Single nuclei were visualized successfully on LCM slides using PI staining, bright fluorescent and well-spaced nuclei catapulted. To minimize chances of contamination, single nuclei were catapulted in several small batches (Table S2). Photographs recorded before and after each catapulting event confirm isolation of single nuclei (Fig. 2).

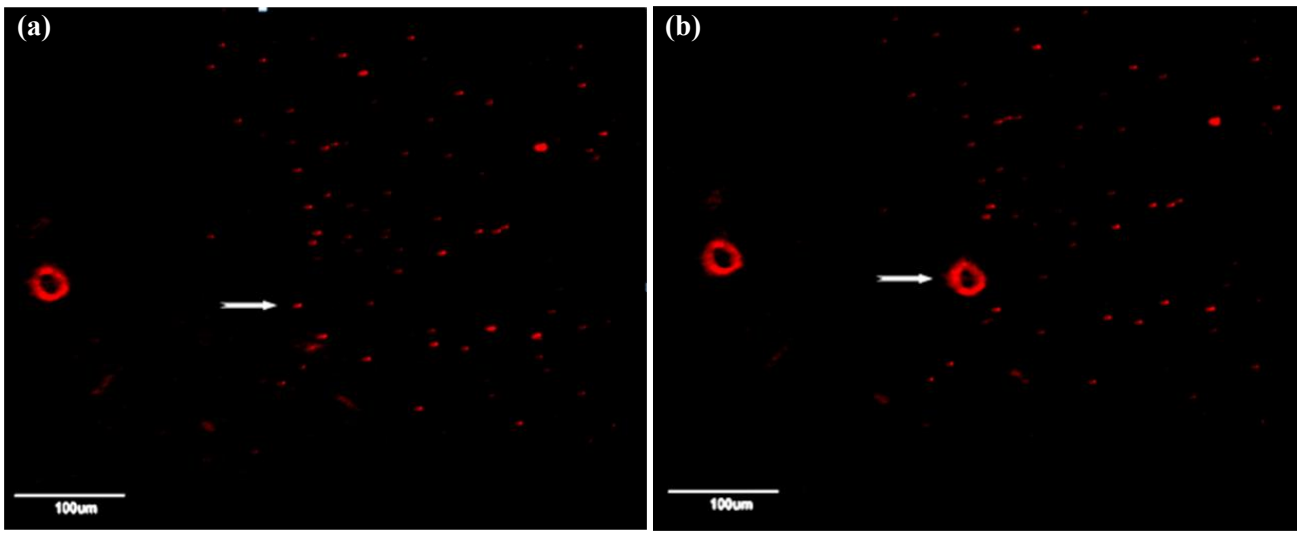

Fig. 2. Stained single nuclei on LCM slide before (a) and after catapulting (b). Red spots show stained single nuclei from spores.

Table S1. Details of MDA performed in different volumes during the study.

Reagents used	Reaction volume given by supplier (Qiagen)	Reaction volumes used during this study			
Reaction volume used	40µl	2µl	5µl	10µl	15µl
D2 solution	3.5µl	0.20µl	0.6µl	1.2µl	2µl
Vortex, centrifuge briefly and keep it on ice for 10 minutes					
Stop solution	3.5µl	0.20µl	0.6µl	1.2µl	2µl
Vortex and centrifuge for 5-10 seconds					
Composition of master mix					
Water	3µl	0µl	0µl	1.2µl	1.4µl
Repli-g Mini reaction buffer	29µl	1.45µl	3.48µl	5.8µl	8.7µl
Vortex and centrifuge for 5-10 seconds before adding DNA Polymerase					
Phi29 DNA Polymerase	1µl	0.15µl	0.36µl	0.6µl	0.9µl

Table S2. Number of nuclei isolated by LCM and success of PCR amplification of marker genes during multiplex PCR.

Source for isolation of single nuclei	Catapulting of single nuclei done in different batches	Number of single nuclei catapulted by LCM	Number of single nuclei that gave amplification of BiP gene during PCR	Number of single nuclei that gave amplification of ITS (ITS4, 5.8S) gene during PCR
Spores	Batch-1	24	6	NA*
	Batch-2	16	9	NA
	Batch-3	27	16	0
	Batch-4	31	14	Contamination occurred here
Hyphae	Batch-5	24	12	12
	Batch-6	26	15	11

*Those nuclei from which only *BiP* was amplified

Table 1. The status of hyphal growth and autofluorescence observed during mini-tests.

