Madras Agric. J. 90 (10-12) : 579-588 October-December 2003

https://doi.org/10.29321/MAJ.10.A00140

Nucleic acid studies in the phylogenies and strain identification of cyanobacterial taxa

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Abstract: Cyanobacteria though traditionally have been distinguished on the basis of phenotypic properties, structure and physiology, employment of molecular biological methods results in greater phylogenetic resolution than the use of morphological data alone. Recently, molecular sequence analyses have been viewed as highly superior to structure-based phylogenies in cyanobacteria. Restriction fragment length polymorphisms (RFLP) analyses have been used to evaluate intergeneric, interspecific and intraspecific variation in algae. Restriction fragment length polymorphisms have been used extensively as an efficient DNA fingerprinting method to identify symbiotic cyanobacteria. The RAPD technique in conjunction with PCR has been employed to identify cyanobacterial groups to the strain level of classification. The choice of primers with high G+C content provided good DNA profiles at a low concentration. Neilan (1995) reported the use of RAPD-PCR to generate uniqueness in phylogenetic analysis of toxic cyanobacteria. Lakshmanan and Kumar (2003) employed RAPD-PCR technology using decamer and tandem repeat primers to identify the cyanobacterial cultures of the composite inoculum upto strain level by detecting the genetic heterogeneity. One area in which a significant application has been developed recently is in the phylogenetic characterization of natural cyanobacterial communities by 16S rRNA sequence analysis. The use of DNA-DNA hybridizations as a tool in cyanobacterial phylogeny was pioneered by Stam (Stam and Venema, 1975 and Stam, 1980). ITS sequencing has been used commonly in intrageneric phylogenetic studies of algae. Peters (1997) used ITS sequences to compare species within the widespread algal family Desmarestiaceae.

Key words : Algae, Cyanobacteria, Morphology, Nucleic acid, Phylogeny, Polymorphism, Taxa.

Introduction

The phylogenies of various cyanobacterial cultures were extensively explored only with the use of morphological characters prior to the development of technologies that have made the acquisition of molecular sequences relatively easy. Ecologists who identify species from natural collections typically rely upon the species concept, often using pigmentation and structural information to deduce ecologically important algal functions. Therefore, taxonomists have historically used morphological features to define and to identify cyanobacterial taxa, including variations in cyano-bacterial thallus structure, which include occurrence as unicells, colonies, unbranched filaments, or branched filaments. In many cases such procedures may be justified, but sometimes significant physiological attributes of algae (including nitrogen metabolism, toxicity, vitamin require-ments, bioluminescence and growth dependencies) can vary considerably among isolates of the same species. Frequently the physiological behaviours considered typical of algal species have been defined for only one isolate and thus cannot be reliably attributed to all members of the same morphological species. In addition, some morphological species have been observed to undergo seasonal succession at the clonal level, *i.e.* physiological behaviour may not be consistent throughout the year. Intraspecies variation in physiological behaviour is thought to be more common and ecologically significant than realized (Wood and Leatham, 1992).

Recently, molecular sequence analyses have been viewed as highly superior to structure based phylogenies because the former offered many more characters, which were thought to be less subject to the confounding effects of parallel or convergent evolution. Some workers suggested that the use of structural characters to deduce phylogenies, which would in turn

Table 1. Generation of RAPD-PCR products for different cyanobacterial strains of composite culture inoculum using decamer primers of high G+C content

Primer	Sequence	G+C content	RAPD products generated by cyanobacterial strains								Total no of RAPD products
			A	B	C	D	E	F	G	H	
CRA 22	CCGCAGCCAA	70	3	4	3	3	4	3	2	2	24
CRA 23	GCGATCCCCA	70	4	5	4	5	5	2	2	2	29
CRA 25	AACGCGCAAC	60	2	1	2	3	1	3	3	6	21
CRA 26	GTGGATGCGA	60	2	3	2	1	1	4	4	5	22
OPA 8	GTGACGYAGG	60	3	4	3	4	5	4	1	3	27
OPA 11	CAATCGCCGT	60	7	6	6	6	5	2	5	6	43
OPA 13	CAGCACCCAC	70	3	5	1	1	5	3	4	5	27
Total			24	28	21	23	26	21	21	29	193

