

PCR-RFLP of 16s rRNA Amplification Techniques and Utilization of Different Carbon Sources Used for Identification of *Frankia* Spp. Isolated from Different Egyptian Governorates

¹Jamal S.M. Sabir, ^{1,2,4}S.E.M. Abo-Aba, ²M.M. Mohamed, and ^{1,3}A.M. Gomaa

¹Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

²Faculty of Biotechnology, Misr University for Science and Technology (MUST). Cairo, Egypt.

³Agricultural Microbiology and ⁴Microbial Genetics Departments, National Research Centre, Cairo, Egypt.

Abstract: Symbiotic *Frankia* form specialized structures (nodules) along the root system of the host plants. The occurrence of *Frankia* inside root nodules providing the host plant with fixed nitrogen. The Molecular biology technique (PCR-RFLP) was used to detect the polymorphism of 16s rRNA in addition to the traditional physiological tests for the detection and distinction between the isolated *Frankia* strains. In this study, *Frankia* strains were isolated from ecologically different Egyptian governorates. According to their physiological properties i.e., use efficiency of different carbon sources e.g., sodium propionate, sodium pyruvate, sodium acetate, sucrose, glucose and mannitol in addition to the molecular biology technique, the *Frankia* isolates were identified.

Key words: *Frankia*, carbon sources, 16S rRNA, polymerase chain reaction PCR, Restriction Fragment Length Polymorphism RFLP.

INTRODUCTION

Bacteria are the most dominant group of microorganisms in the soil which reach to several hundred million per gram of soil, depending upon the physical, chemical and biological conditions of the soil. Actinomycetes including *Frankia* sp. are taxonomically belonging to the group of bacteria. *Frankia* are sporulating, Gram-positive filamentous bacteria of the Actinomycetales capable of fixing atmospheric nitrogen, forming symbiotic relationship with different dicotyledonous plants forming symbiotic nodules. This symbiosis termed as actinorhizal by analogy to the mycorrhizal association (Tjepkema and Torrey, 1979). *Frankia* can be differentiated from other actinomycete genera on the basis of morphology, where they form three different types of cells i.e., vegetative hyphae, sporangia, and vesicles (Lechevalier and Lechevalier, 1990). These various cell types can be produced in pure culture, in planta and presumably in soil. Hyphae (0.5-2 µm diam.) are the actively growing cell type; they branch to form a mycelial mat and may differentiate to form vesicles and sporangia. Sporangia which contain multiple ovoid spores commonly develop as pure cultures enter stationary phase. In pure culture vesicles are spherical, thick-walled structures borne on short stalks from the hyphae Gomaa *et al.* (2008) whereas in plants their shape may vary and can be determined by the host. *Frankia* form inside nodules of some, but not all, actinorhizal plants (Schwintzer, 1990).

The ability of *Casuarina* species to form symbiotic N fixing association with *Frankia* is one attribute which makes these tree species potentially important for fuel-wood production, agroforestry and reclamation of infertile soils in the tropics, subtropics and arid zones. Enhancing symbiotic nitrogen fixation of *Casuarina* plantings involves the selection of compatible host plants and effective strains of *Frankia* that fix high rates of atmospheric nitrogen (Dommergues *et al.*, 1984).

Pérez *et al.* (1999) tested various typing methods to develop genetic markers for ecological studies of *Frankia* isolates from Mexico. DNA extracted from clonal cultures of native strains or from reference cultures of *Casuarina*-infective *Frankia* strains was used as the template in polymerase chain reactions (PCR) with primers targeting different DNA regions. *nifH* and 16S rDNA probes from the reference strain *Frankia* Br were

Corresponding Author: Abu-Bakr Gomaa, Agricultural Microbiology Department, National Research Centre, Cairo, Egypt. E-mail: abgomaa@yahoo.com

utilized to authenticate the isolates. Polymorphisms of the restricted fragments of the intergenetic spacer between the 16S-23S rDNAs were analyzed. Repetitive extragenic palindromic sequences (rep-PCR) (BOXA1R primer) were used to generate genomic fingerprints. All studied strains showed two copies of the ribosomal operon and a single copy of the *nifH* gene. PCR - restriction fragment length polymorphism patterns of the 16S-23S intergenetic spacer (IGS) were similar for all *Frankia* isolates; however, the rep-PCR technique was sensitive enough to distinguish between some of these *Frankia* strains. The Mexican cultured strains of *Frankia* nodulating *C. equisetifolia* appeared to be closely related to the isolated and nodular *Frankia* from trees growing outside Australia.

