Journal of Entomological Society of Iran
2011, 31(1), 57-69

Characterization of *Xenorhabdus* (γ-Proteobacteria) strains associated bacteria with the *Steinernema* (Nematoda: Steinernematidae) isolates from Iran

J. Karimi[1&*], A. Kharazi-Pakdel[2], T. Yoshiga[3], M. Koohi Habibi[2] and M. Hassani-Kakhki[1]
1. Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, 9177948974, Iran, 2. Department of Plant Protection, Campus of Agriculture and Natural Resources, University of Tehran, Karaj, Iran, 3. Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Saga, Japan.
*Corresponding author, E-mail: jkb@um.ac.ir

Abstract

The gram-negative bacterium *Xenorhabdus* is mutualistically associated with entomopathogenic nematode, *Steinernema*. Three bacterial strains were isolated from the entomopathogenic nematodes, *Steinernema glaseri* (Steiner) and *S. carpocapsae* (Weiser), of the white grubs, *Polyphylla olivieri* (Laporte de Castelnau) in Iran. This study was focused on the characterization of the bacterial strains by using phenetic characters and 16S ribosomal RNA gene sequence. This polyphasic approach led to the identification of all strains as *Xenorhabdus* spp., separated as *X. nematophila* and *X. poinarii*. The phylogenetic relationship between Iranian and global strains of *Xenorhabdus* was analyzed and discussed. Characterization of symbiotic bacteria associated with the entomopathogenic nematodes is a main step to the study of different aspects of this complex. These results provide new insights for the biodiversity of bacto-helminthic complex in Iran.

Key words: *Xenorhabdus poinarii*, *Xenorhabdus nematophila*, *Steinernema glaseri*, *Steinernema carpocapsae*, characterization, Iran

چکیده

باکتری‌های *Xenorhabdus* همزیست اختصاصی نماتدهای بیمارگرحشرات در جنس *Steinernema* می‌باشند. در این بررسی مشخصات سه جدایه‌ی باکتری جدا شده از دو گونه نماتد بیمارگر حشرات *Steinernema glaseri* (Steiner) و *S. carpocapsae* (Weiser) تعیین شده است. این دو گونه‌ی بیمارگر از جمعیت طبیعی لاروهای کرم سفید ریشه، *Polyphylla olivieri* (Laporte de Castelnau)، جداسازی شدند. مطالعه‌ی حاضر بر روی مشخصات اختصاصی این جدایه‌های باکتری متمرکز بود و با استفاده از مشخصات فنتیک و توالی ژن 16S ریبوزومی تعیین هویت صورت گرفت. این بررسی منجر به شناسایی هر سه جدایه به عنوان *Xenorhabdus* spp. و همچنین تفکیک دو گونه‌ی *X. nematophila* و *X. poinarii* از یکدیگر گردید. روابط فیلوژنی در میان ایزوله‌های ایرانی *Xenorhabdus* و نمونه‌های جهانی تجزیه و تحلیل شده و مورد بحث قرار گرفتند. شناخت مشخصات باکتری‌های همزیست نماتدهای بیمارگر حشرات، مرحله‌ای اساسی در مطالعه‌ی این گونه از نماتدها است. نتایج این بررسی، اطلاعات جدیدی را در مورد تنوع این مجموعه نماتد- باکتری بیمارگر حشرات، در ایران فراهم نموده است.

واژگان کلیدی: *Xenorhabdus poinarii*، *Xenorhabdus nematophila*، *Steinernema glaseri*، *Steinernema carpocapsae*، تعیین مشخصات، ایران

Introduction

Entomopathogenic nematodes (EPNs) carry bacterial symbionts that are responsible for killing the insect hosts (Akhurst, 1983; Forst & Clarck, 2002). The bacterial symbionts of the genus *Xenorhabdus* (Gammaproteobacteria: Enterobacteriaceae) thrive in entomopathogenic nematodes of the genus *Steinernema* (Steinernematidae) (Bird & Akhurst, 1983; Boemare *et*

al., 1993; Fischer-Le Saux *et al*., 1999a). In natural populations, a given nematode species carries a specific bacterial species, although one specific bacterial species may be associated with several nematode species (Akhurst & Boemare, 1990; Fischer-Le Saux *et al*., 1999b).