A : *A.azollae* MPK-SK-AM 24
B : *Anabaena variabilis* SA_0
C : *Anabaena* GG-SK-AT
D : *Nostoc muscorum* DOH
E : *Nostoc* RS 60-ST2
F : *Nostoc* GG-SK-AT
G : *Westiellopsis* TR5-ST3
H : *Westiellopsis* GG-SK-AT

Table 2. Generation of rep-PCR products for different cyanobacterial strains of composite culture inoculum using long repetitive sequence primers

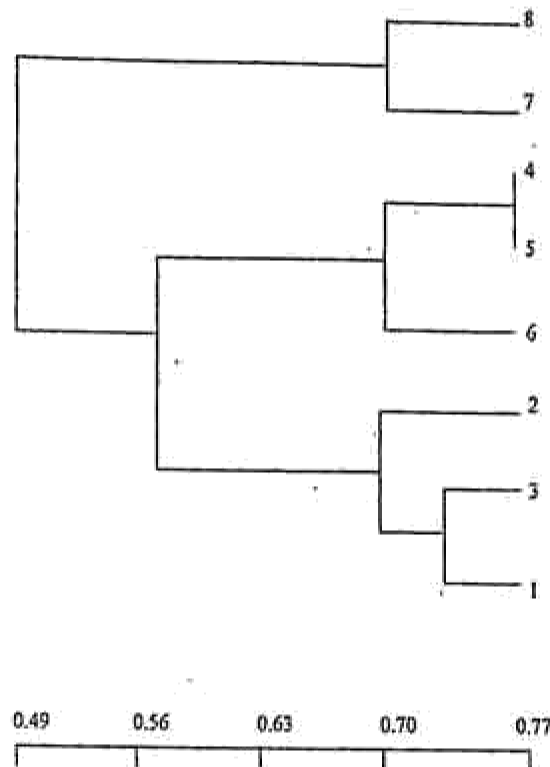
Primer	Sequence	rep-PCR products generated by cyanobacterial strains								Total no.of rep-PCR products
		A	B	C	D	E	F	G	H	
LTRR 1	3'-CAAAATTGATTGTTTTTAGG-5'	1	7	2	3	7	5	7	2	34
LTRR 2	5'-CTATCAGGGATTGAAAG-3'	3	3	2	4	4	4	5	3	28
LTRR consensus	5'-GTTTAACTAACAAAAATCCCTATC AGGGATTGAAAG-3'	3	1	5	1	1	2	2	2	17
Total		7	11	9	8	12	11	14	7	79

A : *A.azollae* MPK-SK-AM 24
B : *Anabaena variabilis* SA_0
C : *Anabaena* GG-SK-AT
D : *Nostoc muscorum* DOH
E : *Nostoc* RS 60-ST2
F : *Nostoc* GG-SK-AT
G : *Westiellopsis* TR5-ST3
H : *Westiellopsis* GG-SK-AT

Table 3. Generation of RAPD-PCR products for different cyanobacterial cultures by multiplex reaction using dual primers

Cyanobacterial strain	RAPD products of dual primer		Percentage of finger printing	
	CRA 25&26	OPA 8&11	CRA 25&26	OPA 8&11
Anabaena azollae-MPK-SK-AM24	3	5	10.71	20.0
Anabaena variabilis SA_0	2	3	7.14	12.0
Anabaena GG-SK-AT	5	5	17.85	20.0
Nostoc muscorum DOH	5	2	17.85	8.0
Nostoc RS-60-ST2	6	2	21.42	8.0
Nostoc GG-SK-AT	5	3	17.85	12.0
Westiellopsis TR5-ST3	2	5	7.14	20.0
Total	28	25	100	100

Fig.1. Phylogenetic relationship (Dendrogram) between cyanobacterial strains of composite culture inoculum



1. *Anabaena azollae - MPK-SK-AM 24*
2. *A. variabilis* SA_0
3. *Anabaena GG-SK-AT*
4. *Nostoc muscorum DOH*
5. *Nostoc RS-60-ST2*
6. *Nostoc GG-SK-AT*
7. *Westiellopsis TR5-ST3*
8. *Westiellopsis GG-SK-AT*

be used to understand character evolution, represented circular logic and should thus be avoided (Swofford *et al.* 1996). While employment of molecular sequence methods has not resulted in widespread overturn of previous evolutionary hypotheses based upon structural data (Moritz and Hillis, 1996), its use often results in greater phylogenetic resolution than the use of morphological data alone. In the cyanobacteria, the use of molecular methods to study the genotypic relationships and strain identification is very much underway and in this paper various methods based on nucleic acid analysis for strain identification in cyanobacteria are reviewed.

