Molecular methods for research on arbuscular mycorrhizal fungi in India: problems and prospects

S. Ram Reddy*, Pavan K. Pindi and S. M. Reddy

Department of Microbiology, Kakatiya University, Warangal 506 009, India

Research on arbuscular mycorrhizal fungi (AMF), presently, is at the crossroads. Some aspects like their beneficial role in overall plant growth under diverse edaphic conditions, have been thoroughly investigated and further, there is no second opinion to the advocacy of their development as bioinoculants. However, the biosystematics, culturability and field performance evaluation of this group of fungi are still some areas lagging behind for want of suitable techniques, which have become hurdles for further progress of research. Having exhausted all the options to resolve the issues, the mycorrhizologists are looking towards molecular aspects of this group of organisms. rDNA region of the genome, with its variable and conserved regions, has been found to be an ideal location to offer solution to the issues. PCR-based techniques have become mandatory to obtain sufficient quantities of DNA, as these organisms are nonculturable and thus only a small quantity of DNA could be isolated from spores and infected roots. Employing DNA-based molecular markers, some advancements and success have already been achieved in areas like phylogeny, taxonomy and functional symbiosis. The next one or two decades are going to witness tremendous increases in molecular level research on this group of organisms, which hopefully will help resolve many issues being confronted today. This article reviews the molecular investigations carried out during the last two decades all over the world and also project areas for future research in India.

Keywords: Arbuscular mycorrhizae, molecular markers, PCR, rDNA.

AMONG the different types of mycorrhizae, arbuscular mycorrhizal fungi (AMF) that produce characteristic fungal structures, viz. arbuscules and vesicles in the cortex region of the infected roots are most common. Arbuscular mycorrhizal association is found in 80% of the plant species and most plant families except Cruciferae, Chenopodiaceae, Caryophyllaceae and Cyperaceae[1] are mycorrhizal. In addition to the widespread distribution of AMF throughout the plant kingdom, the association is geographically ubiquitous and occurs in plants growing in the arctic to tropical regions. The association also occurs over a broad ecological range from aquatic to desert environments[2]. A majority of the agricultural and horticultural crops and forest trees belonging to diverse families have been reported to be mycorrhizal. The beneficial effects of AMF on plant growth have been well documented by numerous workers. Phosphorus absorption, uptake of various other minerals, drought tolerance, growth hormones, resistance to soil-borne diseases, nodulation in legumes, transplant shock, plant establishment in disturbed and hostile soils are some of the beneficial effects conferred on the host plant by AMF symbiont[3].

Investigations on AMF pioneered by Mosse[4], and supported by Gerdeman and Nicolson[5] in the early sixties, were vigorously followed by numerous researchers throughout the world for the last more than forty years. Thousands of papers, hundreds of review articles and scores of monographs and proceedings were published from time to time. A large chunk of these papers is redundant and deals with multiple beneficial aspects of AMF on plant growth, stressing the need to develop AMF as bioinoculants for agriculture, forestry and horticulture. It is a gloomy situation to note that few papers appeared dealing with culturability, phylogeny, taxonomic aspects and nature of symbiosis of these organisms. Awful negligence of these aspects assumes greater significance in view of their unequivocal role in plant growth, increasing demand to use them as bioinoculants, constraints to improve them through biotechnological approaches and maintenance of quality control. The reasons attributed for this gross negligence can broadly be categorized into three types: first, AMF are obligate symbionts; hence they cannot be cultured in pure form in the laboratory and large-scale multiplication is not possible. Few workers have made an attempt to culture these organisms on auxenic medium. They have met with little success[6,7]. Till date, solution to this problem remains mystical. Second is the non-availability of reliable characteristics for phylogeny and taxonomy because these fungi do not produce sexual states and exist in imperfect states only. Establishment of phylogenetic relations, identifications and classifications are based on morphological features of the asexually produced propagules. In the absence of spores, the intraradical structures at best allow identification to the family level[8]. These criteria besides being insufficient also added confusion to the existing state. Hence, further

*For correspondence. (e-mail: sanditi_ramreddy@yahoo.co.in)

attempts based on phenetic characteristics are sure to add smoke to the fog. Lastly, identification of actual mycosymbiont confers major benefit to the host, because a variety of AMF spores are commonly encountered in the vicinity of the root. Further, it is possible that more than one AM fungus may colonize the roots simultaneously.

