J. Biol. Control, 21(2): 203-209, 2007

Biochemical and molecular profiling of indigenous *Xenorhabdus* isolates associated with *Steinernema* spp.*

H. S. VIDYA and M. NAGESH
Project Directorate of Biological Control, Post Box No.2491,
H. A. Farm Post, Bellary Road, Bangalore – 560024, Karnataka, India
E mail: vidyapragu@yahoo.com

ABSTRACT: Bacterial symbionts were obtained from entomopathogenic nematodes *Steinernema carpocapsae, S. riobrave, S. feltiae* and *S. tami*, and identified as belonging to *Xenorhabdus* species by subjecting to biochemical characterization. The *Xenorhabdus* isolates were further characterized for their interspecific variation by protein profiling and RFLP analysis of 16S rDNA. The protein profiles recorded discernible differences with protein distribution ranging from 97Kda to 14Kda, but most of the fragments were common to all isolates. RFLP analysis of 16S rDNA of the isolates using eight restriction enzymes showed the distinctness of isolates and based on the restriction enzyme patterns, the isolates were grouped into two clusters. The study showed that a combination of biochemical and molecular techniques could be used for the identification and characterization of *Xenorhabdus* isolates.

KEY WORDS: Indigenous isolates, PCR-RFLP, *Steinernema*, *Xenorhabdus*, 16S rDNA

INTRODUCTION

Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae carry in their intestine species-specific bacterial symbionts belonging to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Akhurst and Boemare, 1990). The third stage infective juveniles of the nematodes enter the insect's haemocoel and release their symbiotic bacteria. These bacteria grow and reproduce in the haemocoel and produce toxins to kill the host and antibiotics to prevent the putrefaction of the host. In these mutualistic associations the nematodes transport their bacterial symbionts between insect host, protects the bacteria from the soil environment and carry the bacteria into the haemocoel of insect hosts, whereas the modified cadavers provide the suitable nutrients for the reproduction of the nematodes.

Studies on the taxonomic identification and specificity of *Xenorhabdus* and *Photorhabdus* bacteria belonging to the family Enterobacteriaceae are important as they kill a variety of insect pests. Various workers have reported the identification and classification of *Xenorhabdus* isolates by conventional phenotypic and physiological tests. However, these tests failed to distinguish the isolates from one another (Akhurst and Boemare,

* Part of research work of the senior author for Ph.D. program at University of Mysore, Mysore, India

1988). Accordingly, for accurate identification and delineation of isolates, molecular techniques like SDS-PAGE, PCR-RFLP and DNA-DNA hybridization have routinely been employed and proved to be successful tools for the identification of *Xenorhabdus* isolates (Phyllis *et al.*, 1984; Brunel *et al.*, 1997). 16S rDNA gene sequences were found to be useful in analyzing the phylogeny of *Photorhabdus* species (Brunel *et al.*, 1997). We recently reported that Rapid Amplification of Polymorphic DNA (RAPD) analysis could be used to differentiate four indigenous *Xenorhabdus* isolates (Vidya and Nagesh, 2006). In the present study, the work involved studies on biochemical and molecular characterization of local *Xenorhabdus* isolates and their genetic relatedness were assessed by profiles generated by SDS-PAGE and PCR-RFLP of 16S rDNA.

MATERIALS AND METHODS

Bacterial sources and maintenance

The bacterial isolates studied previously for RAPD analysis (Vidya and Nagesh, 2006) were utilized in the present investigation. *Xenorhabdus nematophilus* (SCX3) was isolated from *S. carpocapsae* from Bangalore. *Xenorhabdus* sp. (SCX6, SCX8) was isolated from *S. riobrave* and *S. tami* from Gujarat and Jorhat, respectively. *S. feltiae* was used for the isolation of *Xenorhabdus bovenii* (SCX7) from Bangalore. The nematode isolates were obtained from Nematode biological control laboratory of the Project Directorate of Biological Control, Bangalore. The bacterial isolates were maintained on Mac-conkey agar plates at 4-8°C with a subculture interval of approximately one month. The cultures were checked periodically for purity on NBTA plates. Blue colored colonies were picked and streaked onto plates of nutrient agar. Colonies that had grown at 25°C for 24 hrs on nutrient agar plates were used to make the bacterial suspensions for all the tests in this study.

