

## Molecular Analysis of Phenotypic Diversity among Four *Frankia* Isolated from *Casuarina* Nodules in Egypt. 2- Molecular Diversity among Four *Frankia* Isolates.

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**Abstract:** Four *Frankia* isolates (Fc, Fe, Fh and Fg) isolated from nodules of four species of *Casuarina* plants formed abundant terminal vesicles on filamentous; showed typical morphological characteristics of *Frankia* growth pattern and metabolic activities are differed. The growth pattern of the four isolates was found to be growing exponentially and their metabolic activity in BAP-PCM liquid medium under stirred conditions. All isolates bound the universal probe, was showing variation in hybridization intensity against RNAs immobilized on H bond N nylon membrane through all of the cell spots contained the same amount of RNA. These RNAs were strongly hybridized with the general *Frankia* probe (PF) 16s RNA from isolate Fg. Only RNA isolated from *Frankia* isolate Fc show weakly significant hybridization with this probe. DNA fingerprinting was used for molecular analysis of somaclonal variations among four *Frankia* isolates. Three arbitrary base primers from ten primers were successfully used to amplify DNA extracted from 4 *Frankia* isolates. RAPD-PCR revealed polymorphism 63, 36 and 73% primer 1, 2 and 3 primers of Fc, Fe, Fg and Fh, respectively. The frequency of genetic variability was detected in 4 *Frankia* isolates dependent variations in growth pattern, 16s RNA and metabolic activities. The similarity percentage was 52, 48, 52 and 44% in Fc, Fe, Fg and Fh, respectively according to PAF.

**Key words:** *Frankia*, *Casuarina* spp., Oligonucleotide probes rRNA, DNA fingerprint, RAPD-PCR.

### INTRODUCTION

Characteristics of *Frankia* such as, their slow growth rate relative to other *Frankia* strains, the endophytes from *Casuarina* nodules were obtained in pure culture only in 1981 (Gauthier *et al.*, 1981). During the last decade hundreds of *Frankia* isolates have been obtained from *Casuarina* nodules using different isolation techniques (Baker and Mullin, 1989). All isolates obtained from nodules were assigned from nodules were assigned to the genus *Frankia* on the basis of (i) morphological features, such as sporangium and vesicle formation in submerged liquid culture, (ii) chemical composition of certain cell constituents such as cell wall type III, phospholipids type PI and the presence of the diagnostic sugar 2-O-methyl-mannose and (iii) the ability to fix nitrogen and to nodulate plants (Lechevalier, 1986 and Mort *et al.*, 1983). Many isolates lacking some of these morphological and physiological characteristics of typical *Frankia* have been obtained from actinorhizal nodules (Mirza *et al.*, 1991). However, the application of the above mentioned criteria in the characterization of these atypical isolates is therefore not sufficient to identify these isolates as members of the genus *Frankia*.

Genetic and phenotypic diversity among *Frankia* strains are well known (Lalonde *et al.*, 1988 and Gardes and Lalonde, 1987), but ecological and systemic assessments have been problematic because readily used morphological, biochemical, or molecular markers have largely been lacking. Analysis of the nucleotide sequence of RNA from the small ribosomal subunit (i.e. 16s RNA) has gained rapid and wide acceptance in systematic, evolutionary and ecological studies of various microorganisms (Hahn *et al.*, 1989 and Woese, 1987).

RAPD analysis, a technique used for DNA fingerprinting and genetic mapping (De Gioia *et al.*, 2005). In RAPD technique, a single 10 mer of arbitrary sequence is used as a primer in PCR to amplify genomic DNA. Genomic DNA from different individuals gives different PCR products allowing the identification of DNA polymorphisms (Puente-Redondo *et al.*, 2000), as well as elucidation of their phylogenetic relationships is widely used. Sharma (2003) and Swelim (2005) recorded that, the utility of DNA markers as RAPD-DNA in detecting genetic variability.

The aim of this investigation is to molecularly analyze 4 *Frankia* isolates obtained from root nodules of *Casuarina* spp. on the genetic basis of using 16s rRNA hybridization against oligonucleotide probes and RAPD-PCR.

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## MATERIALS AND METHODS

The isolates of *Frankia* were inoculated at a rate of 1 µg protein/ml into BAP-PCM medium and incubated at 28 ± 2°C at 2 weeks under stirred conditions as described by Schwencke (1991). The developed mycelia were harvested by centrifugation at 5000 rpm and determined cellular proteins by the methods of Bradford (1976) using bovine serum albumin (BSA) as a standard protein.