Problems associated with identification of different taxa based on spore morphology are brought forth elegantly[9]. Further, it was felt that molecular identification approaches have the potential to revolutionize our understanding of AMF. Molecular techniques based on DNA analysis seem to offer a wide range of advantages. The knowledge of mycorrhizal functioning at molecular level could be used for the sustainable improvement of crop plants. Besides the utility for application, molecular studies on AMF are necessary and interesting *per se*. Sound basic research about their evolution and the molecular crosstalk between symbiotic partners is the basis for future progress in the field of plant–microbe interactions[10]. Indeed, the need for molecular approaches was felt increasingly. Studies were initiated in the eighties and intensified with added vigour during the last two decades. Significant successes have also been achieved. India has made significant contributions to the field of arbuscular mycorrhizae. Different laboratories from various parts of the country have worked on distribution, ecology, symbiosis and biotechnological aspects of these fungi and as such considerable information got accumulated[11–16]. Few studies have also been made on taxonomic aspects of this group of fungi[17,18]. Though several methodologies have been followed in understanding the biology of these organisms, molecular marker-based studies are almost missing. A welcome feature in this respect is that recently a new AM fungal species has been described based on molecular details[19]. This article reviews the molecular approaches made on different aspects of AMF during the last few decades and affords insights for directions for future research in India.

Genetic diversity

Molecular analyses have become commonplace in the last two decades in order to get greater insight regarding the structure and function of different organisms and also to explore a range of questions that are being addressed[20]. Molecular approaches primarily rely upon exploitation of genetic variation[21]. Study of the AMF genome has been modest, considering the fact that these fungi possess large genomes compared to other zygomycetes, ranging from 0.13 to more than 1.0 pg DNA per nucleus[22]. Analysis of DNA base composition in nine glomalean species demonstrated a low GC content with high levels of methylcytosine[23]; furthermore, the genomes contain extensively repeated DNA sequences[24].

Molecular methods have been particularly successful for studying rDNA sequences from AMF[25–27]. Several investigators have reported that individual spores of AMF which are multinucleate, show a high level of genetic diversity in the internal transcribed spacer (ITS) region of the nuclear rRNA genes[28,29]. Ribosomal-based DNA sequence analysis has revealed genetic variation both within and between AMF species[30–32]. Further, the genes of this region are available in high copy number and possess highly conserved as well as variable sectors, which facilitate differentiation of taxa at different levels. The nuclear SSU rDNA sequences (16S-like) evolve relatively slowly and are useful for studying distantly related organisms, whereas the mitochondrial rDNA genes evolve more rapidly and can be useful at the ordinal or family level.

ITS are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITSI) and between the 5.8S and 28S rRNA coding regions (ITS2; Figure 1). Studies on restriction site variation in the ribosomal DNA (rDNA) in populations have shown that while coding regions are conserved, spacer regions are variable. These spacer sequences have high evolution rate and are present in all known nuclear rRNA genes of eukaryotes[33]. They are useful for phylogenetic analyses among related species and/or among populations within a species.

Molecular markers

Molecular research involving identification of genetic diversity demands sufficient quantities of genomic DNA. However, the biggest constraint in case of AMF is their non-culturability due to their obligate symbiotic nature. This road-block to obtain sufficient quantities of DNA for DNA-based molecular identification techniques was removed by introduction of polymerase chain reaction (PCR). PCR amplification of the desired part of the DNA has become a mandatory step in any molecular technique (Table 1). With PCR, it is possible to amplify the genome or part of it from a single spore, colonized root or soil sample, directly.

There are three main categories of molecular research on AMF, namely genes and their expression, genomics and genetic variation. Although gene cloning from AMF species has proved successful, genome size and structure could not be elucidated. Cloning of arbuscule related genes has been reviewed by Burleigh[34]. Zeze *et al.*[35] cloned *Eco*RI digests of DNA from *Scutellospora castanea* into pUC18 and about 1000 recombinant DNA clones were obtained. Further, Marianne *et al.*[36] constructed genomic libraries of *Glomus versiforme* and *Gigaspora margarita*.

In genomics, array technology is particularly powerful and involves synthesis of small filters (macro-arrays) or glass slides (micro-arrays) on which a comprehensive set of genes involved in specific metabolic function, or gene (genome) or transcript clones are spotted. Although preparation of arrays is expensive, subsequent processing of these arrays requires less time and material than a traditional library screen and provides information about considerably larger

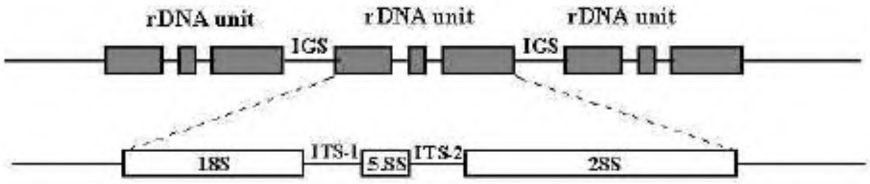

Figure 1. Location and details of rDNA genes. IGS, Intergenic space; ITS, Internal transcribed spacer.