Mini-tests	Auto fluorescence	Hyphal growth across LCM / plain glass slides
Test-1	Reduced*	High**
Test-2	Reduced	Low***
Test-3	Reduced	High
Test-4	Reduced	High
Test-5		No colonization occurred
Test-6	Reduced	High
Test-7	Reduced	High

* Autofluorescence was called reduced when the nuclei were clearly seen on the slides (without any background)
** Hyphal growth was called 'high' when a dense network of hyphae was viewed on the slide under the microscope
*** Hyphal growth was called 'low' when 1-2 hyphae of very short length were observed under the microscope

Isolation of single nuclei from hyphae: Thinner hyphae grown on LCM and plain glass slides in fungal compartment of a bicompartmental Petri plate provided the best material for the isolation of nuclei. About 10-25 single nuclei can be catapulted from hyphae grown across an LCM slide. This study catapulted 24 single nuclei from the hyphae grown across the LCM slide.

MDA: Specific amplification from MDA-1 was observed from spores and hyphae, even with increasing reaction volumes (Fig. 3). Expected size amplicon was observed for known genes in subsequent multiplex PCR. Although the quantity of DNA increased about 2-3-fold in MDA-2 and MDA-3 measured by Nanodrop (Thermo Scientific, Inc.) but highly non-specific amplification was observed for the studied genes in subsequent PCR (Fig. S1).

Multiplex PCR: Among the genes studied, only *BiP* amplified successfully from about 50% of the isolated single nuclei from spore nuclei. Non-specific and inconsistent amplification for Glomus specific genes observed from MDA-2 and MDA-3 products (Fig. S1).

Amplification of *BiP* and *ITS* (*ITS4, 5.8S*) genes from hyphal nuclei was successful in about half of the isolated nuclei. Some nuclei allowed PCR amplification of the *BiP* gene but not *ITS* and vice versa. However, a few nuclei were found to have allowed amplification of both marker genes-shown by arrows. No amplification of the marker genes was observed from nuclei catapulted from the plain glass slides.

Cloning, restriction digestion, and sequencing of *BiP* from single nuclei: Seventeen alleles were recorded from the clone sequences of *BiP* gene using *G. irregulare* isolate DAOM 181602 across pre-identified 45 SNP positions from direct sequence traces. Sequencing of cloned sequences showed some nuclei contain single sequence variants, while others contain more than one sequence variant. Similar information can be observed from the clone library of *BiP* gene and direct sequence traces. Identical alleles were observed from a few single nuclei catapulted. In our study, RFLP patterns was called simple when nuclei exhibited one major RFLP pattern and 1-2 unique patterns, and mixed showing 2-3 major patterns and 1-2 unique patterns. NJ tree of *BiP* sequences (Fig. 5) showed that alleles under study are nearly similar to those reported by Kuhn *et al*., (2001).

Relationship of RFLP patterns with sequences in the phylogeny: RFLP pattern from both restriction enzymes was nearly the same for all *BiP* clones; therefore, data presented for *AluI*. Three restriction patterns (a1, b1, c1) were observed from the cloned sequences (Fig. 4, Table 2). Among the restriction patterns, a1 & b1 correspond to *BiP* sequences clustered in the upper part of phylogeny and the c1 to lower part of phylogeny.