### **Extraction of Ribosomal RNA:**

Four *Frankia* isolates were grown in BAP-PCM as described above. Nine days post, the filamentous mats are harvested by centrifugation 5000 rpm for 15 min. The pellets were washed twice with PBS buffer (145 mM Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and stored at -70°C. The pellets were resuspended in 500 µl 7 M guanidine-hydrochloride solution 1 M Tris pH 7.4 buffer and sonicated on ice three times for 30 Sec. at 60 W to disrupt *Frankia* cells. Extraction of RNA was done according to the method of Hahn *et al.* (1989). The yield of RNA was measured by reading at wave lengths of 260 nm and 280 nm using spectrophotometer isolated RNA were diluted to 10 ng, 100 ng and 1 µg/10 µl in TE-buffer. RNA isolated were separated by agarose 0.8 % gel electrophoresis and visualized by attaining with ethidium bromide (0.5 µg/ml). Ten µl RNA solution of 1 µg/10 µl mixed with 1 µl of sample buffer (0.25 % bromophenol blue; 0.25 % xylene cynol; 30% glycerol in H<sub>2</sub>O) were loaded in each slot. The gel was run for 60 min at 60 V and Lambda Hind III was used as marker; after running the gel was stained by ethidium bromide (0.5 µg/ml) for 15 min. The RNA bands were visualized by examining the gel with uv-LAMP (354NM).

### **Oligonucleotides Probe:**

The eubacterial primer 111.5 (Emblet *et al.*, 1988, 5' AGGGTTGCGCTCGTTG) was used as universal probe and probe FP, designed as *Frankiagenus* probe (Hahn *et al.*, 1990, 5' ATAAATCTTTCCACACCACCAG), were used as specific probes.

### **Southern Blot:**

RNA separated by gel electrophoresis were transferred to a Hybond N nylon membrane and immobilized by using Southern blot (Southern, 1975). The gel is placed on a nylon membrane. Hybond N nylon membrane was cut with exactly the size of the gel and wetted in sterile water. The VacuBlot apparatus was used according to the manual. The filter was denaturated by 0.5 M NaOH/1.5 M NaCl for 5 min, neutralized by 1 M Tris pH 8.0/1.5 M NaCl for 5 min and washed by 10x SSC buffer (0.15 M NaCl; 0.015 M sodium citrate pH 7.0) for 5 min subsequently with 10x SSC for 40 min. Shortly, the filter was dried between two sheets of 3 MM paper and the isolated RNA was fixed by UV-treatment for 2 min.

### **Dot Blot Hybridization:**

The fixed target RNA was hybridized with *Frankia* probe (PF) labeled probe and autoradiography was carried out to locate the position of 16s rRNA band which it was complementary to the PF radioactive probe. Target nucleic acids could be applied 100ng directly by spotting aliquots of the target solution onto the nylon membrane, each spot containing identified cell muss 1 µg protein (Kafatos *et al.*, 1979). By this technique targets RNA from pure cultures could be analyzed qualitatively for the presence of a specific target sequence. Hybond N nylon membrane was cut of correct size, wetted in distilled water and putted in the HybridDot manifold. Ten µl of sample contain 100 ng of RNA was applied per dot after the working vacuum pump. Pure 16s rRNA isolated from effective *Alnus Frankia* strain Ag45/Mut 15 (Hahn *et al.*, 1989) was used as positive control. Then, the filter was dried and the RNA was fixed treatment the filter 2 min under the UV-source (354 nm).

### **Preparation of Total Genomic DNA:**

Total genomic DNA was isolated using a lysozyme-dedocyl sulfate lysis procedure (Owen and Borman, 1987) modified by (Lea *et al.*, 1990). *Frankia* isolates were cultured in BAP-PCM broth at 28°C, with shaking at 200 rpm and harvested by centrifugation at 5000 rpm. The filamentous mats were lysed by the addition of 10% SDS solution, followed by incubation with 100 µl RNase A, 25 µl of proteinase K solution (100mg/ml) was added and the mixture incubated for 1 h at 37°C. The DNA was then extracted using the phenol extraction method. The DNA in aqueous phase was precipitated with 95% ethanol, followed by washing with 70% ethanol, the DNA pellet was allowed to dry, then dissolved in TE buffer (pH, 8.0) and stored at -20°C.