Table 1. Details of some PCR-based molecular studies on AMF

Amplification region	Molecular marker	Primer/s	Target organism	Reference
SSU rDNA	PCR	VANS1	Glomales	109
Genomic DNA	PCR–RADP	OPA-02 and OPA-04 OPA-18 and P124 OPA-18 and P124	*Glomus versiforme, Gl. mosseae* *Gl. caledonium, Acaulospora laevis,* *Gigaspora margarita, Scutellospora gregaria*	39
SSU rDNA	PCR	VANS1 and NS21	*G. intraradices*	83
Genomic DNA	Competitive PCR	PO and M3	*G. mosseae*	46
ITS	PCR–RFLP	ITS1 and ITS4	*Glomus* sp., *Scutellospora* sp., *Gigaspora* sp.	41
SSU 1492′	PCR	NS71 and SSU1492′	*Gigaspora* sp.	75
Partial rDNA	PCR-partial	SS38 and VANS1 VANS1 VAGIGA	Roots and spores of AM, *Scutellospora* and *Glomus* Gigasporaceae	30
ITS1 and ITS2	PCR	ITS1 and ITS2	*G. margarita*	32
ITS	PCR	ITS1 and ITS4	*G. mosseae* and *Gigaspora margarita*	29
SSU rDNA	PCR–RFLP PCR-nested	LR1 and FLR2 FLR2-5.23 and FLR2-8.23 LR1-23.46	Subgroups of Glomales *G. mosseae, G. intraradices* *G. roseae*	45
28S rDNA	PCR–SSCPs	LSU-Primers	*Glomus* sp.	82
SSU rDNA	PCR	NS31 and AM1	*Glomus* sp.	43
SSU rDNA	PCR–SSCP Nested–PCR	VANS1 ITS, AM1	Subgroups of Glomales	60
ITS	PCR–RFLP	ITS1 and ITS4	*G. mosseae*	74
ITS	Nested PCR–SSCP	Eukaryotic universal primer *Glomus*-specific ITS primer	*Glomus* sp.	110
ITS	Nested-PCR	ITS 5 and ITS4	Glomeromycota (except Archaeosporaceae)	44
ITS	PCR	SSU-Glom/LSU-Glom1 ITS5 and ITS4	Major groups within Glomeromycota	44

PCR, Polymerase chain reaction; SSU rDNA, Smaller subunit ribosomal DNA (18 S); ITS, Internal transcribed spacer; RFLP, Restriction fragment length polymorphism; RAPD, Random amplified polymorphic DNA; SSCP, Single-strand conformation polymorphism.

number of genes at one time. To date, only one genome project on mycorrhizae has been completed[37].

Molecular research on exploration of genetic variation in AMF varies from systematics and taxonomy through development of tools for field sample identification[38]. This broad research area almost entirely relies on PCR amplification of rDNA sequences, with nuclear encoded small subunit (17S/18S rDNA) being the most common. However, mitochondrial SSU-rDNA (16S), nuclear encoded large subunit 28s RNA and nuclear encoded rDNA internal ITS are also increasingly exploited (Table 2). Random amplified polymorphic DNA (RAPDs) and microsatellites as molecular markers have been far less popular[39,40]. Amplified fragment length polymorphism (AFLP) analysis was also investigated. It is assumed that the analysis will provide a method for investigation of a broader range of loci from the genome than the single gene locus of rDNA work[20,41–44]. Availing the facilities and advantages with PCR, several investigators employed extended versions of the basic technique. Nested-PCR, a highly sensitive procedure that uses two sets of primers is being employed for monitoring the species of AMF and for determining their abundance in plants and soil[44,45]. Another version of PCR, competitive PCR, which co-amplifies the target template with an internal standard was also developed to quantify the AM fungus within the roots[46]. Simon *et al.*[47] coupled the techniques of PCR and SSCP (single strand conformation polymorphism) to detect sequence differences existing among AMF in roots collected from field.