The symbiosis between both partners is a fascinating model of co-speciation. The bacteria are carried monoxenically in a differentiated vesicle within the gut of infective juveniles (IJs) and are released into the insect hemocoel after the nematode invasion (Adams & Nguyen, 2002; Adams *et al*., 2006). Death of the insect occurs within 48 hours (Forst *et al*., 1997; Burnell, 2002). As they are of potential use against some important lepidopteran, dipteran and coleopteran pests, this symbiosis provides an efficient biological control of commercial crop pests (Hazir *et al.*, 2003). It appears that the symbiont transmission becomes more efficient as the nematode carries more *Xenorhabdus* cells, although in facultative interactions between the nematode and the bacteria lower number of bacteria are carried (Sicard *et al*., 2003). The identification and characterization of EPNs in Iran has started since 2000 and several species of *Steinernema* and *Heterorhabditis* have been already isolated (Parvizi, 2003; Tanha Ma'afi *et al.*, 2006; Eivazian Kary *et al*., 2009; Karimi *et al*., 2009a, 2009b). The study of diversity of Iranian species of *Steinernema* in Mazandaran, Tehran, West-Azerbaijan, East-Azerbaijan and Ardabil provinces led to the discovery of following species: *S. carpocapsae* (Weiser), *S. feltiae* (Filipjev), *S. glaseri* (Steiner), *S. monticolum* Stock *et al.* and *S. bicornutum* Tallosi *et al*. The genus *Heterorhabditis* is represented only by the species *H. bacteriophora* Poinar in Iran. Based on few studies, phylogenetic position of native EPNs species/isolates has been studied. Similar to EPNs, there is little knowledge about their symbiotic bacteria. In any comprehensive study on the characterization of EPNs, isolation and identification of their symbiont is a necessary step. Moreover, from practical point of view, reliable information about bacterial symbiont of any EPN has to be acquired through mass production systems and gnotobiological surveys. Here, several isolates of *Steinernema* were isolated from the larvae of white grub, *Polyphylla olivieri* (Laporte de Castelnau) (Col.: Scarabaeidae) in Tehran province (Karimi *et al*., 2009a, 2009b) and the associated symbiont with Iranian *Steinernema* was characterized.

Materials and methods

Phenetic characterization

Three isolates of *Steinernema* species, including *S. carpocapsae* (Iran 4 and Iran 6) and *S. glaseri* (Iran2) were isolated from larval stages of the white grubs as natural pathogens in Tehran

province, Iran. Bacteria were extracted from 100 freshly emerged IJs (Akhurst & Boemare, 1988). The IJs were immersed in sterile Ringer's solution with 10% w / vol sodium hypochlorite for 10 minutes to avoid possible external contamination from the tegument. The IJs were then rinsed twice in sterile Ringer's solution and crushed in 1 ml of sterile PBS buffer lacking Mg^{2+} and Ca^{2+} salts (8 gr NaCl, 0.2 gr KCl, 1.15 gr Na_2HPO_4, 0.2 gr KH_2PO_4, 1 l H_2O sterile). One hundred ml of the suspension streaked onto NBTA medium (Akhurst & Boemare, 1988, 2005). After examining different methods for isolating the symbiont of *S. glaseri*, we decided to use freshly harvested IJs from cadaver washing them three times using centrifugation with sterilized water. A small number of IJs were transferred to a drop of physiological saline (M9) solution in sterile Petri dishes and then transferred to 5% of chlorox. After incubation for 2 minutes, IJs were transferred to sterilized M9 drop and then cut with a sterile platinum wire. After serial dilution of M9, they placed into NBTA plates (Poinar & Thomas, 1966).

All *Xenorhabdus* strains were cultured at 28°C on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA medium), in order to control the presence of phase I and phase II variants (Akhurst & Boemare,1988; Volgyi *et al*., 1998).

Colony morphology and dye uptake

The LB, NBTA and MacConkey agar plates were used as described by Akhurst & Boemare (1988). Cell morphologies were examined under light microscopy. Physiological properties were determined for all strains of the other *Xenorhabdus*. The cells adsorbing bromothymol blue from NBTA were used as inoculums for all tests after preincubation overnight on tryptic soy agar (TSA) (Akhurst, 1980). Conventional biochemical tests were performed according to standard methods. Utilization of substrates was tested in mineral salt solution including 5 gr NaCl l 1. Acid production from carbohydrates was tested under aerobic conditions on mineral salts agar with 0.2 gr yeast extract and 10 gr of the respective carbohydrate per liter (Akhurst, 1980, 1982).

