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Molecular Characterization of *Azotobacter chroococcum* Strains Isolated from Different Agro-climatic Zones of Karnataka, India

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ABSTRACT

Azotobacter chroococcum is an aerobic, free-living bacterium capable of synthesizing various plant growth-promoting substances and is useful in crop improvement for biological nitrogen-fixing activity. Ten *A. chroococcum* strains were isolated and purified from different agro-climatic zones of Karnataka, India by Waksman No. 77 N-free agar by a serial dilution plate technique. Molecular diversity of the isolates was estimated by using ten selected RAPD primers. A total of 103 bands were scored out of which 87 were found to be polymorphic (84.97%). A dendrogram divided the isolates into two groups separated by 37 linkage distances and the dissimilarity matrix showed a maximum difference of 64% between the isolates of the North eastern transition zone and the Central dry zone and a minimum difference of 18% between the isolates of the Eastern dry zone and the Hilly zone. The isolate from zone 4 was clustered separately from the group. Thus, RAPD markers analysis proved to be a quick, simple and significant testing method to assess genetic diversity among *A. chroococcum* isolates.

Keywords: cluster analysis, dissimilarity matrix, genetic diversity, RAPD marker, STATISTICA

INTRODUCTION

Azotobacter chroococcum is a beneficial bacterium known to fix nitrogen and improve crop growth and yield (Naik *et al.* 2007). It is a free-living aerobic bacterium dominantly found in soils, associated with the plant rhizosphere and providing a beneficial effect on plant growth. Hence, it is used as a bio-fertilizer for improving agriculture productivity, the focus of a number of studies (Dobereiner 1997; Cakmakc *et al.* 2001). A large number of experiments have shown that in many cases nitrogen (N) concentration in plants is increased by inoculation with *Azotobacter* sp. (Kumar *et al.* 2001; Emtiazi *et al.* 2004: Naik *et al.* 2007). Studies have also shown the importance of *A. chroococcum* as a soil inoculant, effective not only in N-fixation but also in increasing the production of growth hormones like ethylene, auxins and gibberellin (Remus *et al.* 2000), fungicidal substances (Lakshminarayana 1993), siderophores (Suneja *et al.* 1994) and the possibility of solubilizing phosphates (Narula *et al.* 2000).

According to Bhattarai and Hess (1993) indigenous isolates might be preferred in the selection of bacteria for inoculation of crop plants, as they adapt to local ecological conditions and can be more competitive than the non-indigenous strains. Therefore, the objectives of the study were to isolate *A. chroococcum* strains from Karnataka soils and to compare the diversity among the indigenous *A. chroococcum* The geographical area of Karnataka is classified into ten agro-climatic zones, namely: 1) North eastern transition zone, 2) North eastern dry zone, 3) Northern dry zone, 4) Central dry zone, 5) Eastern dry zone, 6) Southern dry zone, 7) Southern transition zone, 8) Northern transition zone, 9) Hilly zone and 10) Coastal zone. Each zone has its own characteristic features in relation to climatic conditions, soil types and vegetation which influence the establishment of a diversified flora and fauna. The zonal classifications among agro-climatic regions of Karnataka are based on the reports of Mathimaran (2001) and Veena (2005).

Molecular analysis of genomic DNA of microorganisms is useful for better distinguishing bacterial strains at an intra-species level (Kumar *et al.* 2005). These techniques provide valuable information on the magnitude of genetic variability within and between organisms of different species (Bert *et al.* 1996). Random Amplified Polymorphic DNA (RAPD) has been used for typing and identification of a number of closely related species of bacteria and to assess genetic relationships. The results of DNA-DNA homology studies can be used successfully to estimate genetic distances (Kang and Mills 2004).