Differential mRNA display (DD) has been used successfully to isolate fungal genes expressed during a func-

tioning symbiosis[48]. It offers an opportunity to compare simultaneously the mRNA transcripts of colonized and non-colonized root systems and at the same time allows for isolation of mRNA transcripts of genes being expressed within the fungus during a functional symbiotic relationship. This method combined with RT–PCR has been employed to detect symbiosis-related genes induced in arbuscular mycorrhizae[49]. Differential screening is based on differences in the concentration of nucleic acid species between two or more samples (i.e. mycorrhizal and non-mycorrhizal) and is aimed at isolating differentially transcribed mRNAs[50]. This powerful technique, extensively employed earlier in plant studies, has been used successfully to isolate novel plant and AM fungal genes differentially expressed during AM symbiosis[51,52]. Antibodies with fluorescent labels were also employed as protein markers in some investigations. Antibodies corresponding to fungal proteins have been utilized in an attempt to identify specific fungal species and/or to detect AMF in plant root and soil systems[53–55]. Isozyme patterns have also been used to identify AMF spores and even mycelium in symbiotic structures[56]. Through this technique, it was possible to distinguish different fungal isolates, but it has not been possible to outline any distinct level of similarity at the species level[57].

Phylogeny

The fossil record and molecular data show that evolutionary history of AMF (Glomales) dates back at least to the Ordovician, coinciding with the colonization of the terrestrial environment by the first land plants. The earliest evidences for AM symbiosis were fossil arbuscules found in specimens of the early land plant *Algaophyton* from the Devonian Rhynie Chert[58]. Fossil spores and hyphae resembling those of present glomalean fungi had been detected in plant materials from this site[59]. It has been observed that Ordovician spores and hyphae are remarkably similar to today's *Glomus* type[60]. These findings prove that glomalean fungi were present at a time when the land flora comprised of plants of the bryophyta class, lending strong support to the notion that AM fungi were instrumental in the succession of early land plants.

Of special interest in the phylogeny of AMF is *Geosiphon pyriformis*, a fungus that lives symbiotically with cyanobacterium, *Nostoc punctiforme*. Mollenhauer[61] first speculated that *Geosiphon* might be related to the genus *Glomus* and therefore AMF. A subsequent study comparing morphological and ultrastructural details of *Geosiphon* spores with those of AMF revealed striking similarities between the two[62]. SSU rRNA sequence analysis also further confirmed this relationship[63] and a monophyletic clade with them was suggested. The phylogenetic tree showed that the AMF include *Geosiphon* as a basal branch, and form a distinct clade not clustering with other zygomycetes. Based on the increasing amount of molecular data, the natural relationships within the AMF are now becoming clear[64]. Recently, two new families, the Archaeosporaceae and Paraglomaceae were described, representing ancestral lineage within the AMF[65]. Paraglomaceae is indicated as the most ancestral lineage within the AMF in phylogenetic analysis, but with only low bootstrap support for this. Based on SSU rRNA sequence analysis, Schüβler *et al.*[66] put forward valid arguments to justify creation of a new phylum, Glomeromycota to accommodate monophyletic AMF and related fungi like *Geosiphon*. In the light of information available especially at DNA level, it has been felt that the definition of Glomales will have to be modified to accommodate non-mycorrhizal fungi, because it is likely that the transition from ectomycorrhizal to other nutrition modes occurred more than once, as has already been shown for ectomycorrhizal fungi[67]. Redecker *et al.*[68] using both new and existing 18S rRNA sequence data, confirmed that at least five species of glomalean fungi lie outside the previously defined families and diverged early in the evolution. It is also further suggested that dimorphic spores are the ancestral state for the order and that one or the other morphology was lost in various lineages. Dataset comprising 5.8S rDNA showed that *Scutellospora castanea* most likely originated from ascomycetes.

Thus, molecular data available on different taxa of AMF have allowed the redefinition of different taxa and have established new phylogenetic relationships also, to deeply divergent lineages within Glomales[60,69].

Table 2. Primers for amplification of fungal nuclear ribosomal RNA genes

rRNA	Gene primer[a]	Product size (bp)[b]
Nuclear small		
NS1	5′ GTAGTCATATGCTTGTCTC	555
NS2	5′ GGCTGCTGGCACCAGACTTGC	
NS3	5′ GCAAGTCTGGTGCCAGCAGCC	597
NS4	5′ GTTCCGTCAATTCCTTTAAG	
NS5	5′ AACTTAAAGGAATTGAGGGAAG	310
NS6	5′ GCATCACAGACCTGTTATTGCCCTC	
NS7	5′ GAGGCAATAACAGGTCTGTGATGC	377
NS8	5′ TCCGCAGGTTCACCTACGGA	
Nuclear ITS		
ITS1	5′ TCCGTAGGTGAACCTGCGG	290
ITS5	5′ GGAAGTAAAAGTCGTAACAAGG	315
ITS2	5′ GCTGCGTTCTTCATCGATGC	290
ITS3	5′ GCATCGATGAAGAACGCAGC	330
ITS4	5′ TCCTCCGCTTATTGATATGC	580
ITS1F	5′ CTTGGTCATTTAGAGGAAGTAA	700
ITS4B	5′ CAGGAGACTTGTACACGGTCCAG	
TW13	5′ GGTCCGTGTTTCAAGACG	1200

[a]All odd-numbered primers are 5′ primers; even numbers indicate 3′ primers. Sequence are written 5′–3′.