Biochemical tests

All tests were conducted at 28°C and each colony type of four *Xenorhabdus* isolates, *viz.*, SCX3, SCX6, SCX7, and SCX8 were subjected to catalase test, oxidase test, gelatin hydrolysis test, casein hydrolysis test, citrate utilization test and sucrose fermentation. Catalase activity was determined by inoculating single isolated colonies of bacteria on a glass slide and flooding with 10 per cent hydrogen peroxide. Casein hydrolysis test was conducted by growing colonies on nutrient agar containing 10 per cent skimmed milk and by observing the zone of hydrolysis. The method of Dye (1968) was followed for determination of oxidase, starch hydrolysis reduction of nitrate, production of urease and release of reducing sugars from sucrose and utilization of organic acids. Lecithinase acitivity was tested on yeast extract salts agar containing fresh egg yolk emulsion. Peroxidase enzymatic activity was done as determined by Anderson (1930). The activity of cytochrome c oxidase was estimated according to Schaeffer (1961).

Analysis of total protein by electrophoretic technique

Preparation of soluble proteins

Bacterial cultures were centrifuged at 2500g for 10 mins. The pellets were resuspended in Tris / Hcl pH 7.5, 50 mM Nacl and sonicated on ice for 20 s.

Electrophoretic techniques

SDS-PAGE was performed according to Laemmli (1970). Gels and buffers were used as described by Davis (1964). Soluble proteins (300-400ug) were loaded on 1:25mm thick, vertical slab gels (Bio-Rad). Proteins were electrophoresed at 20mV and increased to a constant of 45mV for approximately two hours. Protein profiles were visualized by staining gels with 10 per cent (W/V) comassie brilliant blue in 50 per cent methanol /10 per cent glacial acetic acid at 50°C for 1 hr. Gels were destained in 25 per cent methanol / 10 per cent glacial acetic acid and stored in the solution. Molecular weight of desired proteins in kilodaltons (Kda) was determined by comparing the distance between the gels and their protein bands to logarithmic transformation of molecular weight plotted against the distance traveled by standards (Weber and Osborn, 1969).

DNA Extraction, PCR Conditions and RFLP analysis

Total genomic DNA was extracted from the cultures according to Sambrook *et al.* (1989). PCR cycling conditions were optimized as previously described (Vidya and Nagesh, 2006). The genetic distance and per cent similarity among the isolates were determined based on Nei and Li (1979) distance.

RFLP of PCR-amplified 16SrDNAs

The 16S rDNA region was amplified by using the universal 16S rDNA primer pair designed by Weisburg *et al.* (1991) in the amplification of the almost complete 16S rDNA. RFLP analyses (Fisher le-saux *et al.*, 1998) were performed by utilizing 5-15μl of purified PCR products. The eight enzymes were used according to the recommendations of the manufacturer (Genei, India). *HinfI, AluI, MspI, SmaI, NheI, KpnI, HindIII* and *EcoRI* DNA digests and DNA molecular weight markers were analyzed by horizontal electrophoresis at 4Vcm^{-1} in 2% (w/v) in agarose gel (AMRESCO) with TBE buffer. For each restriction enzyme, presence of band at each position was recorded as 1 and absence as 0 (zero) and using this data pairwise squared Euclidean distance was calculated. Based on this distance matrix, cluster analysis was done using minimum variants algorithm using the software for cluster analysis (STATISTICA).

RESULTS AND DISCUSSION

Biochemical tests

The differentiating characteristics for the study of *Xenorhabdus* isolates are summarized in Table 1. All four isolates gave positive results for gelatin liquefaction, casein hydrolysis, and