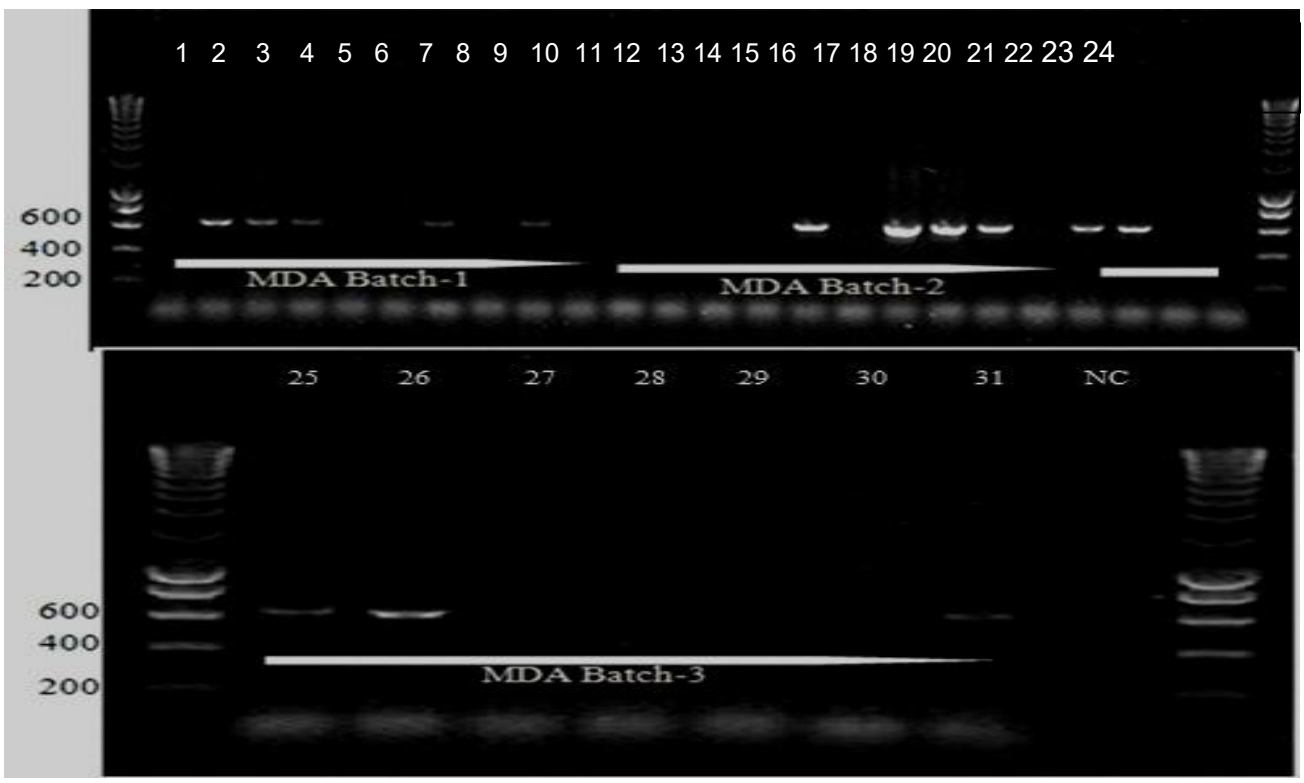

Fig. 3. PCR amplification of *BiP* gene using MDA product as template. NC indicates negative control; long narrow pentagon indicates amplification of *BiP* gene performed under different reaction volumes.

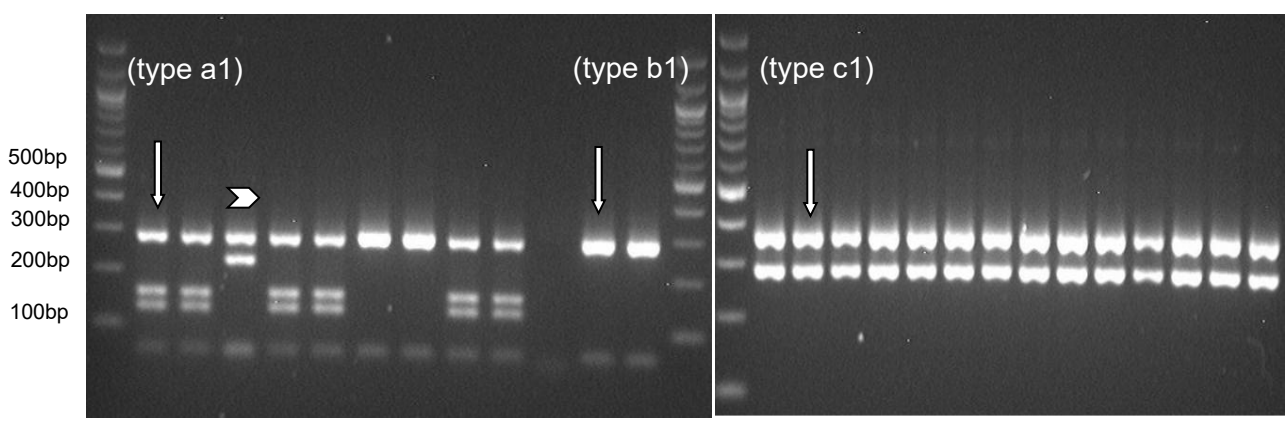

Fig. 4. RFLP of *BiP* gene amplified from the DNA of single nuclei, restricted with *AluI* enzyme. Different patterns shown by arrows, and a unique pattern with an arrowhead.