#### **RAPD-PCR Analysis:**

RAPD was performed for 4 *Frankia* isolates, using the ten primers (operon RAPD 10-mer kits), B-05, 07, 08, 09, 10, 11, 12, 13, 15, 20. 50 ng of DNA of each isolate was amplified by the polymerase chain reaction (PCR) using 25 µl reaction mixtures under the following conditions: 200 mM of dNTPs, 1x Taq polymerase buffer, 1.2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH, 8.0), 50 mM KCl, 0.01% gelatin, 200 ng oligonucleotide primer and 2.5 unit Taq polymerase (Promega Co., USA). Amplifications were carried out in DNA thermal cycler (MWG-BIOTECH primus) programmed according to Weisburg *et al.* (1989) as follows: One cycle at 72°C for 4 min and then 40 cycle at 34°C for 30 sec., 35°C for 1 min and 72 °C for 2 min., one cycle at 72 °C for 5 min, then 40 °C for 10 min infinitive.

The PCR products were subjected to electrophoresis on 1% agarose in TAE buffer was prepared and a total vol. of sample 6 µl (1µl of miniprep, 4 µl d-H<sub>2</sub>O and 1 µl6x loading dye) of each DNA extract was loaded in each well. The gel was electrophoresed in 65 V for 1.5 h and then stained with ethidium bromide solution (10mg/ml) for around 10-15 mins. DNA fractions visualized on a UV transilluminator. These bands were scored as either 10 or 0 for their presence or absence across isolates, and a binary matrix generated, which was further analysis using the NISYS program.

### **RESULTS AND DISCUSSION**

Four isolates of *Frankia* were isolated from nodulated trees of *Casuarina sp.* They were namely Fc, Fe, Fg and Fh according species to the system described by Shash (2009).

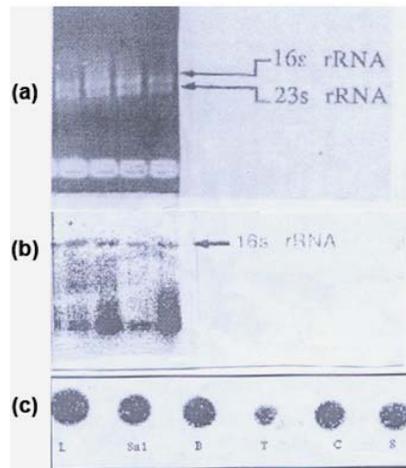
Generally, all the tested isolates showed filament diameter 1-2 µm. Abundant sporanges were formed and varied in size from early (subdivided filaments) to late stages (swollen spore-filled structures up to 60 µm in length). In N-free BAP-PCM medium vesicles formation occurred within a 2 days and increased with time. High variability in the counts of vesicles was observed between the isolates (Shash, 2009).

#### **Molecular Detection of rRNA:**

Ribosomal RNA was isolated from *Frankia* isolates. The amount of RNA was calculated at wave length of 260 nm and 280 nm which allow to concentration in the samples. The position of separated rRNA bands (16s and 23s rRNA) were clearly located in the gel in all the extracts of *Frankia* isolates (Fig. 1-A).

By using Southern blot technique, the specific hybridization was carried with 16s rRNA band, while hybridization with small degradation products fragments from RNA was also observed (Fig. 1-B) may be due to the sonication step. Strong hybridization signals were obtained using Dot-blot technique with the general *Frankia* probe against various RNAs immobilized on Hybond N nylon membrane (Fig. 1-C), indicated that RNA was presented in all RNA preparations. All isolates bound the general probe, was showing variation in hybridization intensity though all of the cell spots contained the same amount of RNA (i.e., 100ng). Weakly and strong hybridization signal were detected with general *Frankia* probe against 16s rRNA from 4 *Frankia* isolates indicated that there was some differences in the nucleotide sequence in their variable regions. Moreover, this probe has shown strong hybridization against 16s RNA isolates from the effective *Casuarina-Frankia* isolates Fc. Good results were reported by using the *Frankia* specific probe (PF) against 16s rRNA to distinguish between effective and ineffective *Alnus* strains (Hahn *et al.*, 1989). Experiments using *Frankia* restriction DNA fragments from seven infective isolates showed essentially identical hybridization patterns against nif A, B, D, K and H probes from *Alnus* (Nazaret *et al.*, 1989). Further, 6 infective strains of *Casuarina* were all of them found in a single genomeic species according to their 16s ribosomal RNA sequences homology (Nazaret *et al.*, 1991).