Murry *et al.* (2006) utilized the polymerase chain reaction (PCR) to amplify regions of the *Frankia* genome, they allowing analysis of the microsymbiont genome without first isolating the microbe in pure culture. Root nodules were collected from six *Ceanothus* spp. common to the coastal regions of the Santa Monica Mountains of southern California. Individual lobes were surface-sterilized, total DNA was extracted and amplified using prokaryotic-specific primers. To assess the genetic diversity of *Frankia* endophytes in the population studied, the BOX primer was used to generate genomic fingerprints of prokaryotic nodule inhabitants using rep-PCR. Fingerprint patterns fell into twelve distinct groups indicating the occurrence of genetic diversity of *Frankia* in the nodules sampled. DNA extracts of individual lobes that gave distinct BOX-PCR fingerprints were also amplified by PCR using primers directed against conserved regions of the 16S ribosomal RNA gene. The nucleotide sequences of the PCR products were determined and aligned with the corresponding region from other taxa for phylogenetic analysis. The sequences from *Ceanothus* nodules share a common ancestor to that of the *Elaeagnus*-infective strains.

Gtari *et al.* (2006) studied the occurrence and diversity of *Frankia* nodulating *Elaeagnus angustifolia* in Tunisia were evaluated in 30 soils from different regions by a *Frankia*-capturing assay. Despite the absence of actinorhizal plants in 24 of the 30 soils, nodules were captured from all the samples. Eight pure strains were isolated from single colonies grown in agar medium. On the basis of 16S rRNA and GlnII sequences, seven strains were clustered with *Frankia*, colonizing *Elaeagnaceae* and *Rhamnaceae* in two different phylogenetic groups while one strain described a new lineage in the *Frankia* assemblage, indicating that *Frankia* strains genetically diverse from previously known *Elaeagnus*-infective strains are present in tunisian soils. Genomic fingerprinting determined by rep-PCR, and tDNA-PCR-SSCP, confirmed the wide genetic diversity of the strains.

Gtari *et al.* (2007) *Elaeagnus* compatible *Frankia* isolates from Tunisian soil have been previously clustered with *Frankia*, colonizing *Elaeagnaceae* and *Rhamnaceae* in two different phylogenetic subgroups, while strain BMG5.6 was described as a new lineage closely related to *Frankia* and *Micromonospora* genera. In this study we further assess the diversity of captured *Frankia* and the relationship with BMG5.6-like actinobacteria, by using *nifH* gene sequences. Using PCR-RFLP screening on DNA extracted from lobe nodules, additional microsymbionts sharing BMG5.6 features have been detected proving a widespread occurrence of these actinobacteria in *Elaeagnus* root nodules. Neighbour-Joining trees of *Frankia nifH* sequences were consistent with previously published 16S rRNA and *GlnII* phylogenetic trees. Although four main clades could be discerned, actinobacterial strain BMG5.6 was clustered with *Frankia* strains isolated from *Elaeagnus*. The present study underscored the emanation of new diazotrophic taxon isolated from actinorhizal nodules occupying intermediate taxonomic position between *Frankia* and *Micromonospora*. Moreover, its aberrant position in *nifH* phylogeny should open network investigations on the natural history of nitrogen-fixing gene among actinobacteria.

José *et al.* (2003) Concluded that polymerase Chain Reaction (PCR)-based genomic fingerprints of 16 *Frankia* isolates were obtained using two different primers. rep-PCR DNA fingerprints were obtained by using DR1R primer, and Randomly Amplified Polymorphic DNA (RAPD) fingerprints by using a large primer derived from the 16S rDNA sequence of *Escherichia coli* (879F primer). According to the results obtained, primer DR1R generates strain-specific patterns. However, primer 879F yielded an identical band patterns in two *Frankia* strains, CcI3 and UGL 020603, isolated from different *Casuarina* species and geographical origins, indicating that it could identify genomic fingerprints at a higher taxonomic level (subspecies or species) than DR1R primer.