Table 1. Biochemical tests on four indigenous isolates of *Xenorhabdus*

Biochemical characteristics	*Xenorhabdus nematophilus* (SCX3)	*Xenorhabdus* sp. (SCX6)	*Xenorhabdus* sp. (SCX8)	*Xenorhabdus bovenii* (SCX7)
Catalase	-	-	-	-
Oxidase	-	-	-	-
Cytochrome oxidase	-	-	-	-
Peroxidase	-	-	-	-
Gelatin liquefaction	+	+	+	+
Casein Hydrolysis	+	+	+	+
Reducing compounds from	-	-	-	-
Sucrose	-	-	-	-
Potato Starch hydrolysis	-	-	-	-
Nitrate reduction	+	+	+	+
Utilization of organic acids	+	+	+	+
Growth on the Mac-conkey agar				
Pigmentation	Buff	Light pink	Yellow	light pink
Urease	-	-	-	-
Lecithinase	+	+	+	+

utilization of organic acids, lecithinase activity and growth on the Mac-conkey agar. The isolates gave negative results for reducing compounds from sucrose and potato starch hydrolysis. The isolates varied in their pigmentation with *Xenorhabdus nematophilus* (SCX3) showing buff color, *Xenorhabdus* sp. (SCX6) showing yellow, *X. bovenii* (SCX7) and *Xenorhabdus* sp. (SCX8) showing pink colonies. These characters confirmed that the indeginous isolates belong to the genus *Xenorhabdus* (Buchanan *et al.*, 1974).

SDS-PAGE profiles of total cell proteins

The whole-cell protein patterns of four indigenous isolates were analyzed by SDS-PAGE (Fig. 1). A band of approximately 43 Kda was common to all *Xenorhabdus* isolates and few other bands were considered common between isolates. For example, 20kda and 66 kda band was exhibited by *X. nematophilus* (SCX3) and *Xenorhabdus* sp. (SCX6), respectively. Also, banding pattern of *X. nematophilus* (SCX3) and *Xenorhabdus* sp. (SCX6) revealed protein sizes ranging from 97Kda to 14Kda and that of *X. bovenii* (SCX7) and *Xenorhabdus* sp. (SCX8) ranged from 66 Kda to 14 Kda, respectively. Very little qualitative differences were observed and they were not significant enough to separate the isolates into clusters.

PCR amplification of 16S ribosomal DNA

PCR-based RFLP analysis of 16S rDNA is a practical molecular technique that is free of most subjective interpretation and allows efficient identification of bacterial isolates. DNA isolated from the four isolates was amplified with universal 16S ribosomal primers. They produced a single band of about 1600 bp (Fig. 2). The size of the obtained fragment was in accordance with the expected sizes of the published data of 16S rDNA sequences of *Xenorhabdus* (Brunel *et al.*, 1997).

RFLP analysis

Restriction analysis of 16S rDNA and bacterial species complement each other well and hence RFLP typing of 16S rDNAs was successfully used to identify the species. The PCR amplified products of 16S rDNA were cleaved by eight tetrameric restriction enzymes. Six enzymes, viz., *HinfI*, *AluI*, *MspI*, *SmaI*, *KpnI* and *EcoRI* were

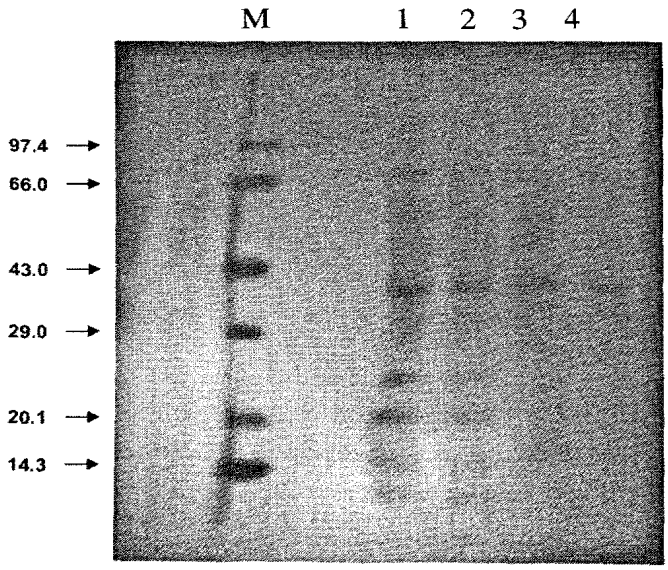


Fig. 1. Protein profile of *Xenorhabdus* isolates, M- Medium Range Marker. Lane -1 SCX3, Lane -2 SCX6, Lane -3 SCX7, Lane- 4 SCX8,