[b]Product sizes are approximate based on rRNA genes of *Saccharomyces cerevisiae*.

Table 3. Classification of order Glomales[70]

Order:	Glomales Morton & Benny		
	Suborder:	Glomineae Morton & Benny	
		Family:	Glomaceae Pirozynski & Dalpe
			Genus: *Glomus* Tulasne & Tulsane
			Genus: *Sclerocystis* (Berkeley & Broome) Almeida & Schenck
		Family:	Acaulosporaceae Morton & Benny
			Genus: *Acaulospora* (Gerdemann & Trappe) Berch
			Genus: *Entrophospora* Ames & Schneider
	Suborder: Gigasporineae Morton & Benny		
		Family:	Gigasporaceae Morton & Benny
			Genus: *Gigaspora* (Gerdemann & Trappe) Walker & Sanders
			Genus: *Scutellospora* Walker & Sanders

Taxonomy

Around 150 known species of AMF assigned to six genera are included in class Zygomycota, single order Glomales (Table 3). This order consists of the suborder Glomineae, with two type families Glomaceae (*Glomus* and *Sclerocystis*) and Acaulosporaceae (*Acaulospora* and *Entrophospora*) and the suborder Gigasporineae, with the type family, Gigasporaceae (*Gigaspora* and *Scutellospora*[70]. Apart from two new families, Archaeosporaceae and Paraglomaceae, a new family Diversisporaceae could be created based on full-length SSU rRNA gene sequences[71]. Identification of taxa in the Glomales has relied extensively on the morphology of the spores. The disadvantage of morphological characters is that they hide all of the diversity that occurs within strains of the species. It has been stressed that new genetic and molecular characters must be searched for fungal systematics and they must offer answer at different resolution levels from species to genus and family.

Here are a few examples wherein molecular studies resolved the controversies arising from morphological observations. Spores of *Acaulospora gerdemanii* and *Glomus leptotrichum* were observed to be formed on the same hypha, therefore, the two spore types were termed 'synanomorphs'. However, their 18S rDNA sequences proved that this dimorphic organism neither belongs to *Glomus* nor to *Acaulospora*, nor to the any established families[72]. Instead, it belongs to one of several ancestral, deeply divergent lineages within the Glomales[68]. Similarly, the dispute of two more dimorphic spore-forming fungi, one forming spores of acaulosporoid and another producing *Glomus*-type spores was also settled. The genus *Glomus* had been inadequately defined and represents a conglomerate of fungi that are morphologically hard to separate and which have genetic distances as large as those between the AMF families Gigasporaceae and Acaulosporaceae. In the light of the SSU rDNA sequence analyses, proposals for new classification of AMF are being seriously thought of[73].

The intraspecific diversity of *G. mosseae* revealed by vegetative incompatibility test was confirmed by total protein profiles and ITS–RFLP profiles[74]. Studies conducted by Antoniolli[29] using rDNA ITS sequences demonstrated that a high degree of variation exists in natural populations of *G. mosseae*, perhaps necessitating the splitting of this species. Grouping of *Gigaspora* based on six nucleotide-long molecular signature was confirmed by SSU analysis and isozyme profiles[75]. Analysis of AFLP fingerprints of isolates from three genera, namely *Glomus*, *Gigaspora* and *Scutellospora* justified the placement of *Gigaspora* and *Scutellospora* in the same family.

Analysis of the 16S ribosomal subunit of *Glomus sinuosum* and *Sclerocystis coremioides* showed that these two species are the closest relatives and fall within a monophyletic clade. This justified the transfer of all the species of *Sclerocystis* into *Glomus* based on the information that formation of complex sporocarps is an advanced character of *Glomus* species, but the sporocarpic trait is not sufficiently unique to group these species into a separate genus *Sclerocystis*. *Entrophospora infrequens* has always presented an enigma. Clapp *et al.*[76] reported that single spores of *E. infrequens* contain rDNA genes from different species of *Glomus* and Gigasporaceae separately or even in combination. It is presumed that *E. infrequens* does not belong to the typical AMF and may not be mycorrhizal at all. Published ITS sequences of *E. contigua* and *Acaulospora lacunosa* reveal their similarity to basidiomycetes than to members of Acaulosporaceae.