Analysis of the nucleotide sequence of RNA from the small ribosomal subunit (i.e., 16s rRNA) has gained rapid and wide acceptance in systematic so that the present work aims at isolation of effective pure strains of *Frankia* from ecologically different Egyptian soils. Differentiation between these strains according to their use efficiency of different carbon sources, in addition to genetic characterization using 16s rRNA and restriction enzyme pattern of 16s rRNA PCR RFLP product were done during these study.

MATERIALS AND METHODS

Isolation of *Frankia* sp:

Frankia strains were isolated and identified according to Gomaa *et al.*,(2008) as shown in Table (1).

Use Efficiency of Certain Carbon Sources by *Frankia* Isolates:

A number of carbon sources i.e., sodium pyruvate, sodium acetate, glucose, sucrose and mannitol was tested as substitutions to sodium propionate that is considered the recommended carbon source for *Frankia* growth. BAP medium (Fontaine *et al.*, 1986), without carbon source was autoclaved at 121°C for 15 minutes, while the various tested carbon sources were sterilized separately by filtration through sterile membrane filter of 0.45 µm pore size supplemented to sterile Buckner set. After sterilization both BAP medium and various tested carbon were mixed together to give the required concentration (0.2 g carbon/L). Conical flasks of 250 ml capacity containing 100 ml of BAP medium including the respective carbon source were inoculated with homogenized 0.1 ml of freshly prepared *Frankia* isolates (10 days-old) and the reference strain. After incubation at 28±2°C for 10 days on a reciprocal shaker, each culture was counted using the serial dilution method.

DNA Extraction:

Frankia strains were grown in 5 ml of liquid LB culture, 1.5 ml of the culture were centrifuged for 2 min until a compact pellet forms, the supernatant was discarded. The pellet washed by one ml of SET buffer (20% sucrose, 50mM EDTA, 50mM Tris HCl, pH: 8.0) and re-centrifuged as above. 100 ml SET buffer and 200 ml of SL buffer (SET buffer contains 25 mg/ml lysozyme) were added to the pellet, the tubes inverted several times gently and placed on a water bath on 37°C for 60 min. An equal volume of phenol/chloroform/isoamyl alcohol was mixed thoroughly and spind 5 min in a microcentrifuge. Aqueous viscous supernatant was removed to a fresh microfuge tube, leaving the interface behind, After that an approximately equal volume of chloroform/isoamyl alcohol was added and spind for 5 min. The supernatant was transferred to a fresh tube and equal volume of isopropanol was added to precipitate the nucleic acids. The DNA pellets were washed with 70% ethanol and the pellets were dissolved in 100µl d H₂O.

PCR Analysis:

Primer sequences used to isolate the 16SrRNA gene fragment were : F-968 GC (59-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-39) and R-1346 (59-TAG CGA TTC CGA CTT CA-39) or R-1401 (59-CGG TGT GTA CAA GAC CC-39). The PCR master mix. contained 0.01nM primer 50 nM dNTPs, 1U Taq DNA pol., 3µl of 10x buffer, mixed with 1µl of DNA template , the sterile dH₂O was added to a final volume of 25 µl. Program of the thermal cycler (Biometra, Germany) was programed to 94 °C for 4 min., 94°C for 1 min., 55°C for 1 min., 72°C for 1.5 min, the number of cycles was 35 cycle and the final extension time was (5 min.) at 72°C.

Analysis of the PCR Product:

PCR product was resolved by the use of 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Restriction Enzyme Digestion:

Isolated PCR fragment was digested with the restriction enzyme *CfoI* (Jena Bioscience, Germany) as described by the manufacturer.

Gel Visualization and Documentation:

DNA bands represents the 16 S rRNA gene fragment as well as the restriction digestion pattern was visualized under the UV light and the documentation was carried out using the UV band gel analysis software (Jena lab., UK) in the presence of a 100bp molecular weight DNA standard.

RESULTS AND DISCUSSION

Isolation, Locations and Identification of *Frankia* Sp:

The most important actinorhizal plants are *Casuarinaceae* family which has economic importance in wood production. The ability of *Casuarina* species to form symbiotic N fixing association with *Frankia* is one

attribute which makes these tree species potentially important for fuel-wood production, agroforestry and reclamation of infertile soils in the tropics, subtropics and arid zones. *Frankia* were isolated from different Egyptian locations and identified according to Gomaa *et al.* (2008). The various locations of sampled *Casuarina* nodules and their characteristics are listed in Table (1).