1. Analysis of nucleic acid

1.1. Restriction fragment-length polymorphisms

Restriction fragment-length polymorphisms (RFLP) estimate DNA sequence divergence by detecting variation due to nucleotide base substitutions, deletions, or insertions that have occurred over the course of evolutionary history. RFLP markers have proven to be a reliable and highly informaive tool for characterizing genetic diversity. Among the various molecular markers developed, RFLPs were the first to be used in human genome mapping and later they were adopted for plant genome mapping.

RFLP analyses have been used to evaluate intergeneric, interspecifc and intraspecific variation in algae (Manhart and McCourt, 1992). A good example of such a study is the RFLP analysis of chloroplast DNA of U.S. coastal *Codium* species and subspecies performed by Goff *et al.* (1992). Disadvantages of RFLP analyses are that they are labour-intensive and require relatively large amounts of purified DNA. Further, some exports recommend that RFLP data could not be used to infer phylogenies because the assumption of character independence is violated. For example, if a new restriction site were

to evolve between two pre-existing sites in a particular taxon, this would change RFLP patterns such that it and a related species may show no fragments in common even though they may share the original restriction sites (Swofford *et al.* 1996).

Restriction fragment length polymorphisms have been used extensively as an efficient DNA fingerprinting method to identify symbiotic cyanobacteria (Nierzwicki-Bauer and Haselkorn, 1986). The widespread occurrence of the heptamer repeats in the *Nostoc* strain suggests that they may be useful diagnostics in the future. However the taxonomic value of southern hybridization by using the randomly repeated sequences as a phylogenetic tool may be restricted to the identification of identical strains (Mazel *et al.* 1990).

Planktonic filamentous cyanobacterial strains belonging to the genera *Anabaena, Nodularia, Nostoc* and *Aphanizomenon* were characterized by SDS-PAGE of whole cell proteins and PCR/ RFLP of the 16S rRNA gene (Lyra *et al.* 1997). They reported that RFLP of 16S rRNA is advantageous because the sequencing of 16S rRNA gene is tedious and time consuming especially when one needs to classify a large number of strains.

In the case of angiosperm symbiosis, twelve free-living cultures derived from symbionts of eight *Gunnera* species were characterized by Zimmerman and Rosen (1992) using the RFLP analysis with heterologous probes from *Anabaena* sp. strain PCC 7120 and the immunostaining of the protein profiles.

1.2. Randomly amplified polymorphic DNA (RAPD)

Another approacht to the study of speciation, as well as strain identification is the use of randomly amplified polymorphic DNAs (RAPDs) Advantages of using RAPDs include the fact that only small amounts of DNA are required and procedures can also be done with relative ease, speed and low cost.

The RAPD technique in conjunction with PCR has been employed to identify many organisms to the strain level of classification (Welsh and McClelland, 1990). This technique is sensitive and specific because the entire genome of an organism is used as the basis for generating a DNA profile. The combination of PCR and random oligonucleotide primers has provided a method for the rapid and sensitive delineation of animal, plant, fungal, algal and bacterial strains (Akopyanz *et al.* 1992; Kresovich *et al.* 1992 and Scott *et al.* 1992). Eskew *et al.* (1993) used DNA amplified fingerprinting by using single short nucleotide primers of arbitrary sequences to direct amplification of characteristic set of DNA products by Taq DNA polymerase. They used this technique to differentiate the isolates in *Azolla-Anabaena* symbiosis.

DNA amplification fingerprinting (DAF), a PCR based method using short oligonucleotides for production of characteristic banding patterns, has been used by Eskew *et al.* (1993) to study the cyanobacterial symbionts of *Azolla* sp. ferns. The authors generated fingerprints that were unique to the symbionts from three different *Azolla* sp. and were able to show the maternal tranmission of one symbiont.