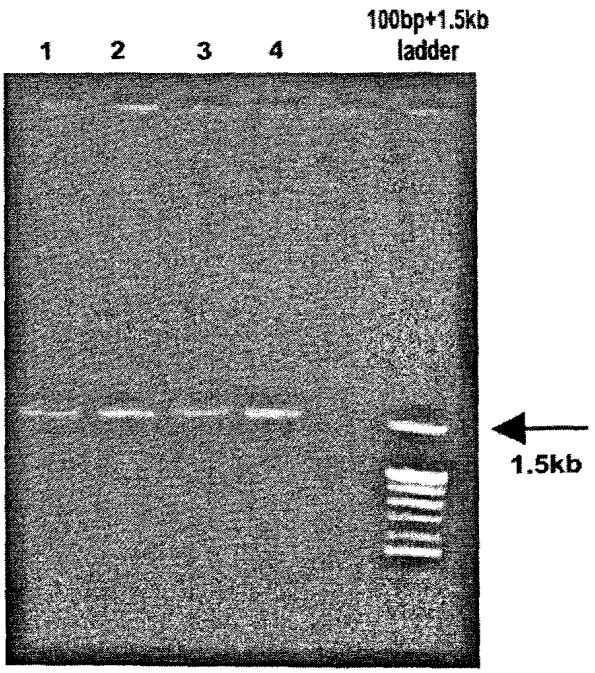


Fig. 2. Amplified products of 16S rDNA. Lane -1 SCX3, Lane -2 SCX6, Lane -3 SCX7, Lane- 4 SCX8, Lane- 5 Marker- 100bp+1.5KB DNA ladder

polymorphic and two enzymes were monomorphic (*NheI, HindIII*). The banding pattern is as in Fig. 3 and 4. A total of 43 fragments were visually observed, of which 26 were polymorphic.

The number of polymorphic bands observed was more with the enzymes *HinfI, AluI,* and *MspI* and hence they can be used as a basis for differentiation of indigenous isolates of *Xenorhabdus*. The average per cent similarity obtained is shown in Table 2. Similarity matrix obtained indicated that the isolates *X. nematophilus* (*S.carpocapsae)* and *Xenorhabdus bovenii* (*S. feltiae)* are the closest (Fisher le-saux *et al.,* 1998) at 86.90 per cent and *Xenorhabdus* sp. (*S. riobrave*) and *Xenorhabdus* sp. (*S. tami*) were the farthest with the per cent similarity of 57.24.

The genetic distances were calculated and clustered by unweighted pair group method using arithemetic averages. The dendrogram obtained is as in Fig 5. Obvious correlation can be derived between the geographic origin of the isolates and their phylogenetic relationships based on RFLP pattern. Two isolates, *i.e. X. nematophilus* (SCX3) and *Xenorhabdus bovenii* (SCX7), isolated from Karnataka formed one group, whereas *Xenorhabdus* sp. (SCX6) and *Xenorhabdus* sp. (SCX8) from Gujarat and Assam formed the other group.

Among the four isolates analyzed by RFLP (Fig. 5) and RAPD (Vidya and Nagesh, 2006), we found that the topologies of two dendrograms were different. The cluster analysis derived from the RFLP was not congruent with that estimated from RAPD patterns. The reason being, probably, RAPD fragments are effective in identification of isolates belonging to the same species. RFLP analysis of amplified fragment of 16S rDNA is more effective for the study of interspecific isolates as the repeat unit of ribosomal DNA contains potentially highly variable and conserved regions (Nadler, 1990).