As rightly pointed out by Clapp *et al.*[77] 'all that glistens is not necessarily glomalean', molecular analysis has also opened up new controversies. Redecker *et al.*[78] created a sensation by reporting that *Scutellospora castanea* borrows sequences of ITS from ascomycetes.

Identification and quantification in roots and soil

A major problem in studies on AMF is that the fungi cannot be easily identified within roots. Traditionally, spores were used to determine which species was present and active in the root. However, it is well known that spore formation is highly dependent on physiological parameters and often not correlated with root colonization. Further, spore numbers

do not necessarily reflect the extent to which different fungal species colonize the root system[79]. Species determination from spores also has many pitfalls. Since the fungi are obligate symbionts, the actual fungi colonizing roots are likely to be functionally important.

Fungal structures can be detected in roots, but their morphology is in general similar and not sufficient for identification at species level. In order to understand relationships between fungal populations in soils and in roots, it is necessary to be able to identify species or isolates in the different compartments of the ecological niche. Therefore, molecular tools that would allow species identification of AMF *in situ* independent from spore formation were developed. In this regard, PCR is an indispensable tool. A few authors designed PCR primers from random sequences, while most used a systematic approach, comparing sequences of known function (Table 4). A randomly amplified fragment from *G. mosseae* was used to generate specific primers for this species[80] and to establish a quantitative PCR system to measure root colonization[46]. Clapp *et al.*[79] performed the first molecular study of a field population of AMF. Their PCR results for *Acaulospora* and *Scutellospora* species were mainly in agreement with spore counts, but interestingly, there was a strong discrepancy between strong root colonization by *Glomus* and absence of sporulation. An influence of canopy type on this cryptic colonization was also detected. Moreover, concurrent colonization of the root by all the three genera was demonstrated. These results reiterated the need to apply molecular methods in order to obtain more reliable data of AMF populations in roots.

Many authors targetted different parts of the ribosomal genes, for example, the large subunit[81,82] or the ITS[9,27]. In some cases, nested-PCR procedure was employed to circumvent the problem of PCR inhibitors present in many root samples. These authors designed primers specific for different species to study AMF populations in greenhouse as well as field-collected samples. These studies also revealed that root fragments of even 1 cm are colonized by more than one fungus. A synergestic interaction within the root was also suggested. Kjøller and Rosendahl[82] combined nested-PCR and SSCP and differentiated isolates of *G. mosseae*, *G. caledonium* and *G. geosporum* side by side in colonized roots.

Jacquot *et al.*[45] suggested that nested-PCR with taxon-specific primers could be used in order to monitor the effect of abiotic or biotic factors in different compartments of the root. Using the primer combination NS31–AMI, which amplifies SSU rDNA gene sequences of all three well-established families of the *Glomales*, Daniell *et al.*[43] amplified and developed restriction fingerprints of *Glomus* species colonizing arable crops. Through these studies, they were able to monitor AMF diversity in soil. A simple method to detect and quantify *G. intraradices* in roots of different plant species was developed[83] employing taxon-specific primer VANSI paired with universal primer NS21. Some investigators demonstrated that competitive PCR can be used for the quantification of *G. mosseae* colonization within roots with accuracy and sensitivity[46]. Competitive PCR provides information on the relative abundance and spatial distribution of a mixed population of AMF within a root system, allowing the study of interspecific interaction. Employing PCR, RFLP and sequencing, Helgason *et al.*[84] compared the diversity of AMF between a woodland and adjacent arable sites. All these studies demonstrate the power of molecular identification methods to elucidate the species composition of active AMF communities.

Table 4. Primers used to amplify LSU rDNA sequences from mycorrhizal roots[72]

LSU 0061[1,2]	5′ AGCATATCAATAAGCGGAGGA
LSU 3f[1]	5′ AGTTGTTTGGGATTGCAGC
LSU 4f[3]	5′ GGGAGGTAAATTTCTCCTAAGGC
LSU 6f[1]	5′ AAATTGTTGAAAGGGAAACG
LSU 9f[1]	5′ ATTCGTTAAGGATGTTGACG
LSU 5r[1]	5′ CCCTTTCAACAATTTCACG
LSU 7r[3]	5′ ATCGAAGCTACATTCCTCC
LSU 8r[1]	5′ GGGTATCCGTTGCAATCCTC
LSU 0599[2]	5′ TGGTCCGTGTTTCAAGACG
LSU 0805[1,2]	5′ CATAGTTCACCATCTTTCGG

[1]Primers used for sequencing.
[2]Primers used for primary PCR amplification.
[3]Primers used for the second nested-PCR amplification.