Prabina *et al.* (2003) employed 11 decamer primers to amplify the genomic DNA of symbiotic and free living forms of 17 cyanobacterial isolates and obtained fingerprinting pattern characteristic to each culture. Balasubramani (1999) demonstrated that DNA amplification fingerprinting could be a useful tool to study the phylogenetic relationship among the *Anabaena azollae* isolates and confirmed the inheritance pattern of algal symbionts in a sexual hybrid of *Azolla*.

In a recent study carried out in our laboratory, RAPD-PCR technology was used to identify the cyanobacterial cultures of the composite inoculum upto strain level by detecting the genetic heterogeneity. Seven decamer primers with G+C content ranging from 60-70% were used to generate DNA fingerprint. The criteria for choosing these primers was the generally accepted concept towards oligonucleotides of high G+C content (Yu and Paul, 1992). These seven primers produced informative and reproducible genetic markers for the cyanobacterial strains of the composite culture inoculum (Table 1). Genetic diversity among the strains

was determined using the banding patterns from the RAPD reaction. The primer CRA 22 produced 24 RAPD products for the eight cyanobacterial strains and the fingerprint showed 100% polymorphism. The molecular weight of the strain specific bands ranged from 300-1500 base pairs. The amplicon with a molecular weight of 700 bp for the primer CRA 25 was unique for the cyanobacterium *Anabaena* GG-SK-AT. The primers of OPA sequence also generated reproducible genetic markers to all the cyanobacterial strains investigated (Lakshmanan and Kumar, 2003).

Current RAPD technology has been optimized for the identification of cyanobacterial cultures upto the strain level. The choice of primers with high G+C content provided good DNA profiles at a low concentration. Neilan (1995) reported the use of RAPD-PCR to generate uniqueness in phylogenetic analysis of toxic cyanobacteria. Nishihara *et al.* (1997) reported the use of RAPD analysis to discriminate genotypes in 5 species of the cyanobacterium *Microcystis.*

1.2.1. rep-PCR

Repetitive sequences constitute an important part of the prokaryotic genome and for cyanobacteria, a distinct family of repetitive sequences have been identified in a number of filamentous heterocystous groups. The conserved status of repetitive sequences dispersed in the cyanobacterial genome make them methodologically important tools for diversity studies and strain identification.

In our laboratory, rep-PCR was employed to identify eight cyanobacterial cultures belonging to the genera *Nostoc, Anabaena* and *Westiellopsis* (Lakshmanan and Kumar, 2003). The DAF was generated using six repetitive sequence oligonucleotides as primers in the PCR (Table 2). The rep-PCR generated reliable fingerpring with 100% polymorphism. The use of the primer STRR 1A in the PCR on the eight cyanobacterial strains yielded multiple distinct DNA products ranging in size from approximately 300-1200 bp. The amplified PCR products obtained by using the LTRR primers ranged in size from approximately 300-5000 bp. The banding pattern obtained by RAPD-PCR was in turn used to infer the phylogenetic relationship between the cyanobacterial strains of composite culture inoculum (Fig.1).

Rasmussen and Svenning (1998) demonstrated the use of short tandemly repeated repetitive (STRR) and long tandemly repeated repetitive (LTRR) sequences in the genome of cyanobacteria to generate a fingerprint method for symbiotic and free-living isolates. The results showed a genetic similarity as well as heterogeneity among the isolates from different *Gunnera* species. The method was applied to non heterocystous cyanobacteria from which a finger print pattern was obtained indicating that the technique is useful for clustering of even closely related strains.

Tandeau De Marsac (1990) reported the characterization of three distinct families of repeated seqeuences in the genome of the cyanobacterium, *Calothrix* sp. PCC 7601. These repeated sequences named short tandemly repeated repetitive are present at a level of about 100 copies per genome and consisted of tandemly amplified hepta nucleotides. Three short tandemly repeated repetitive (STRR) sequences from *Calothrix* sp. strain PCC 7601 hybridized only with the digested genomic DNA of the heterocystous strains tested.

It is noteworthy that repetitive elements have also been used to generate species-and strain-specific fingerprints in eubacteria (Versalovic *et al.* 1991). The taxonomic value of two insertion elements from *Calothrix* sp. strain PCC 7601 appeared to be restricted to the identification of identical strains (Mazel *et al.* 1990).