Wang *et al.* (1993) showed that RAPD tests with just one or a few short arbitrary primers are more sensitive than conventional multilocus enzyme electrophoresis to detect differences among closely related *E. coli* isolates. Picard *et al.* (2000) analysed the biodiversity of 150 strains of *Pseudomonas* spp. using RAPD markers. Similarly, Babalola *et al.* (2002) analysed genetic diversity among three bacteria, namely *Pseudomonas* sp. *Enterobacter sakazakii* and *Klebsiella oxytoca* from the rhizospheres of maize and sorghum by RAPD markers. Genetic diversity of rhizobacterial isolates form different countries were analysed using RAPDs (Hafeez *et al.* 2006). Therefore, in the present study, an attempt was made to elucidate the genetic diversity of *Azotobacter chroococcum* strains isolated from different agro-climatic zones of Karnataka using RAPD markers.

MATERIALS AND METHODS

Isolation and purification of strains

A. chroococcum strains collected form 10 different agro-climatic conditions were isolated on Waksman No.77 N-free agar by serial dilution plate technique (Wu *et al.* 2006), identified and maintained at Department of Microbiology, University of Agricultural Sciences, Bangalore. Then plates were incubated for 72 h at 28 ±

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Table 1 Morphological character. Growth characters of *A. chroococcum* isolates on Waksman No.77 N-free medium

A. chroococcum isolates (Zones)	*Cultural characters	**Pigmentation	Oxygen requirement	Shape	Motility	Capsule	Microcyst	Gram reaction
1	Good growth, raised slimy colony	Light brown	Aerobic	Ovoid	+	+	+	Gram -ve
2	Moderate growth, flat entire slimy colony	Black	Aerobic	Ovoid	+	+	+	Gram -ve
3	Good growth, flat entire slimy colony	Pale brown	Aerobic	Round	+	+	+	Gram -ve
4	Good growth, raised slimy colony	Dark brown	Aerobic	Blunt ended	+	+	+	Gram -ve
5	Good growth, raised slimy colony	Light brown	Aerobic	Ovoid	+	+	+	Gram -ve
6	Good growth, flat entire slimy colony	Light brown	Aerobic	Ovoid	+	+	+	Gram -ve
7	Moderate growth, flat entire slimy colony	Dark brown	Aerobic	Round	+	+	+	Gram -ve
8	Moderate growth, raised slimy colony	Black	Aerobic	Blunt ended	+	+	+	Gram -ve
9	Moderate growth, flat entire slimy colony	Pale brown	Aerobic	Ovoid	+	+	+	Gram -ve
10	Moderate growth, flat entire slimy colony	Light brown	Aerobic	Ovoid	+	+	+	Gram -ve

* Cultural characters were observed 3 days after incubation.
** Pigmentation observed 7 days after incubation.
+ Presence of motility, microcyst and capsule.

Table 2 Growth characters. Effect of different carbon sources on growth of *A. chroococcum* isolates.

A. chroococcum isolates (Zones)	Carbon source					
	Mannitol	Glucose	Fructose	Sucrose	Rhamnose	Glycolate
1	+	+	+	+	–	+
2	+	+	+	+	–	+
3	+	+	+	+	–	+
4	+	+	+	+	–	+
5	+	+	+	+	–	+
6	+	+	+	+	–	+
7	+	+	+	+	–	+
8	+	+	+	+	–	+
9	+	+	+	+	–	+
10	+	+	+	+	–	+

+ Growth; – No growth

2°C. The plates were checked for *A. chroococcum* growth and pigmentation on prolonged incubation for a week. The isolated colonies of *A. chroococcum* were re-streaked for purification and the pure isolates thus obtained were maintained on agar slants prepared with Waksman No. 77 medium (glucose 10.0 g, NaCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, K_2HPO_4 0.5 g, 2 drops of 1% (w/v) $FeCl_3$ and 1% (w/v) $MnCl_2$ solution, 1% (v/v) Congo Red solution 5 mL, agar 20.0 g, distilled water 1 L, pH 7.0) for further characterization. Each isolate was identified by conducting the test according to specific characters described in Bergey's Manual of systematic Bacteriology (**Tables 1, 2**).