Phospholipase test was conducted on yolk agar using a superficially disinfected egg with 70% ethanol. Under the laminar flow, the yolk was collected aseptically and poured into an equal volume of 0.% NaCl sterile saline solution. After homogenization, 10% v/v of this yolk solution was added to nutritive agar at 45°C. An opaque halo around the inoculation line meant a positive lecitinase reaction.

For proteolysis test on gelatin agar, 12 gr/liter of gelatin were added and the plates streaked. After incubation, plates were flooded with a solution of 12 gr $HgCl_2$, 16 ml of 12N HCl and 80 ml distilled water. A clear halo due to the proteolysis was evident.

For gnotobiological assay, a colony of the stock culture was transferred into 5 ml of LB media. The overnight culture was sealed on one half of TSY agar plate. Surface sterilized IJ larvae were transferred to the bacterium-free part of the agar plate. This was considered positive if the majority of the dauer larvae molted into L4 and grew to fertile adults producing viable progeny. *Spodoptera littoralis* (Boisd.) (Lep.: Noctuidae) larvae were injected with a range of bacterial concentration (100-10000 cells) as axenic to test their pathogenicity.

DNA extraction

The Petri dishes were incubated at 24 h. Colony Forming Units, then counted and some of them taken for molecular analysis. In order to determine 16S rDNA gene sequences, DNA was isolated from the cells harvested from 24 h nutrient broth and bacterial culture lysed in TKE buffer (0.1 M Tris-HCl, 0.1 M KCl and 20 mM EDTA-Na, PH 8.0) containing 0.1 mg lysozyme and 10 µg RNase A at 37°C for 20 min. After addition of 0.25 ml of 10% sarkosyl to the bacterial lysate, DNA was purified by phenol-chloroform extraction and ethanol precipitation and later dissolved in 500 µl TE buffer.

Genomic and phylogenetic characterization

The 16S rRNA gene of Iranian strains of *Xenorhabdus* was amplified .The total volume of the PCR mixture was 100 µl using TaKaRa kit, containing 10 µl of 10x PCR buffer, 8 µl of dNTP mixture, 2.5 unit µl of Ex *Taq*, 1 µM of each primer, and 2 µl of template DNA solution. To amplify the gene, the primer set of 16S-F (5'-GAA GAG TTT GAT CAT GGC TC-3') and 16S-R (5'-AAG GAG GTGATC CAG CCG CA-3') was used (Fischer-Le Saux *et al*., 1999b). The PCR conditions were denaturation at 95 for 10 min, followed by 35 cycles of 94 for 1 min, 65 for 1 min, 72 for 2 min and a final extension at 72 for 8 min. The DNA band was excised and extracted from the gel by using the Gel-M Gel Extraction System (Viogene). The 16S gene sequence was determined using PCR primers and internal primers described in Fischer-Le Saux *et al*. (1999b). The resulted sequences of three bacterial strains were deposited in the gene bank, The National Center for Biotechnology Information (NCBI) (table 1). Multiple-sequence alignments of the 16S rDNA gene sequences were created by the

software CLUSTAL X (version 1.81) with 15.0 gap opening penalties and 6.66 gap extension penalties (Thompson *et al*., 1997).

Table 1. Sources and strains of *Xenorhabdus* species.

Species	Strain	Accession number	Nematode host (*Steinernema* spp.)	Country
Xenorhabdus poinarii	Iran2	EU250472	*S. glaseri*	Iran
X. poinarii	AZ26	DQ211703	*S. glaseri*	Portugal
X. poinarii	CU01	DQ211706	*S. cubanum*	Cuba
X. poinarii	SRK 1	EU513180	*Steinernema* sp.	India
X. nematophila	Iran4	FJ640983	*S. carpocapsae*	Iran
X. nematophila	Iran6	GU270840	*S. carpocapsae*	Iran
X. nematophila	YL001	EU124381	*Steinernema* sp.	China
X. nematophila	ES96	DQ211707	*S. carpocapsae*	Spain
X. nematophila	A24	FJ860883	*S. carpocapsae*	Russia
X. nematophila	K102	FJ860891	*S. carpocapsae*	Mexico
X. nematophila	BE06	DQ211704	*S. carpocapsae*	Belgium
X. nematophila	K97	FJ860884	*S. carpocapsae*	USA
X. budapestensis	DSM 16342T	AJ810293	*S. bicornutum*	Serbia & Montenegro
X. indica	OM01	DQ211718	*S. abbasi*	Oman
X. innexi	DSM 16336T	AJ810292	*S. scapterisci*	Uruguay
X. szentirmaii	DSM 16338T	AJ810295	*S. rarum*	Argentina
X. bovienii	AiAt199	AB243430	*S. litorale*	Japan
*P. luminescens luminescens**	DSM 3368	X82248	-	-