1.2.3. Multiplex PCR

The simultaneous use of more than one pair of carefully designed primers (multiplex) allows identification of multiple organisms in one PCR assay. The multiplex RAPD-PCR is a modification of standard RAPD protocols, where the PCR reaction is performed by multiplexing the oligonucleotide primers as against the single primer employed in normal PCR reaction. Neilan (1995) developed a method based on the combination of two 10-mer oligonucleotides in a single PCR to provide specific and repeatable DNA finger prints for cyanobacterial

isolates *Anabaena* and *Microcystis*. The strain specific randomly amplified polymorphic DNA profiles made it possible to discriminate among all toxigenic cyanobacteria studied to the three taxonomic levels of genus, species and strain. The markers produced for each strain were also applied to a phylogenetic analysis to infer the genetic relatedness in this group of prokaryotes.

Multiplex PCR was also performed in our laboratory using CRA (CRA 25 and 26) and OPA (OPA 8 and 11) primers to identify the cyanobacterial strains of composite culture inoculum. The combination of PCR and random oligonucleotide primers of 10 nucleotide provided a method for the rapid delineation of cyanobacterial strains. The primers with high G+C content provided good DNA profiles at low concentration (Table 3). The molecular weight of strain specific bands generated by the dual primers CRA 25 and 26 ranged between 300-2200 bp.

1.3. Sequencing of ribosomal genes

According to Woese (1987), the 16S rRNA is a good molecular chronometer as it is an effective molecule for measuring the overall rate of evolutionary change in a line of descent. One area in which a significant application has been developed recently is in the phylogenetic characterization of natural microbial communities by 16S rRNA sequence analysis (Pace *et al.* 1980). For the analysis of natural microbial populations in which unknown diversity must be anticipated, the rRNA is being focussed because they are functionally and evolutionarily homologous in all organisms (Stakebradt and Woese, 1981). Moreover, the rRNAs are ancient molecules and extremely conserved in overall structure. Hence, homologous rRNAs are readily identifiable by their sizes (Lane *et al.* 1988).

The first global evolutionary scheme of cyanobacteria comprising 29 partial 16S rRNA sequence (about 700 positions) was published by Giovannoni *et al.* (1988), who suggested that the rise of the oxygen concentration in the precambrian atmosphere allowed the colonization of new biotypes and probably led to extensive divergence of the cyanobacteria. Most of the branches were long and unbranched, reflecting the selection of strains generally representing the different genera and not expected to be close relatives.

Molecular study of evolutionary divergences that took place greater than 500 million years ago (which include separation of the major algal phyla) requires sequencing of very slowly evolving genes, such as nuclear-encoded genes for small and large subunit ribosomal RNA (SSU) and LSU rDNA. SSU is more highly conserved than LSU and hence is more useful for this type of analysis. Buchheim *et al.* (1997) used SSU rDNA sequence data to demonstrate that the genus *Chloromonas* is not monophyletic.

The Ribosomal Database Project (RDP) at the University of Illinois contains a large array of sequences for both prokaryotes and eukaryotes and additional rDNA sequences can be found in Gen Bank (Maidek *et al.* 1997)

Most of the studies of cyanobacteria phylogeny with 16S rDNA sequences (Giovannoni *et al.* 1988) were based on isolates available as axenic cultures. Nelissen *et al.* (1996), constructed a cyanobacteria specific oligonucleotide probe which distinguished between the 16S rRNA genes from those of bacterial contaminants

A diagnostic system using the sequence polymorphism within the 16S rRNA variable regions V6, V7 and V8 for individual strain characterization and identification of toxin producing organisms belonging to the genera *Nostoc, Anabaena, Microcystis, Planktothrix* and some non toxin producing strains has been developed (Rudi *et al.* 1997).

Rudi *et al.* (2000) developed a method to analyze genetically the cyanobacteria composition in natural water bodies, using multiple sequence specific labelling of oligonucleotide probes targeted to 16S rRNA and subsequent hybridization of the labelled probes to their respective complements spotted onto a solid support. This array made it possibly to accurately analyze the whole microbial communities and to directly relate sequence information obtained from organisms in type culture collections to the biodiversity that exists in nature.

2. DNA-DNA Hybridizations

Wayne *et al.* (1987) proposed the use of DNA-DNA hybridizations as a criterion for

the definition of eubacterial species. For members of the same species, atleast 70% hybridization is required and 20% is required for congeneric strains. The use of this method for the cyanobacteria was pioneered by Stam (Stam and Venema, 1975 and Stam, 1980), who used a filter-hybridization technique.