Table 2. Percentage of RFLP patterns in the cloned six single nuclei.

Enzyme used	SN. 1	SN. 2	SN. 3	SN. 4	SN. 5	SN. 6
AluI	c1= 96% unique pattern= 4%	a1=98% unique= 2%	a1= 72% b1= 26% unique pattern= 2%	c1= 100%	c1= 100%	c1= 100%

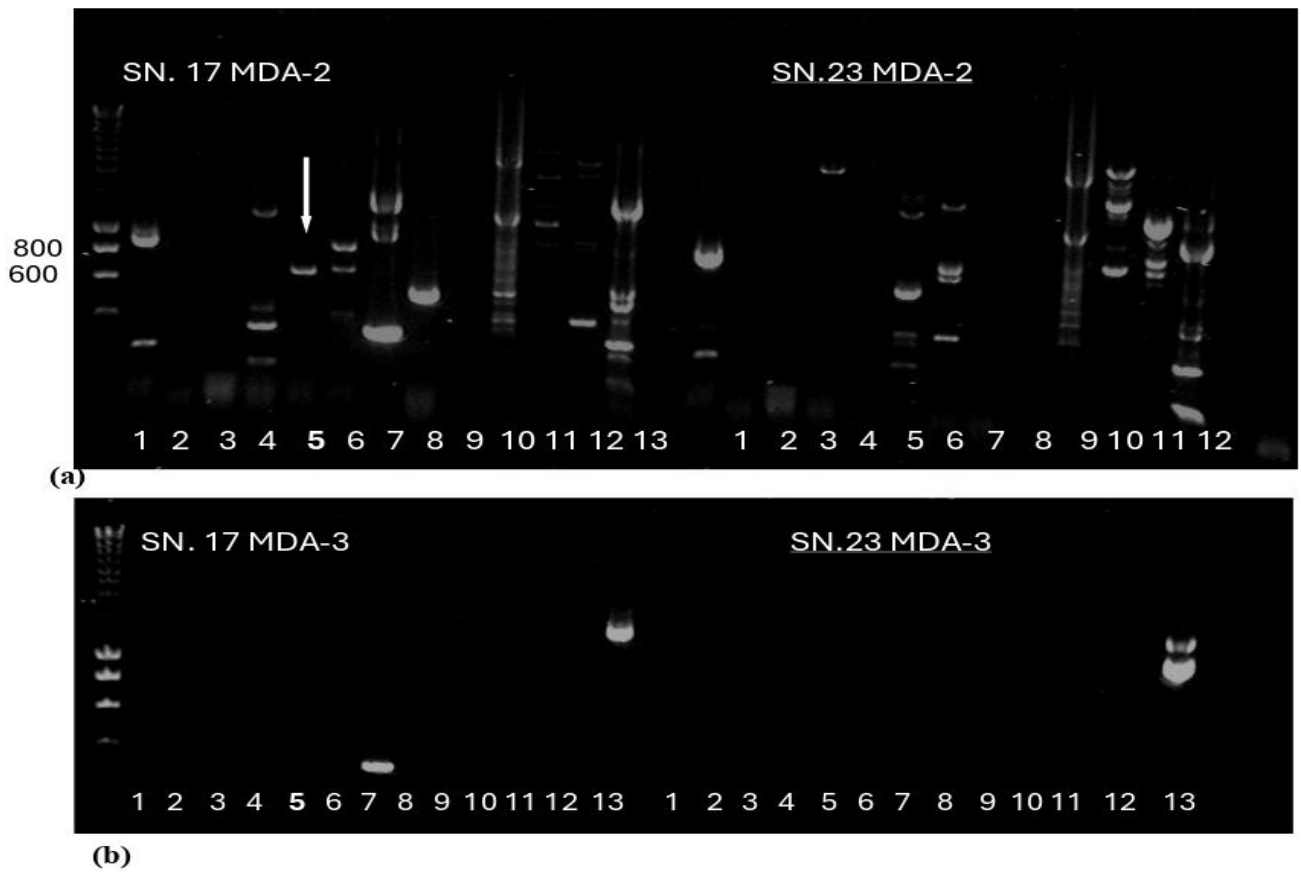

Fig. S1. Multiplex PCR based on MDA-2 (a) and MDA-3 (b) products obtained from 2 microdissected single nuclei using multiple glomus specific primers. Arrow indicates the only correct size band in lane # 5, NC indicates negative control.