**P. = Photorhabdus*

The score of DNA weight matrix were assigned by IUB ambiguity codes. The transition weight was set to 0.5. Pairwise sequence divergence (the evolutionary distances) was calculated using the Kimura two-parameter distance model (Kimura, 1980), and the distance tree was calculated using the Kimura two-parameter model, the neighbor joining (NJ) (Saitou & Nei, 1987) and maximum parsimony (MP) methods (Eck & Dayhoff, 1966) included in the MEGA4 (Tamura *et al*., 2007). Bootstrap analysis was carried out with 100000 datasets (Felsenstein, 1985). To establish a root for the analysis, the sequence of the 16S from the subspecies *Photorhabdus luminescens luminescens* (strain DSM 3368) were aligned as the outgroup.

Results and discussion

All isolates shared all the common phenotypic characters of the *Xenorhabdus* genus. Cells were rod-shaped and motile. The phylogenetic analyses led to bacterial species assignation of the new isolates or to define the closest species previously described.

Reaction of isolate Iran2 was negative for catalase and oxidase activities and nitrate to nitrite reduction. It hydrolyzed gelatin of the Kohn's method and produced antimicrobial compounds (Boemare *et al*., 1993). As other *Xenorhabdus* species, it produced acid from fructose, glycerol, N-acetylglucosamine and maltose but not from L-arabinose and cellobiose. This strain (Iran2) had a negative response to the Simmons' citrate reaction. On the basis of the cell and colony morphology, catalase negativity, dye uptake, exo-lecitinase, and exo-lipase activities all the three bacterium strains belonged to the *Xenorhabdus* genus. Maximum temperatures of growth were 40°C for Iran 2 and 35°C for Iran4 and Iran6 strains. DNAse activity was negative for Iran2 and positive for Iran4 and Iran6 strains.

The pathogenicity of Iran2 strain of *Xenorhabdus* was negative against *S. littoralis* larvae while both strains of Iran4 and Iran6 had pathogenicity against *S. littoralis*. The injection of approximately 1000 cells was necessary to reach the LD_{50}. This rate was high, compared to other *Xenorhabdus* strains. The *Xenorhabdus* strains, associated with Iran2 isolate had the LD_{50} in a range of 1000-10000 cells and are considered slightly pathogenic to lepidopteran species.

The phylogenetic analyses of 16S rRNA genes of partial sequences indicated that Iran4 and Iran6 strains were closely related to the type strain of *X. nematophila*, while Iran2 strain can be a member of *X. poinarii*. The 16S rRNA gene sequences of three isolates were aligned to the homologous sequences of *Xenorhabdus* type strains. Homology values of 95-98.1% clearly placed the sequences of the isolates among those of *Xenorhabdus*. The length of the DNA fragment of this excluding primers were 1500, 1502 and 1500 bp for Iran2, Iran4 and Iran6 strains, respectively. The sequences are available under the NCBI accession numbers EU250472, FJ640983, GU270840. The sequences (> 1500 nucleotides) of the three isolates discovered their relations to the genus *Xenorhabdus* and revealed the existence of several lineages. The sequences helped to identify interspecific nucleotide differences (table 2). All strains of *Xenorhabdus* consistently formed clusters in both neighbor joining and maximum parsimony methods. To determine optimal outgroups for the phylogenetic analysis of *Xenorhabdu*s species, we tested the partial 16s rRNA gene sequences of the subspecies *P. luminescens luminescens* (Lengyel *et al*., 2005; Somvanshia *et al*., 2006). The bacterial species *Proteus vulgaris* was used as the outgroup (Rainey *et al*., 1995). The phylogenetic relationships among the 17 studied *Xenorhabdu*s species are presented in figures 1 and 2. The sequences alignment was deposited in TeeBASE (S10987). The MP (fig. 1) and NJ (fig. 2) phylogenetic tree diagrams are extremely similar to each other and their monophyly are

strongly supported by high values of bootstrap in both trees. A total of 1415 nucleotides were used in the analysis, of which 1247 characters were constant. The parsimony-uninformative characters were 78 and 90 characters were parsimony-informative.