In order to comprehensively study the genetic relationships of the isolates and to assign their taxonomical status, a combination of data from different approaches was investigated and it was evident that all the four indigenous bacterial isolates had similar biochemical characteristics. They were genotypically different as revealed by the variations in SDS-PAGE total proteins, RFLP patterns of 16SrDNA gene and RAPD markers

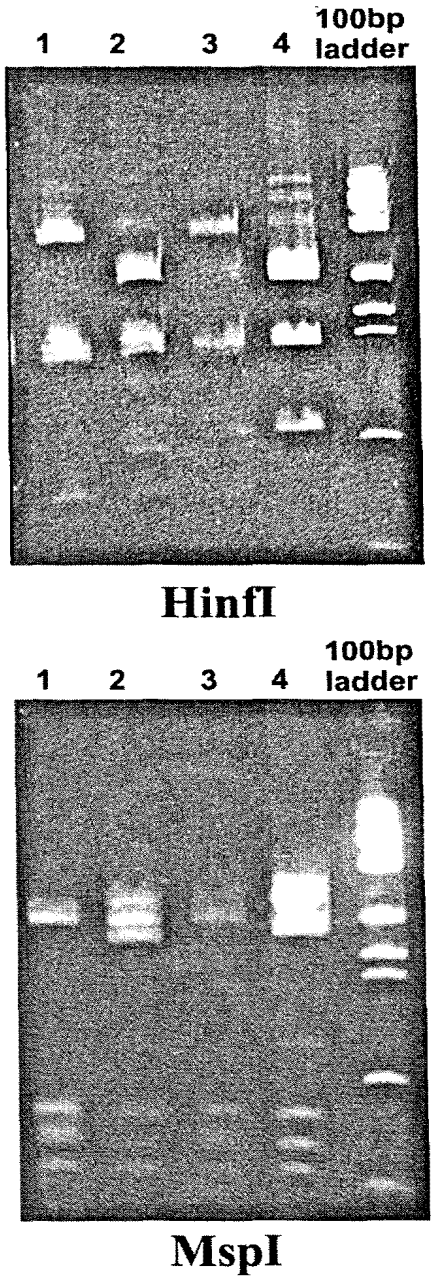
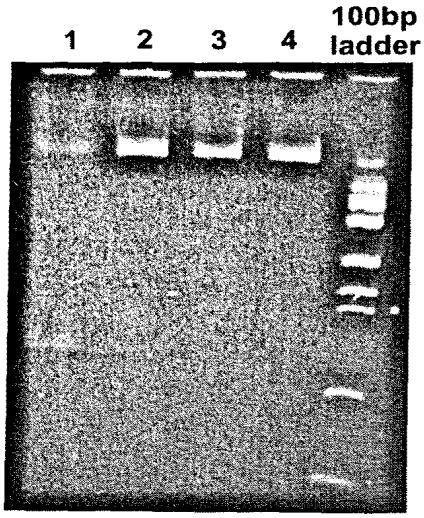


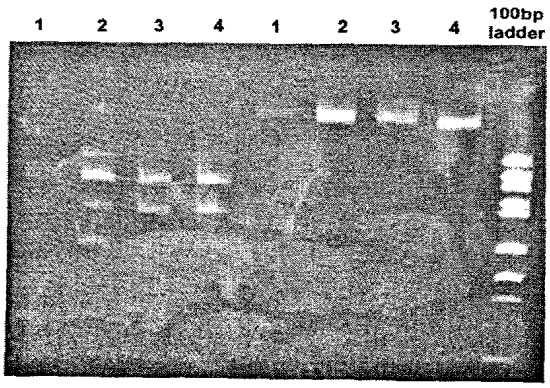
Fig. 3. Restriction Pattern of PCR-Amplified product of 16Sr DNA digested with *AluI, HinfI* and *MspI*. Lane -1 SCX3, Lane -2 SCX6, Lane -3 SCX7, Lane- 4 SCX8, Lane- 5 Marker 100bp ladder

Table 2. Average per cent similarity among the four *Xenorhabdus* isolates

Bacterial isolates	*Xenorhabdus nematophilus* (SCX3)	*Xenorhabdus* sp.		*Xenorhabdus bovenii*
		(SCX6)	(SCX8)	(SCX7)
SCX3	100.0	63.02	57.24	86.90
SCX6	63.02	100.0	77.65	59.24
SCX7	86.90	59.24	58.0	100.0
SCX8	57. 24	77.65	100.0	58.0



NheI



SmaI and HindIII

Fig. 4. Restriction pattern of PCR-amplified product of 16S rDNA digested with *EcoRI*, *KpnI*, *NheI*, *SmaI* and *HindIII*. Lane -1 SCX3, Lane- 2 SCX6, Lane 3- SCX7, Lane-4 SCX8, molecular weight Marker -100bp ladder