Discussion

The isolation and genetic analysis of single nuclei from multinucleate AM fungi has been a significant technical challenge. In this study, we developed a robust method using Laser Capture Microdissection (LCM) to successfully isolate single nuclei from both spores and hyphae of *Glomus irregulare*, effectively overcoming the pervasive issue of autofluorescence that has hindered previous attempts (Bago *et al*., 1999; Bago *et al*., 1998; Bianciotto & Bonfante, 1992; Giovannetti *et al*., 1999).

A critical step was optimizing conditions to minimize autofluorescence (Ames *et al.*, 1982; Dreyer *et al*., 2006; Helber & Requena, 2008; Vierheilig *et al.*, 2001). For spores, gentle crushing and Propidium Iodide (PI) staining on LCM slides allowed clear visualization and successful catapulting of individual nuclei, as confirmed by pre- and post-catapult imaging. We found that growing hyphae directly on LCM slides in bicompartmental plates provided the best material for single nuclei isolation. A series of optimization tests (Table 1) established that this approach significantly reduced autofluorescence and promoted excellent hyphal growth, enabling clear differentiation of nuclei from debris. This direct-growth method eliminated the need for laborious manual transfer of hyphae.

Genetic analysis of the isolated nuclei provided key insights into the genome organization of *G. irregulare (*Jansa *et al*., 2002; Sokolski *et al*., 2010). MDA effectively amplified the minimal DNA from single nuclei, allowing for subsequent PCR (Gadkar & Rillig, 2005; Lee & Young, 2009; Mathimaran *et al*., 2008). We observed specific amplification from MDA-1 even with increasing reaction volumes (Fig. 3). Although, the DNA quantity increased in MDA-2 and MDA-3, but amplification became highly non-specific, highlighting the importance of using MDA-1 products for reliable analysis. Among the genes studied, the *BiP* gene was successfully amplified from approximately 50% of nuclei.

Cloning and sequencing of *BiP* from 6 single nuclei revealed a striking range of 1 to 7 allelic variants per nucleus (Fig. 5). This variation was corroborated by RFLP analysis, which showed "simple" and "mixed" patterns. Direct sequencing traces further confirmed that some nuclei contained a single *BiP* sequence while others harbored multiple variants. The presence of multiple alleles within a single nucleus suggests a minimum gene copy number and does not support a simple haploid, heterokaryotic model where each nucleus contains a single allele.

However, the evidence regarding the overall ploidy state remains ambiguous. Our multiplex PCR was only

able to amplify, at most, two genes (*BiP* and *ITS*) from any single nuclei, and this amplification was inconsistent. This is likely a technical artifact of working with minute starting DNA, preventing a definitive conclusion on whether the nuclei are haploid or polyploid. The inconsistent amplification means we cannot rule out the presence of other genes that failed to amplify.

Phylogenetic analysis provided a broader context for our findings. The Neighbour-joining tree of *BiP* sequences (Fig. 5) grouped the cloned alleles from single nuclei into four major clades (A-D), which correlated strongly with the RFLP patterns; alleles in clades A and B corresponded to RFLP patterns a1 and b1, while clade C alleles corresponded to the c1 pattern. Notably, the alleles we identified were nearly identical to those previously reported by (Kuhn *et al*., 2001.), from multinucleate spore extracts, indicating that our single nuclei approach captures the known genetic diversity of the isolate. In contrast, the *ITS* gene phylogeny was simpler but revealed significant intra-isolate diversity, with one sequence (SN.8) forming a distinct branch (Sedzielewska, 2011; Renker *et al*., 2003). This contrasts with the findings of (Pawlowska & Taylor, 2004), who reported limited variation in microdissected nuclei, and highlights the genetic complexity within a single fungal isolate.