By using specific nucleic acid hybridization probe in detection and identification of *Frankia* isolates could be a powerful tool in ecological investigations. Specificity of probe, the low detection limit of target sequences and simple application could avoid problems of reisolation and identification in pure cultures. The results showed here indicated that the application of oligonucleotide probes in the detection of specific *Frankia* strains does not only depend on the development of a reliable isolation method for target RNA sequences but also depend on the specificity of the probes. However, could be concluded that design new synthetic oligonucleotide probes by sequencing the variable regions of the 16s rRNA from *Casuarina*-compatible *Frankia* is very essentially to discriminate between *Casuarinaceae* strains.



**Fig. 1:** Molecular detection of 16s rRNA of 4 *Frankia* isolates 1 (Fc), 2 (Fe), 3 (Fg) and 4 (Fh).

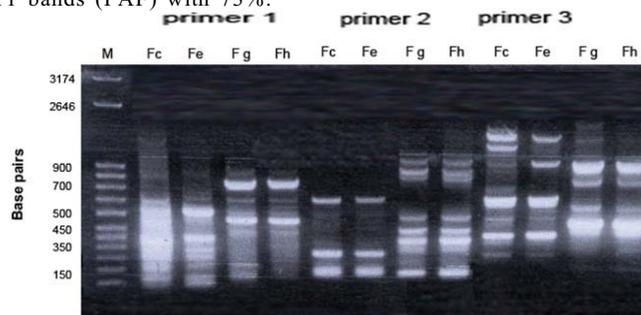
A-16s and 23s rRNA bands of 4 *Frankia* isolates for each isolate 25  $\mu$ l of RNA were loaded on 5 % acrylamide gel electrophoresis.

B-Southern blot hybridization with general *Frankia* primer. The specific hybridization was occurred with 16s rRNA bands.

C-Dot blot hybridization of RNA prepared from 4 *Frankia* isolates hybridized with general *Frankia* probe EP. For each isolate 100 ng of RNA was spotted.

(L) Positive control.

DNA prepared was found crucial for fingerprint of 4 *Frankia* isolates. The yields of DNA were determined spectrophotometrically as 10 15  $\mu$ g/0.05 g of mycelia tissues. The purity of DNA genome as indicated by  $A_{260}/A_{280}$  was 1.8 and DNA quality was evaluated by agarose gel electrophoresis. The PCR conditions for DNA fingerprint were optimized by investigated each factor individually. This included genomic DNA quality and concentration, primer annealing and extension temperature as well as denaturing temperature and time. The optimized conditions were detailed in material and methods section. It was found that quality of genomic DNA extracted was a good template for PCR amplification. However DNA treated with RNase gave sharp and clear amplification products. Ten random primers screened (operon random primer) were surveyed. For the reproducibility of RAPD primers, three independent experiments were performed for each primer. Of the ten primers that were screened in RAPD analysis, 3 primers namely OPA8, OPD and OP were stable, reproducible and gave sufficient polymorphism among four *Frankia* isolates. The distribution of the polymorphic bands which were generated using 3 selected primers among 4 *Frankia* isolates are summarized in Table (1 and 2) and Fig. (2). The results represented that, the primer-1, revealed 16 band total amplified fragment (TAF), 10, specific amplified fragment (polymorphic band) with 63% (PAF) and 6 common amplified fragment (monomorphic band) with 37% (CAF). Primer-2 showed 11 bands, (TAF), 7 bands (CAF) with 64% and 4 bands with 27% and 11 bands (PAF) with 73%.