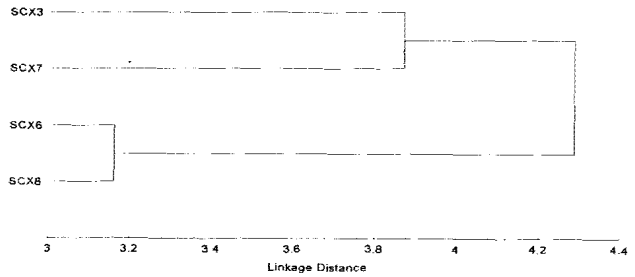


Fig 5. Cluster analysis (UPGMA) of the four PCR - RFLP genotypes of 16S rDNA

(Vidya and Nagesh, 2006). Hence, molecular data provide support for the study of interspecific variation in indigenous *Xenorhabdus* isolates.

ACKNOWLEDGMENTS

We are grateful to Dr. R. J. Rabindra, Director, Project Directorate of Biological Control and Dr. S. S. Hussaini, Principal Scientist for constant help and guidance. The senior author would like to acknowledge Project Directorate of Biological Control and University of Mysore for providing an opportunity to pursue the Ph. D. program. Financial support for this study through a project grant from the Department of Biotechnology, New Delhi, is gratefully acknowledged.

REFERENCES

Akhurst, R. J. and Boemare, N. E. 1988. A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology*, **134**: 1835-1845.

Akhurst, R. J. and Boemare, N. E. 1990. Biology and taxonomy of *Xenorhabdus*, pp. 75-90. In: Gaugler, R. and Kaya, H. K. (Eds.), *Entomopathogenic Nematodes in Biological Control*. CRC Press, Boca Raton, Florida, USA.

Anderson, L. R. 1930. Peroxidase in relation to bacterial growth, with special reference to the influenza bacillus. *Journal of Bacteriology*, **20**: 371-379.

Brunel, B., Givaudan, A., Lanois, A., Akhrust R. J. and Boemare, N. E. 1997. Fast and accurate identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR-amplified 16SrRNA genes. *Applied Environmental Microbiology*, **63**: 574-580.

Buchanan, R. E. and Gibbons, N. E. (ed.). 1974. *Bergey's manual of determinative bacteriology*. 8th ed. The Williams and Wilkins Co., Baltimore.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, **121**: 404-427.

Dye, D. W. 1968. A taxonomic study of the genus *Erwinia*. I. The "*Amylovora*" group. *New Zealand Journal of Science*, **11**: 590-607.

Fisher-le-saux, M., Mauleon, H., Constant, P., Brunel B., and Boemare N. E. 1998. PCR ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from Caribbean region to the taxonomy and geographic distribution of their nematode hosts. *Applied and Environmental Microbiology*, **64**: 4296-4254.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Nadler, S. A. 1990. Molecular approaches to studying helminth population genetics and phylogeny. *International Journal of Parasitology*, **20**: 11-29.

Nei and Li, 1979. Mathematical model for studying genetic variations in terms of restriction endonucleases. *Proceedings, National Academy of Science USA* **74**: 5267-5273.

Phyllis, G., Hotchkin and Kaya H. K. 1984. Electrophoresis of soluble proteins from two species of *Xenorhabdus*, bacteria mutuallistically associated with nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Journal of General Microbiology*, **130**: 2725-2731.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA.

Schaeffer, E. 1961. The use of the cytochromoxidase reaction for detecting *Pseudomonas aeroginosa* in mixed cultures. *Roentgen lab. Prax.* **14**: 42-146.

Vidya, H. S. and Nagesh, M. 2006. RAPD analysis of Indian isolates of *Xenorhabdus* species associated with entomopathogenic nematode *Steinernema* spp. *International Journal of Nematology*, **16**: 178-185.

Weber, K. and Osborn, M. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry*, **244**: 4406-4412.

Wiesburg, G. W., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, **173**: 697-703.

(Received: 11.11.2006; Revised: 22.06.2007; Accepted: 02.07.2007)