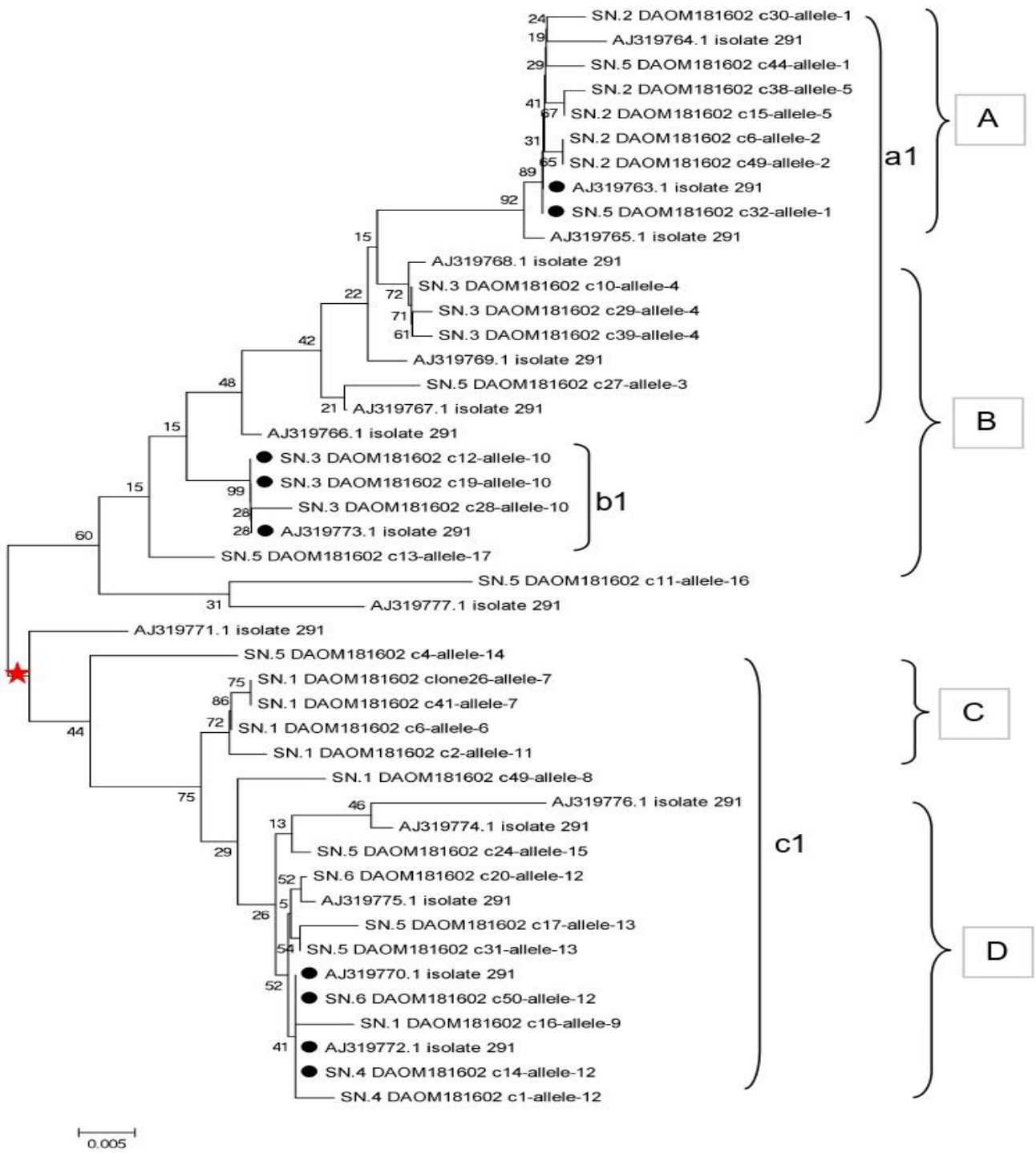

Fig. 5. Mid-point rooted Neighbour-joining tree showing multiple alleles of *BiP* gene from cloned single nuclei. Alleles from isolated single nuclei and Kuhn *et al.*, *BiP* sequences are shown; "SN" indicates single nucleus; Circles indicate alleles identical to those of Kuhn *et al*; "C" indicates a given clone; star indicates midpoint or half way between two distant group of sequences.

Conclusion

The LCM-based method provides a robust tool for isolating single nuclei from AM fungi, paving the way for more precise genetic studies. The integration of data from MDA, multiplex PCR, cloning, RFLP, and phylogeny demonstrates substantial genetic variation at the single-nucleus level for the *BiP* gene. However, the full resolution of the genome organization whether heterokaryotic, polyploid, or a more complex mix requires future studies with more comprehensive genomic analyses, such as single-nucleus sequencing, to overcome the limitations of low DNA template and PCR bias.

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