**Fig. 2:** DNA fingerprint of four *Frankia* isolates (Fc, Fe, Fg and Fh) using primers: 1, 2, 3 by RAPD-PCR analysis.

**Table 1:** DNA fingerprint of four *Frankia* isolates using RAPD-PCR.

Primers	Primer-1				Primer-2				Primer-3			
	Fc	Fe	Fg	Fh	Fc	Fe	Fg	Fh	Fc	Fe	Fg	Fh
Molecular Weight (pb)												
1627	-	-	-	-	-	-	-	-	+	+	+	-
1383	+	-	-	-	-	-	-	-	+	+	-	-
1236	-	-	-	-	-	-	+	+	-	-	+++	+++
1125	-	-	+	+	-	-	-	-	-	-	+	+
1053	-	-	-	-	-	-	+	+	+	+	-	-
925	+	-	-	-	+	+	+	+	-	-	-	-
852	+	+	+	+	-	-	+	+	-	-	+	+
740	-	-	+	+	-	-	-	+	+	-	+++	+++
655	-	+	-	-	-	-	-	-	-	-	+	+
591	+	+	+	+	-	-	-	-	+	+	+	+
460	+	+	+	+	+	+	+	+	+	+	+	+
413	+	+	+	+	-	-	-	-	+	+	-	-
355	+	+	+	+	+	+	+	+	+	+	+	+
307	+	+	-	-	+	+	+	+	-	+	-	-
275	+	+	+	+	+	+	+	+	+	+	+	+
255	-	-	-	-	-	-	-	-	+	+	-	-
232	+	+	-	-	+	+	+	+	-	-	-	-
207	+	+	+	-	-	-	-	-	-	-	-	-
191	-	-	-	-	+	+	+	+	-	-	-	-
176	+	+	+	-	-	-	-	-	-	-	-	-
156	+	+	-	-	-	-	-	-	-	-	-	-
Total No.	13	12	10	8	7	7	10	11	10	10	10	9

Total number of bands; MW = 70 bp; Fc, Fe, Fg and Fh *Frankia* isolates; + Weak amplification; ++ Moderate amplification; +++ Strong fragments.

**Table 2:** Genetic markers and polymorphism among four *Frankia* isolates using random primers by PCR.

<i>Frankia</i> isolates	Polymorphism			Genetic markers				
	TAF	CAF	PAF	Molecular weight (bp)	Fc	Fe	Fg	Fh
Random primers								
Primer-1	16	6	10	1383	+	-	-	-
GTTTCGCTCC				1125	-	-	+	+
				925	+	-	-	-
				740	-	-	+	+
				655	-	+	-	-
				307	+	+	-	-
				232	+	+	-	-
				207	+	+	+	-
				176	+	+	+	-
				156	+	+	-	-
Polymorphic (%)		37	63	10	7	6	4	2
Primer-2	11	7	4	1236	-	-	4	2
AACGCGCAAC				1053	-	-	+	+
				852	-	-	+	+
				740	-	-	-	+
Polymorphic (%)		64	36	4	0	0	3	4
Primer-3	15	4	11	1627	+	+	+	-
CCCCTCAGCA				1383	+	+	-	-
				1236	-	-	+	4
				1125	-	-	+	+
				1053	+	+	-	-
				852	-	-	+	+
				740	+	-	+	+
				655	-	-	+	+
				413	+	+	-	-
				307	-	+	-	-
				255	+	+	-	-
Polymorphic (%)		27	73	11	6	6	6	5

MW: Molecular weight (bp); + Present; - absent of amplified bands. TAF = Total amplified fragments; CAF = Common amplified fragments (monomorphic bands); PAF = Polymorphic amplified fragments (specific bands).

A total of 42 scorable amplified DNA fragments ranging in size from 1383 to 156 bp were observed using the three primers, where as, 25 fragments were polymorphic (PAF) and 17 bands were CAF detected among 4 *Frankia* isolates, (Table 2). The three primers showed polymorphic percentage 60%. As well as among amplified 17 monomorphic bands with the percentage 40%. Interesting to note that, the four *Frankia* isolates were varied in their characterd. Where as, Fc, Fe and Fg isolates revealed 6 while Fh isolate revealed 5 genetic markers.

The genetic variability among bacteria, fungi, actinomycetes isolates and strains for somaclonal variation has yet to be established. Traditional methods based on morphological karyotypic analysis of metaphase chromosome, protein fractions, isozymes and DNA fingerprint have been used to determine genetic variability and identify parental hybrids (Sharma, 2003, Swelim, 2005, Nahid Aiat, 2006 and El-DougDoug *et al.*, 2007). Rapid and unambiguous identification of genetic markers among field isolates has greatly benefited from recent advances in DNA fingerprinting methods based on the polymerase chain reaction (PCR). Random amplified polymorphic DNA (RAPD-PCR) (Sharma, 2003, Swelim, 2005 and Nahid Aiat, 2006).

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