Lachance (1981) investigated the genotypic relationships among the heterocystous genera by DNA-DNA hybridizations. Guglielmi and Cohen-Bazire (1984) used DNA-DNA hybridizations to elucidate the relationships of nine strains belonging to the genus *Pseudanabaena* and the LPP group. They also showed the genotypic divergence of *Pseudanabaena* sp. strain PCC 7403, which correlated well with differences in pigment composition.

The symbionts associated with cycads and angiosperms are classically identified as members of the genus *Nostoc*. Lindblad *et al.* (1989) used heterologous probes from *Anabaena* sp. strain PCC 7120 to characterize the symbionts freshly isolated from five cycad species from Central America.

Stulp and Stam (1984) performed DNA-DNA hybridization studies with twenty strains belonging to the genus *Anabaena* sp. The strains assigned to the same species on the basis of morphology indeed showed very high hybridization percentages (about 100%). On the other hand, strains from different species showed intermediate hybridization values.

By DNA-DNA hybridizations, Wilmotte and Stam (1984) demonstrated that the strains PCC 7942 and PCC 7943 belong to the same species as *Synechococcus* sp. strain PCC 6301, which is often wrongly designated as *Anacystis nidulans* (Komarek, 1970). This result was later confirmed by results of RFLP analysis for strains PCC 6301 and PCC 7942. The genomes of the two strains looked identical, except for a rearrangement (Golden *et al.* 1989, Wood and Townsend, 1990).

DNA-DNA reassociation studies have also been used to investigate the genotypic diversity of *Prochloron* sp. isolates from different locations. The fourteen *Prochloron* sp. isolates appeared to belong to a single species (Stam *et al.* 1985 and Holton *et al.* 1990).

3. Microsatellite (Minisatellite) DNA

Microsatellite DNA consists of regions of variable length having sequences of di or trinucleotides (*e.g.* GAGAGAGAGA...) that are repeated 10-100 times per locus. Such DNA is used to study variation within species, providing a "fingerprint" for each population analyzed.

Variation in the number of repeats is detected by using specific hybridization probes (Oppermann *et al.* 1997). Microsatellite markers in the nuclear genome have been used to study the alga *Laminaria* (Billot *et al.* 1998).

4. Internal transcribed spacer region (ITS)

For studies of species diversification, events assumed to have occurred recently or rapidly evolving sequences are preferred. The regions of the nuclear-encoded ribosomal RNA genes known as the internal transcribed spacers (ITS) are particularly useful in such studies. ITS sequencing has been used commonly in intrageneric phylogenetic studies of algae.

Marks and Cummings (1996) used the ITS region to demonstrate low genetic diversification among freshwater isolates of the common and widespread alga *Cladophora* obtained from a wide range of habitats and geographical locations. Peters (1997) used ITS sequences to compare species within the widespread algal family *Desmarestiaceae*.

Conclusion

Cyanobacteria traditionally have been distinguished on the basis of phenotypic properties, structure and physiology. Despite the availability of many monographs, the identification and classification of cyanobacteria remain difficult and frustrating, resulting more often in uncertain identification (Friedmann and Borowitzka, 1982). Taxonomic characters change so drastically that reliable identification of species become difficult or even impossible (Kumar *et al.* 2000). In this regard, molecular, biochemical and immunological techniques are considered as highly effective to identify cyanobacterial strains in a fast and easy way. In recent years, a number

of new, valuable biochemical and molecular biological techniques for taxonomic purpose have been developed which could also be effectively utilized for checking the genetic purity of cyanobacterial strains.

Presently, the integrated use of phylogenetic and phenotypic characteristics, called "polyphasic" taxonomy is recommended by the cyanobacterial taxonomist (Mann, 2000). The integration of morphological, molecular, biochemical and immunological characteristics of the cyanobacterial strains will form the basis for a polyphasic taxonomy that will not only be of practical use for effective and easy strain idenification but will reflect as much as possible the evolutionary relationship of the strains.

Acknowledgements

The authors are thankful to the Indian Council of Agricultural Research, New Delhi for the financial support extended to some of the investigations carried out in this laboratory and reported in this review under the National Agricultural Technology Project-Team of excellence on biofertilizers for rice based cropping system.

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(Received: August 2003; Revised: September 200