Sixteen isolates of *Frankia* were obtained from nodulated trees of sp. their locations were Kafr El-Sheikh, New valley and Ismailia governorates of Egypt. Soil type and nodules depth and nodule diameter are presented in Table (1).

Table 1: The various locations of sampled *Casuarina* nodules and certain characteristics of soils and nodules.

Governorate	City	Soil type	Host plant	Nodules depth	Nodules diameter
Kafr El-Sheikh	Fac. Agric. Kafr El-Sheikh farm	Clay loam	<i>Casuarina cunninghamiana</i>	20 cm	3 - 5 cm
New valley	Mout City	Clay loam	<i>Casuarina glauca</i>	30 cm	2 - 4 cm
Ismailia	Ismailia Suez Road (10 Km)	Sandy clay loam	<i>Casuarina glauca</i>	15 cm	3 - 4 cm

Differentiation Between Various *Frankia* Isolates:

Distinguishing among the different isolates was accomplished on the base of their use efficiency of certain different carbon sources, and 16s rRNA sequences.

Use Efficiency of Various Carbon Sources:

Different carbon source of some isolates were obtained and were subjected to different formulations of carbon sources without additional exogenous nitrogen source the efficiency of using certain carbon sources i.e., sodium propionate, sodium pyruvate, sodium acetate, sucrose, glucose and mannitol Dada in Table (2) clearly show that the Br reference strain efficiently use glucose as carbon source where it recorded 5.9×10^6 CFU/ml. Furthermore, the isolated *Frankia* sp. I 02 and I 05 preferred application of sodium propionate as a carbon source where their counts reached 1.5×10^6 and 6.5×10^6 CFU/ml. The *Frankia* isolates numbers N 01, N 03, N 04, I 03 and I 04 recorded their highest numbers when sodium pyruvate was applied as a carbon source. Both *Frankia* isolates of K 02 and K 03 efficiently used glucose as a carbon source where their numbers reached 1.6×10^9 and 1.7×10^9 CFU/ml respectively. Of sucrose as a carbon source, it was found that five of the *Frankia* isolates viz. K 01, K 04, K 05, N0 2 and I 01 recorded their growth on sucrose in comparison with the other carbon sources that reached 5.9×10^8 , 3.1×10^8 , 1.2×10^8 , 4.0×10^6 and 1.5×10^6 CFU/ml respectively. Two *Frankia* isolates (N 05 and I 06) were able to efficiently use mannitol more than the other stated carbon sources where their counts reached 2.6×10^6 and 2.7×10^4 CFU/ml consecutively. It was observed that sodium acetate as a carbon source produced the lowest numbers of all tested *Frankia* isolates and the Br reference strain as well when compared with other mentioned carbon sources these results is in agreement with Sarma *et al.* 2006 .

Table 2: Use efficiency of several carbon sources by *Frankia* isolates.

<i>Frankia</i> sp.	Carbon source					
	Sodium propionate	Sodium pyruvate	Sodium acetate	Sucrose	Glucose	Mannitol
	Frankia colony counts (CFU/ml)					
Br	3.8×10^3	3.9×10^3	2.4×10^3	2.3×10^3	5.9×10^4	<30
K 01	7.1×10^4	3.6×10^4	7.0×10^4	5.9×10^6	5.1×10^6	3.7×10^6
K 02	8.4×10^6	4.2×10^4	1.7×10^5	6.0×10^6	1.6×10^8	1.1×10^8
K 03	1.9×10^4	1.6×10^7	4.1×10^4	5.7×10^3	1.7×10^7	9.0×10^4
K 04	1.5×10^5	1.8×10^4	4.9×10^4	3.1×10^6	1.3×10^6	2.5×10^4
K 05	1.9×10^4	5.0×10^4	4.8×10^4	1.2×10^6	9.0×10^5	<30
N 01	2.7×10	6.1×10^3	<30	8.5×10^1	1.0×10^1	<30
N 02	1.4×10^3	5.4×10^3	1.3×10^3	4.0×10^4	2.8×10^3	2.0×10^4
N 03	3.2×10^1	3.6×10^5	<30	6.2×10^4	3.3×10^3	4.4×10^3
N 04	<30	2.2×10^4	<30	4.2×10^3	<30	8.4×10^2
N 05	2.4×10^3	3.7×10^2	2.0×10^2	2.5×10^3	1.5×10^3	2.6×10^4
I 01	3.5×10^1	4.5×10^3	1.6×10^3	1.5×10^4	1.3×10^3	8.5×10^3
I 02	1.5×10^4	4.7×10^3	1.2×10^4	2.1×10^2	5.0×10^2	5.2×10^3
I 03	9.6×10^1	1.0×10^5	3.2×10^2	8.9×10^2	7.2×10^2	5.2×10^3
I 04	8.5×10^2	9.7×10^3	5.3×10^3	1.1×10^3	1.4×10^3	1.6×10^3
I 05	6.5×10^4	4.1×10^3	1.2×10^3	8.6×10^3	3.2×10^3	1.3×10^3
I 06	6.1×10^1	1.7×10^2	7.9×10^1	1.3×10^2	2.3×10^2	2.7×10^2