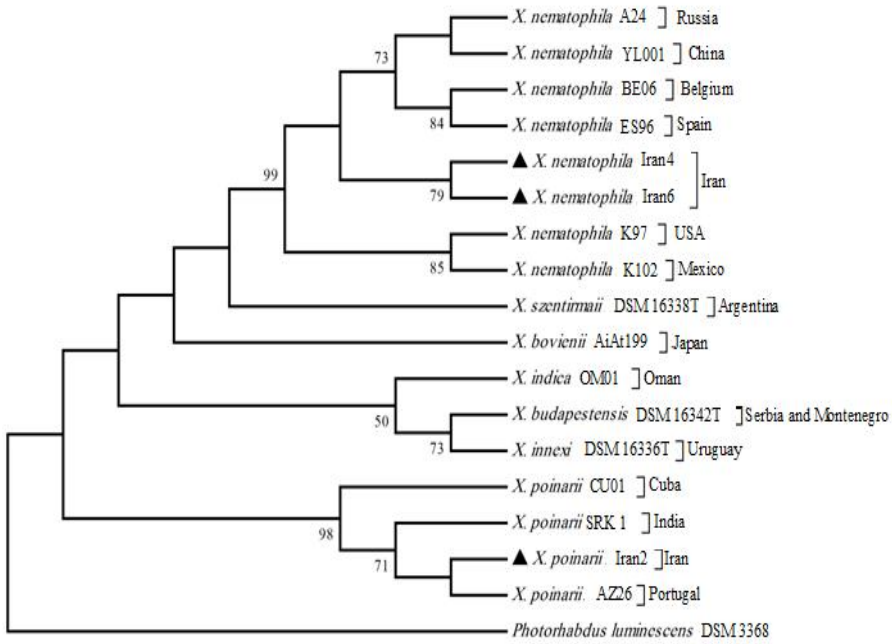

Figure 1. Phylogenetic relationship among *Xenorhabdus* strains, based on the 16S rRNA gene sequences by the maximum parsimony method. The numbers on branch nodes are bootstrap values (100000 resamplings; only values over 50% are given). Tree length = 311, CI = 0.655, RI = 0.7084.

The gnotobiological tests found that the IJs from which they had been isolated, were able to recover, grow and propagate on them in TSY agar plates and produce fertile progeny through generations but the nematodes grew poorly on alternative bacterial symbionts. Being consistent with the previous reports, the bacterial isolates from *S. carpocapsae* was identified as *X. nematophila* and *X. poinarii* from *S. glaseri* (Boemare, 2002). *Xenorhabdus* species/strains are subject to phase variation. Primary and secondary form variants have been described by Akhurst & Boemare (1988).