Functional symbiosis

Understanding how AM symbiosis is established and functions is a key issue in plant development. Therefore, knowledge of the biology of the fungal partner is important. Since the advent of molecular biology and innovative techniques, several problems encountered in the past could be resolved easily. Molecular analyses of the fungus in the asymbiotic and symbiotic stages of development are underway.

In AM symbiosis, both partners display activities during their interaction, which they do not exhibit when they are living alone. This is obvious from the obligate biotrophic nature of AMF. In order to use AMF efficiently in plant production systems, it is necessary to investigate the molecular biology of symbiosis. This comprises the genetic basis of both partners, the expression patterns of their genes during symbiosis and how these patterns are modified and regulated by signals from the respective partner and from the environment. Such studies were initiated eight years ago[85–88]. The major shortcoming of the analysis of AMF gene expression is the obligate symbiotic growth of the fungus and lack of mycorrhizae formation in *Arabidopsis thalliana*. This limits the amount of fungal material available, which can only be obtained either from spores or from extraradical hyphae. In addition, the quantity of living AMF

material inside the root is small in comparison with other plant–microbe interactions. Approximately just 1% of the mRNA extracted from highly colonized roots belongs to the fungal partner and this hinders the discovery of low-expression fungal genes[34]. However, with the help of PCR technology, many of the limitations have been overcome and minute amounts of RNA have served as templates for PCR-based cloning, RNA accumulation analysis or cDNA library construction. One strategy for the identification of functions involved in AM symbiosis is the identification of differentially expressed genes. Differential expression studies on AMF partner genes were first carried out using presymbiotic mycelium from *G. mosseae*[89]. A fungal cDNA fragment encoding the homologue of the fatty acid oxidase FOX2 from yeast and human beings was isolated. The corresponding gene, GmFOX2, was found down-regulated under the influence of the bacterium *Bacillus subtilis*. Another gene isolated was *GmTOR2*, that encodes a homologue protein of yeast TOR2, which is involved in control of the cell cycle and actin cytoskeleton[90].

Other fungal genes have also been isolated by methods that compare RNA accumulation patterns between control roots and mycorrhizas[91]. A cDNA fragment from *G. mosseae* with no similarity to any known sequence was obtained by differential RNA display analysis of *Pisum sativum* wild type mycorrhiza vs a mycorrhiza mutant displaying aborted arbuscule development[92]. Efforts are being made to clone the entire gene in order to understand its function. A phosphoglycerate kinase (PGK) cDNA fragment from *G. mosseae* was identified in tomato mycorrhizas[48]. Further studies revealed a significantly higher accumulation of the encoded protein during symbiosis compared with presymbiotic development.

The arbuscule is thought to be the major site for nutrient exchange between the two symbiotic partners. Therefore, mechanisms should be present to facilitate the transport of these compounds. Utilizing a PCR cloning approach based on the use of highly degenerative primers, five partial genomic clones encoding P-ATPases, the major transport proteins in arbuscules controlling ionic and molecular transport processes, were isolated from *G. mosseae*[93]. Another nutritionally-important AMF gene i.e. the *nr* gene (nitrate reductase gene) was identified in DNA from spores of *Glomus* using a PCR-based strategy[94]. Expression analysis of *chs* genes in *G. margarita* demonstrated that *Gimchs 1* and *Gimchs 3* transcripts were detected during AMF root colonization, while none of these transcripts was detected during spore germination. These results have demonstrated that chitin synthesis in AMF is complex and involves multiple *chs* genes with transcriptional and/or post-transcriptional regulation[95]. DD technique was successfully used to isolate fungal genes expressed during a functional symbiosis[91]. With the same technique, a differential regulation of genes in AMF during symbiosis was also demonstrated.

In mycorrhizal symbiosis, growth and differentiation of the plant root and fungal hyphae must be tightly coordinated. This requires a reciprocal recognition either via the exchange of diffusible signals or a direct cell-to-cell interaction. Towards the characterization of these processes at the molecular level, abnormal AM phenotypes have been identified in mutant plant lines. These include both defective AM phenotypes (Myc^-) and enhanced mycorrhizal phenotypes (Myc^+)[96,97]. Marsh and Schultze[98] discussed the current state of knowledge concerning the different aspects of Myc^- mutants.