PCR Analysis:

DNA from Frankia strains extracted and two universal oligonucleotide primers were used in this study for the detection of the 16S rRNA gene fragment as a characteristic tool for *Frankia* taxonomy. However, those two universal primers generated one common bands in our isolates it was has the same molecular weight (480 bp) as shown in Fig.(1). This result is expected since the used primers are universal used primer for 16s rRNA. Also, this result is in accordance with the results that carried out by Watanabe, 2001 who stated that the universal primers used in this study it generates usually a common DNA band.these results proved that genetic similarities among *Frankia* isolated strains on *Casuarinaceae*, so that theRFLP of generated 16s rRNA PCR products were done to distinguish between isolated strains.

Restriction Enzyme Digestion:

PCR amplification products were analyzed by using *CfoI* restriction endonuclease were done as shown in Fig. (2). Restriction enzyme digestion pattern of the generated PCR fragment with *CfoI* produced one common band along the tested *Frankia* strains with a molecular weight of 290 bp. A second band has a molecular weight of 200 bp. was common at all tested strains except strains K03, K04 and I05. Instead, these strains that mentioned have a unique band of a 90 bp long. RFLP16s rRNA indicated that all isolated strains had similar patterns so that, it belonging to the apparent high homogeneity of *Frankia* strains, these results probably relates to the different locations from which the strains were obtained. Results also proved that the genetic similarities and closely related *Frankia* strains in most of all isolated strains, except K03, K04 and I05 it will be noted seconded band at 90bp. The obtained results demonstrated that some genetic diversity between these strains than others as shown in Fig. (2). these results are in agreement with Rouvier, *et al.*(1996) and El-Sayed, (2008).

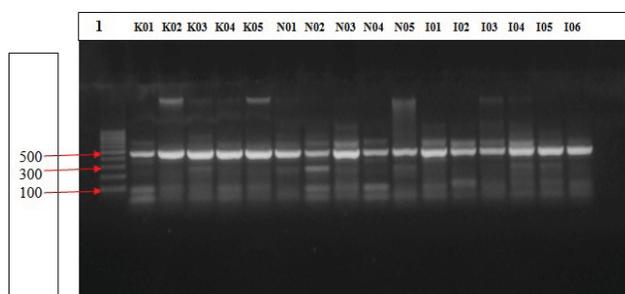


Fig. 1: A- 16 SrRNA gene fragment isolation via PCR.

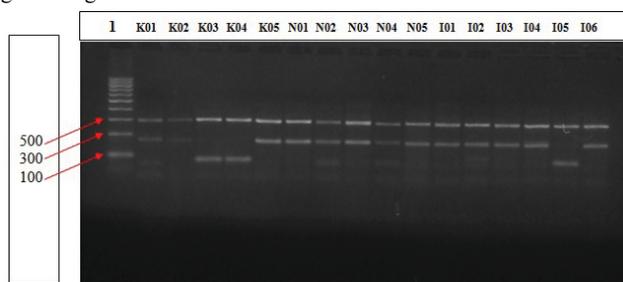


Fig. 2: DNA fingerprinting via PCR-RFLP analysis of restriction analysis of PCR product.