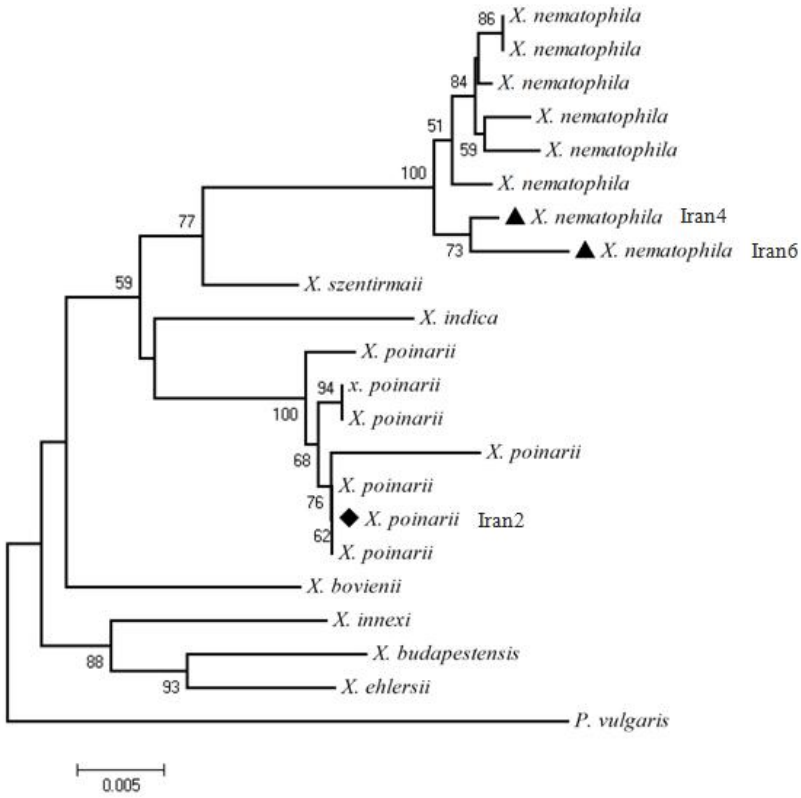

Figure 2. Unrooted neighbor joining tree (with 10000 replicates) constructed with the Kimura two-parameter distance calculation, based on 16S rRNA gene sequences of *Xenorhabdus* strains.

The strains, isolated from infective-stage nematodes, are usually at the primary phase, while the secondary phase may emerge upon subculture. The primary form has a greater ability to provide nutrients for the nematodes and is distinguished from the secondary form by the morphology of the colony and absorption of incorporated dyes in the growth medium. They generally produce a few numbers of antimicrobial factors (lecithinase negative) and differ in antibiotic sensitivities. All experimental cultures were in the primary phase (Gerritsen *et al*., 1992).

Table 2. Pairwise Kimura two-parameter distances between of *Xenorhabdus* strains for 1401 bp of 16S rRNA gene sequences.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*X. bovienii* AiAt199																		
2	*X. budapestensis* DSM 16342T	0.037																	
3	*X. indica* OM01	0.039	0.033																
4	*X. innexi* DSM 16336T	0.037	0.028	0.034															
5	*X. nematophila* A24	0.040	0.048	0.040	0.046														
6	*X. nematophila* BE06	0.041	0.048	0.040	0.047	0.003													
7	*X. nematophila* E S96	0.041	0.048	0.040	0.047	0.003	0.000												
8	*X. nematophila* Iran 4	0.041	0.049	0.040	0.047	0.005	0.005	0.005											
9	*X. nematophila* Iran6	0.044	0.052	0.044	0.050	0.012	0.012	0.012	0.007										
10	*X. nematophila* K97	0.041	0.048	0.039	0.047	0.006	0.004	0.004	0.005	0.011									
11	*X. nematophila* K102	0.041	0.049	0.040	0.047	0.006	0.005	0.005	0.006	0.012	0.002								
12	*X. nematophila* YL001	0.043	0.050	0.041	0.049	0.004	0.004	0.004	0.006	0.014	0.006	0.008							
13	*X. poinarii* Iran2	0.029	0.039	0.028	0.037	0.034	0.035	0.035	0.034	0.037	0.034	0.034	0.037						
14	*X. poinarii* AZ26	0.029	0.039	0.028	0.037	0.034	0.035	0.035	0.034	0.037	0.034	0.034	0.037	0.000					
15	*X. poinarii* CU01	0.030	0.040	0.028	0.040	0.035	0.035	0.035	0.037	0.040	0.034	0.035	0.038	0.004	0.004				
16	*X. poinarii* SRK 1	0.037	0.048	0.036	0.046	0.041	0.043	0.043	0.042	0.046	0.041	0.042	0.044	0.009	0.009	0.013			
17	*X. szentirmaii* DSM 16338T	0.030	0.036	0.023	0.034	0.024	0.024	0.024	0.023	0.029	0.023	0.023	0.025	0.022	0.022	0.024	0.030		
18	*Photorhabdus luminescens*	0.064	0.064	0.065	0.069	0.058	0.058	0.058	0.059	0.064	0.059	0.058	0.061	0.054	0.054	0.055	0.062	0.061	

The knowledge about the diversity of *Xenorhabdus* strains, isolated from *Steinernema* nematodes, has been increasing (Kaya *et al*., 2006; Taillieze *et al*., 2006) and to date, more than 20 isolated species with their diverse host range, host foraging behavior, reproductive modes and environmental tolerance are known. The study of *Xenorhabdus* phylogeny has been historically based on 16S rDNA sequence analyses but recent studies have focused on the data available from the housekeeping genes (Lee & Stock, 2010).