DNA isolation

A. chroococcum strains were grown in Waksman No. 77 N-free medium broth at 28 ± 2°C overnight and genomic DNA was extracted according to the method of Sambrook *et al.* (1989). About 1.5 ml of culture was spun for 7 min in a microcentrifuge tube (Eppendorf, Bangalore, India) and the supernatant was discarded. To the pellet 567 µl of TE buffer, 3 µl of 20 mg/ml proteinase-K and 30 µl of 10% (w/v) SDS were added and incubated for 1 h at 37°C. To this 100 µl of 5 M NaCl and 80 µl of 3% (w/v) CTAB solution were added and incubated for 10 min at 65°C. The solution was centrifuged at 8000 × *g* for 5 min at 4°C and the supernatant was transferred to a fresh tube. An equal volume of chloroform: isoamyl alcohol was added to the supernatant and centrifuged at 8000 × *g* for 5 min at 4°C. The aqueous phase was transferred to a fresh tube and spun with an equal volume of phenol: chloroform: isoamyl alcohol at 8000 × *g* for 5 min at 4°C until a clear supernatant formed. To the extract equal volume of ice-cold isopropanol was added, mixed gently and incubated at -20°C overnight and centrifuged at 10,000 × *g* for 20 min at 4°C. The pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer, analysed on an agarose gel and quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, USA).

PCR amplifications

PCR amplification followed the protocol of Williams *et al*. (1990) with minor modifications. Of the 23 primers screened using the

Table 3 RAPD-PCR primers. The sequence and level of polymorphism of selected polymorphic primers in *A. chroococcum*.

Primers	Sequence (5′-3′)	№ of amplified fragments	№ of monomorpic bands	№ of polymorphic bands
OPD-05	TGAGCGGACA	10	01	09
OPD-20	ACCCGGTCAC	10	00	10
OPH-13	GACGCCACAC	12	04	08
OPH-05	AGTCGTCCCC	09	03	06
OPB-08	GTCCACACGG	04	02	02
OPB-01	GTTTCGCTCC	14	01	13
OPB-12	CCTTGACGCA	11	02	09
OPA-11	CAATCGCCGT	10	01	09
OPD-03	GTCGCCGTCA	15	01	14
OPD-13	GGGGTGACGA	08	01	07
Total		103	16	87

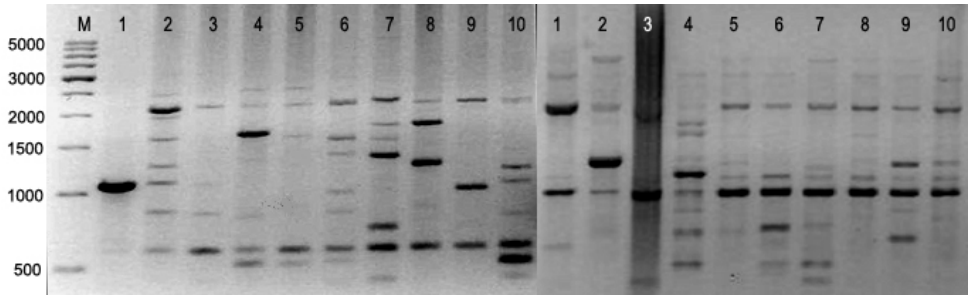

Fig. 1 Gel profiles of *A. chroococcum* isolates according to OPB-01 (left) and OPD-03 (right) primers. Lanes 1-10 contain the amplification profile obtained using the isolates (Zones 1 to 10). Lane M: 500 bp standard DNA marker.

pooled DNA, 10 showing clear and distinguishable bands during screening were selected for RAPD-PCR analysis (**Table 3**). PCR reactions were performed in a final volume of 20 µl containing 30 ηg of template DNA, 200 µM dNTP, 1.5 mM $MgCl_2$, 2µl of 10X reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100), 2.5 µl of 5 pmole primer and 1 unit of *Taq* DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India). Amplifications were performed in an MJ Research PTC 100 thermocycler (Bio-Rad Laboratories, Bangalore, India) with the program consisting of initial denaturation at 94°C for 3 min followed by 45 cycles each consisting of denaturation at 94°C for 1 min, primer annealing at 40°C for 1 min, primer extension at 72°C for 2 min and a final extension of 72°C for 10 min. The PCR reactions were repeated twice to check the reproducibility of the amplification. PCR products were resolved in a 1.5% (w/v) agarose gel visualized and documented using an Alpha Digidoc system (Alpha Innotech, San Leandro, CA, USA).