REFERENCES

Dommergues, Y.R., H.G. Diem, D. Gauthier, B.L. Dreyfus and F. Cornet, 1984. Nitrogen-fixing trees in the tropics: Potentialities and limitations. In: Advances in Nitrogen Fixation Research, pp:7-13. (Eds., Veeger, C. and W.E. Newton). Nijhoff/W. Junk. Pudoc Publ. Wageningen, The Netherlands.

El-Sayed, A.A., 2008. Characterization *Frankia-casuarina* Isolates by Fatty Acid Analysis and Detection of 16s rRNA Against Oligonucleotide Probes. Research Journal of Agriculture and Biological Sciences, 4(6): 832-841.

Fontaine, M.F., P.H. Young and J.G. Torrey, 1986. Effect of long term preservation of *Frankia* strains on infective, effective and *in vitro* nitrogenase activity. Appl. Environ. Microbiol., 51: 694-968.

- Gomaa, A.M., S.E.M. Abo-Aba, and N.S. Awad, 2008. Isolation, Characterization and Genetic Differentiation of *Frankia* sp. Isolated from Ecologically Different Egyptian Locations. *Research Journal of Cell and Molecular Biology*, 2(1): 6-17.
- Gtari, M., L. Brusetti, A. Hassen, D. Mora, D. Daffonchio and B. Abdellatif, 2007. Genetic diversity among *Elaeagnus* compatible *Frankia* strains and sympatric-related nitrogen-fixing actinobacteria revealed by *nifH* sequence analysis *Soil Biology and Biochemistry*, 39(1): 372-377.
- Gtari, M., L. Brusetti, A. Hassen, D. Mora, D. Daffonchio and Abdellatif Boudabous, 2006. Isolation of *Elaeagnus*-compatible *Frankia* from soils collected in Tunisia. *FEMS Microbiology Letters*, 234(2): 349 - 355.
- José, I.M., V. Angel, R. Raul, M.F. Pedro, C. Rodriguez-Barrueco, M. Eustoquio, C. Emilio and V. Encarna, 2003. Genomic fingerprinting of *Frankia* strains by PCR-based techniques. Assessment of a primer based on the sequence of 16S rRNA gene of *Escherichia coli*. *Plant and soil*, 254(1): 115-123.
- Lechevalier, M.P. and H.A. Lechevalier, 1990. Systematics, isolation and culture of *Frankia*, pp: 35-60. In C.R. schwintzer and J.D. 5-Tjepkema (ed.). *The biology of Frankia and actinorhizal plant*. Academic Press, Inc, New York.
- Murry, M., A.S. Konopka, S.D. Pratt and T.L. Vandergon, 2006. The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. *Physiologia Plantarum*, 99(4): 714 – 721.
- Pérez, N., H. Olivera, L. Vásquez and M. Valdés, 1999. Genetic characterization of Mexican *Frankia* strains nodulating *Casuarina equisetifolia*. *Can. J. Bot.*, 77(9): 1214–1219.
- Rouvier, C., O. Yves, P. Prin Reddell, P. Normand and P. Simonet, 1996. Genetic Diversity among *Frankia* Strains Nodulating Members of the Family Casuarinaceae in Australia Revealed by PCR and Restriction Fragment Length Polymorphism Analysis with Crushed Root Nodules. *Applied and environmental microbiology*, 979–985.
- Sarma, H.K., B.K. Sharma, S.S. Singh, S.C. Tiwari and A.K. Mishra, 2006. Polymorphic distribution and phenotypic diversity of *Frankia* strains in nodulated lobes of *Hippöphae salicifolia* D. Don *Current Science*, 90(11): 1561-1521.
- Schwintzer, C.R., 1990. Spore-positive and sporenegative nodules pp: 177-193. In C.R. schwintzer and Tjepkema (ed.). *The biology of Frankia and actinorhizal plant*. Academic Press, Inc, New York.
- Tjepkema, J.D. and J.G. Torry 1979. *Symbiotic Nitrogen Fixation in actinomycetes nodulated plant* preface. Bot. Goz. (Chicago) suppl. 140.
- Watanabe, K., Y. Kodama and S. Harayama, 2001. Design and evaluation of PCR primers to amplify bacterial 16s ribosomal DNA fragment used for community fingerprinting. *Journal of Microbiological Methods*, 44: 253-262.