Acknowledgements

The research project was done at Dr. Yoshiga's lab (Sag University, Japan) and financially supported by the University of Tehran and Iranian Ministry of Science, Research and Technology. My thanks go to my colleagues at the Nematology and Entomology labs, especially Drs R. Kuwata, R. Tanaka, Y. Yoshie, Kondo and Hayakawa, who made the preparation of this work a joyful experience.

References

Adams, B. J. & Nguyen, K. B. (2002) Taxonomy and systematics. pp. 1-33 in Gaugler, R. (Ed.) *Entomopathogenic nematology*. 402 pp. CABI Publishing, Wallinford, UK.

Adams, B. J., Fodor, A., Koppenhöfer, H. S., Stackenbrandt, E., Stock, S. P. & Klein, M. G. (2006) Biodiversity and systematics of nematode-bacterium entomopathogens. *Biological Control* 38, 4-21.

Akhurst, R. J. (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology*121, 303-309.

Akhurst, R. J. (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *Journal of General Microbiology* 128, 3061-3065.

Akhurst, R. J. (1983) Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. *International Journal of Systematic Bacteriology* 33, 38-45.

Akhurst, R. J. & 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. & Boemare, N. E. (1990) Biology and taxonomy of *Xenorhabdus*. pp. 75-90 in Gaugler, R. & Kaya, H. K. (Eds) *Entomopathogenic nematodes in biological control*. 384 pp. CRC Press, Boca Raton.

Akhurst, R. J. & Boemare, N. E. (2005) Genus XL. *Xenorhabdus*. pp. 831-838 in Brenner, D. J., Krieg, N. R., Staley, J. T. & Garrity, G. M. (Eds) *Bergey's manual of systematic bacteriology* (*the Proteobacteria*), *Part B* (*the Gammaproteobacteria*). 1388 pp. Springer, New York, NY, US.

Bird, A. F. & Akhurst, E. J. (1983) The nature of the intestinal vesicle in nematodes of the family Steinernematidae. *International Journal for Parasitology* 13, 599-606.

Boemare, N. (2002) Biology, taxonomy and systematics of *Xenorhabdus* and *Photorhabdus*. pp. 35-56 in Gaugler, R. (Ed.) *Entomopathogenic nematology*. 402 pp. CABI Publishing, Wallinford, UK.

Boemare N. E. & Akhurst, R. J. (1983) Biochemical and physiological of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Journal of General Microbiology* 134, 751-761.

Boemare, N. E., Akhurst, R. J. & Mourant, R. G. (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology* 43, 249-255.

Burnell, A. M. (2002) Genetics and genetics improvement. pp. 241-263 in Gaugler, R. (Ed.) *Entomopathogenic nematology*. 402 pp. CABI Publishing, Wallinford, UK.

Eck, R. V. & Dayhoff, M. O. (1966) *Atlas of protein sequence and structure*. National Biomedical Research Foundation, Silver Springs, Maryland.

Eivazian Kary, N. E., Niknam, G., Griffin, C. T., Mohammadi, S. A. & Moghaddam, M. A. (2009) Survey of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the north-west of Iran. *Nematology* 11, 107-116.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39,783-791.

Fischer-Le Saux, M., Arteaga-Hernández, E., Mráĉek, Z. & Boemare, N. E. (1999a) The bacterial symbiont *Xenorhabdus poinarii* (Enterobacteriaceae) is harbored by two phylogenetic related host nematodes: the entomopathogenic species *Steinernema cubanum* and *Steinernema glaseri* (Nematoda: Steinernematidae). *Federation of European Microbiological Societies* (*FEMS*), *Microbiology Ecology* 29, 149-157.

Fischer-Le Saux, M., Villard, V., Brunel, B., Normand, P. & Boemare, N. E. (1999b) Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens*, subsp. *askurtii*, subsp. *laumondii* and *P. temperate* sp. nov., *P. temperate* subsp. *temperate* subsp. nov. and *P. asymbiontica* sp. nov. *International Journal of Systematic Bacteriology* 4, 1645-1656.

Forst, S. & Clarke, D. (2002) Bacteria-nematode symbiosis. pp. 57-98 in Gaugler, R. (Ed.) *Entomopathogenic nematology*. 402 pp. CABI Publishing, Wallinford, UK.