RAPD profile analysis

Each reproducible band was visually scored as '1' for presence and '0' for absence, and the binary data were used for statistical analysis. The band sizes were determined by comparing with 500 bp DNA ladder (Bangalore Genei, Bangalore, India), which was run along with the amplified products. A Dissimilarity Matrix was developed using Squared Euclidean Distance which estimated all pair wise differences (Sneath and Sokal 1973) and the dendrogram was computed based on Ward's method of clustering using a minimum variance (Wards 1963).

RESULTS AND DISCUSSION

In this work, RAPD-PCR was shown as a simple method to verify the microbiological intraspecific diversity of *Azotobacter chroococcum* isolates. Morphological identification based on growth conditions allowed the confirmation of isolates. Observations on growth characters in W-77 N-free agar medium showed that isolates from 1, 3, 4, 5 and 6 agro-climatic zones showed good growth while the other isolates showed moderate growth on the medium. Similarly, raised slimy colonies were observed in isolates from zones 1, 4, 5 and 8, while flat entire slimy colonies were observed in isolates from zones 2, 3, 6, 7, 9 and 10. A week-old culture isolated from zones 1, 5, 6 and 10 showed light-brown pigmentation; isolates from zones 2 and 8 were black. Isolates from zones 3 and 9 were pale-brown while those from zones 4 and 7 were dark brown. Morphologically, all the isolates were oval to round in shape, motile and Gram-negative. All the isolates produced a cyst and capsule (**Table 1**). All 10 isolates grew on media containing mannitol, glucose, fructose, sucrose and glycolate but not on rhamnose as a carbon source (**Table 2**). All the tests showed positive results for the species belonging to *A. chroococcum* as described in the Bergey's Manual of Systematic Bacteriology (1994). Similar morphological characters were observed by Shankarappa (1996) from the rhizosphere soil of mulberry and a similar study was performed by Callao *et al*. (2000) to identify 14 *Azotobacter* strains from barley rhizosphere.

Twenty three short primers of arbitrary sequence were initially screened using pooled DNA, out of which ten were selected based on their distinct and clear amplification profiles. The screening of primers using pool DNA facilitated the selection of primers amplifying reproducible pattern of fragments. Screening of RAPD primers is essential to save time and cost, and to reject primers which are not informative for the analysis (Prakash *et al.* 2002). About 103 clear, readable and reproducible RAPD markers were produced from the selected 10 primers. The number of bands obtained per primer varied from 4 to 14 with an average of 10.3 bands per primer from 300 bp was obtained. Out of 103 amplification bands, 16 (15.53%) were monomorphic and 87 (84.4%) were polymorphic, which were informative in revealing the relationships among the genotypes (**Table 3**). Similarly, Mathimaran (2001) showed high polymorphism using RAPD characterization of *Glomus mossae* isolated from these agro-climatic zones of Karnataka and Veena (2005) reported 33% polymorphism among *Aspergillus awamori* isolates from these zones using AFLP markers. The polymorphic gel profiles of primer OPB-01 and OPD-03 are shown in **Fig. 1**. The dissimilarity matrix showed a maximum difference of 64% between the isolates of the North eastern transition zone and the Central dry zone and a minimum difference of 18% between the isolates of the Eastern dry zone and the Hilly zone (**Table 4**).