Harrier and Millam[99] felt the need for development of transformation technology for AMF that would greatly aid fundamental biological studies of these fungi in the symbiotic and asymbiotic stages of their life cycle, enabling the targetted upregulation or silencing of specific genes and evaluation of the subsequent effects. They suggested biolistic transformation as a means of studying gene function.

Problems in the use of molecular techniques on AMF

The molecular investigations on AMF have started yielding promising results in some cases and for others, solutions are in sight. Still, several obstacles are to be removed in order to achieve the expected results. Some of the problems are unique to this group, which are not encountered even in related group of organisms. For example, in case of ectomycorrhizae, considerable success has been achieved with these studies; however the same is not true with AMF[100,101], and the reasons for this difference have been amply illustrated[9].

AMF mycelium is embedded deeply within the roots and therefore DNA extraction is a problem. AMF-specific primers are required; otherwise numerous pathogenic and saprophytic fungi will be co-detected. To design one primer for all glomalean fungi excluding plants and other fungi has proven to be difficult. The problem can be avoided using group-specific primers, whose design demands meticulous planning. One piece of root can be colonized by different AMF, and multiple component colonizers have to be separated by cloning the PCR products[79,81]. rDNA of AMF is highly polymorphic in single spores, when compared to many other fungi wherein the variable rDNA sequences (e.g. ITS) are often identical within a species[102]. In addition, identical ITS do not automatically mean that the two fungi are conspecific, only that they are closely related.

The lack of clear species concept and polymorphism of currently used marker genes make it difficult to clearly define AMF species by molecular methods. It has also been felt that a single genetic locus (e.g. rDNA) does not provide clear differentiation of genetic variation intra-species and inter species. This problem was addressed by establishing species concepts based on gene geneology. A concept termed genealogical concordance phylogenetic species

recognition (GCPR) was devised, which detects gene flow caused by interbreeding, and different species are characterized by genetic isolation[103]. This concept has been successfully applied to a wide range of ascomycetes and basidiomycetes. However, it remains to be seen as to whether phylogenetically based species concepts like this are applicable to AMF, because it has been recently reported that nuclei in coenocytic mycelium of AMF are genetically different. The GCPS concept is applicable to truly clonal lineages.

Investigations carried out on *G. coronatum* suggest that AMF spores contain ribosomal genes from different morphospecies. In such cases, the possibility of heterogeneity of the spore material rather than mixed populations of nuclei is not ruled out. Therefore, more work needs to be done to elucidate how the supposed heterogeneity of nuclei is maintained in the mycelium. When phylogenetic analyses show that a sequence falls within a clade of sequences from defined morphogenesis species, it can be identified rather safely. This is only possible in a few cases, where multiple DNA sequence clones are available from isolates of a defined morphospecies.

Perspectives and conclusion

Notwithstanding certain constraints, molecular technologies are definitely making inroads into the problematic and unapproachable areas of AMF. Many exciting and unforeseen details are at anvil. The next one or two decades are going to witness tremendous activity in this area and one can visualize a clear and holistic picture of AMF. The current researchers are likely to provide direction to future research. Following are some of the areas for future line of research.

A new interesting target for PCR cloning is the enzymes involved in lipid biosynthesis. This assumes significance in view of the recent findings that AMF are unable to grow asymbiotically due to the lack of storage lipid biosynthesis[75]. The construction of cDNA libraries followed by random sequencing to obtain expressed sequence tags (ESTs) and screening cDNA assays will also be useful tools for analysis of AMF gene expression. These libraries will form a starting material for more systematic work on gene expression of AMF. For example, they can be sequenced to obtain ESTs. Selection of model isolate is necessary for this type of work. It is also recommended to consider at least one representative from each suborder for the systematic sequencing and expression analysis[10]. As an alternative to rDNA gene analysis, Franken and Requena[10] suggested a method to measure functional biodiversity in ecosystems. This approach has several advantages in that (i) a glomalean-specific primer could be designed; (ii) it detects not only the presence of isolates but their activity, and (iii) it is also useful for formulation and control inocula for field inoculations. Hence, such types of approaches may have to be given top priority in future. Proteome analysis followed by application of reverse genetics to identify genes of interest in AMF is also underway[104,105]. Another emerging technology is laser capture microdissection, which utilizes a laser to remove the contents of the cell of interest[106]. Transcripts from these preparations could be used to generate arbuscule cDNA libraries. Bioimaging using confocal microscopy is another technology recently applied to mycorrhizal research[107], which, when coupled with developing AM transformation techniques[108], may help to link gene expression studies with structural aspects of symbiosis.

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