Forst, S., Dowds, B., Boemare, N. & Stackebrandt, E. (1997) *Xenorhabdus* spp. and *Photorhabdus* spp.: bugs that kill bugs. *Annual Review of Microbiology* 51, 47-72.

Gerritsen, L. J. M., Raay, G. & Smits, P. H. (1992) Characterization of form variants of *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* 58, 1975-1979.

Hazir, S., Kaya, H. K., Stock, S. P. & Keskin, N. (2003) Entomopathogenic neamtodes (Steinermatidae and Heterorhabditidae) for biological control of soil pests. *Turkish Journal of Biology* 27, 181-202.

Karimi, A., Kharazi-Pakdel, A., Yoshiga, T. & Koohi-Habibi, M. (2009a) Report of entomopathogenic nematode, *Steinernema glaseri*, from Iran. *Russian Journal of Nematology* 17, 83-86.

Karimi, A., Kharazi-Pakdel, A. & Yoshiga, T. (2009b) Insect pathogenic nematode, *Steinernema feltiae*, from Iran. *International Organization for Biological Control*/*West Palaearctic Regional Section* (*IOBC/WPRS*) *Bulletin* 45, 409-412.

Kaya, H. K., Aguillera, M. M., Alumai, A., Choo, H. Y., de la Torre, M., Fodor, A., Ganguly, S., Hazir, S., Lakatos, T., Pye, A., Wilson, M., Yamanaka, S., Yang, H. & Ehlers, R.-U. (2006) Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biological Control* 38, 134-155.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111-120.

Lee, M. M. & Stock, S. P. (2010) A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp. (γ-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. *Journal of Invertebrate Pathology* 77, 1-12.

Lengyel, K., Lang, E., Fodor, A., Szállás, E., Schumann, P. & Stackenbrandt, E. (2005) Description of four novel species of *Xenohabdus*, Family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. *Systematic and Applied Microbiology* 28, 115-122.

Parvizi, R. (2003) An evaluation of the efficacy of the entomopathogenic nematode *Heterorhabditis bacteriophora* and *Steinernema* sp. in controlling immature stages of the apple clearwing, *Synanthedon myopaaeformis*. *Iranian Journal of Agricultural Sciences* 34, 303-311. [In Persian with English summary].

Peat, S. M., Ffrench-Constant, R. H., Waterfield, N. R., Marokhuzi, J., Fodor, A. & Adams, B. J. (2010) A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: a case for 16S, gyrB, and glnA. *Molecular Phylogenetics and Evolution* 57, 728-740

Poinar, G. O. & Thomas, G. M. (1966) Significance of *Achromobacter nematophilus* Poinar & Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (*Neoaplectana* sp., Steinernematidae). *Parasitology* 56, 385.

Rainey, F. A., Ehlers, R. U. & Stackebrandt, E. (1995) Inability of the polyphasic approach to systematics to determine the relatedness of the genera *Xenorhabdus* and *Photorhabdus*. *International Journal of Systematic Bacteriology* 45, 379-381.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Sicard, M., Le Brun, N., Pagès, S., Godelle, B., Boemare, N. & Moulia, C. (2003) Effect of native *Xenorhabdus* on the fitness of their *Steinernema* host: contrasting types of interactions. *Journal Parasitology* 91, 520-524.

Somvanshia, V. S., Langb, E., Gangulya, S., Swiderskib, J., Saxenac, A. K. & Stackebrandt, E. (2006) A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh. *Systematic and Applied Microbiology* 29, 519-525.

Tailliez, P., Pagès, S., Ginibre, N. & Boemare, N. (2006) New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. *International Journal of Systematic and Evolutionary Microbiology* 56, 2805-2818.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596-1599.

Tanha Ma'afi, Z., Ebrahimi, N., Abootorabi, E. & Spiridonov, S. E. (2006) Record of two steinernematid species from Iran. *Proceedings of the 17th Iranian Plant Protection Congress, Vol. II, Plant Diseases*, p. 482.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.

Volgyi, A., Fodor, A., Szentirmai, A. & Forst, S. (1998) Phase variation in *Xenorhabdus nematophilus*. *Applied and Environmental Microbiology* 64, 1188-1193.

Received: 20 April 2010
Accepted: 24 May 2011