Table 4 Genetic dissimilarity matrix of 10 *A. chroococcum* isolates based on polymorphism of RAPD markers

Z1	0									
Z2	44	0								
Z3	47	45	0							
Z4	65	56	53	0						
Z5	43	44	24	57	0					
Z6	46	45	25	54	26	0				
Z7	53	56	41	64	35	29	0			
Z8	44	45	23	58	20	25	35	0		
Z9	41	45	29	61	18	30	38	25	0	
Z10	41	45	29	61	22	30	38	25	26	0

Cluster analysis based on 103 RAPD bands revealed that the 10 *A. chroococcum* isolates clustered at a linkage distance of about 36 units on the dendrogram where isolates from zone 4 and zone 1 spanned the extremes (**Fig. 2**). The dendrogram clearly depicts that all 10 *A. chroococcum* isolates formed two major clusters. The major group A consisted of one isolate (zone 4) characterized by a blunt-ended shape and dark-brown colonies, and is linked to group B. The major group B with nine isolates clustered into two subgroups (B_1 and B_2). Subgroup B_1 consisted of one isolate (zone 7) characterized by a round shape and dark-brown colonies. Subgroup B_2 segregated into two minor clusters (B_{2a} and B_{2b}). The minor cluster B_{2a} consisted of two isolates of zone 1 and 2. Both isolates were similar with respect to their ovoid structure but differed in their colony colour and shape. The minor cluster B_{2b} consisted of six isolates. Predominantly the members of the cluster showed an ovoid structure, and flat, entire and slimy colonies. Furthermore, the close relationship between the lines of the remaining zones was due to their identical characters.

All the isolates in the present study were collected from different geographical locations with diverse agro-climatic conditions and hence showed relatively high polymorphism, noted by their growth conditions. The isolate of the North eastern transitional zone and the Central zone were very

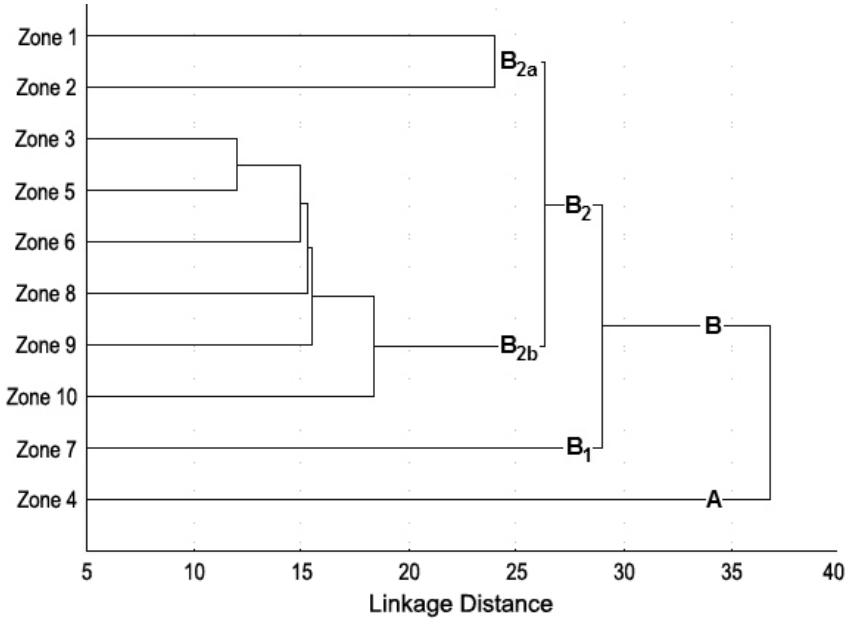

Fig. 2 Cluster analysis. Dendrogram showing RAPD-marker-based genetic relationships among 10 *A. chroococcum* isolates and grouping them into two clusters 'A' and 'B' with one and nine isolates, respectively.

distant geographically. It is also clear that the soil and climatic features of these two regions are characteristically different. Hence, this study has proved efficient to differentiate between the isolates obtained from various agro-climatic zones of the state. In conclusion, RAPD-PCR–based DNA fingerprinting of the genomic DNA and random arbitrary primers are a rapid and sensitive method for the detection of genetic variation among different isolates of *A. chroococcum*.

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