PHYLOGEOGRAPHIC ANALYSIS OF ENTOMOPATHOGENIC NEMATOPHILIC SYMBIOTIC BACTERIA USING 16S RDNA GENE SEQUENCING

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ABSTRACT: In our study, we have isolated and identified three symbiotic bacteria (SRK2, SRK6, SRK8) from the intestine of *Heterorhabditis indica* nematode and two symbiotic bacteria (SRK1, SRK7) vesicle of *Steinernema glaseri* nematode based on their 16s rDNA sequences. Phylogenetic studies were conducted to infer their evolutionary relationships with closely related organisms isolated from entomopathogenic nematodes. These organisms have shown 98-99% sequence similarity with *Photorhabdus* spp. and *Xenorhadus* spp. SRK1 and SRK7 strains (*Xenorhadus* spp.) genetically more diverged than SRK2, SRK6 and SRK8 strains (*Photorhabdus* spp.). SRK2, SRK6 and SRK8 strains were highly grouped with ARG, Iran8, FR42, Az36 and TT01 strains of *Photorhabdus luminescens* in the tree, and phylogeographically related to FR42 and FR32 strains isolated from France. SRK1 strain was phylogenetically related to the *Xenorhabdus poinarii* strain Iran2 whereas SRK7 strain corresponded with CN03 strain from China. Overall, we found SRK2, SRK6 and SRK8 strains showing the strain equivalency to the *P. luminescens*. SRK1 and SRK1 strains were equivalent to the *X. poinarii* and *X. stockiae*, respectively. Thus, a symbiotic association between bacteria and entomopathogenic nematodes is widespread in nature occurring in every type of ecological niche.

KEY WORDS: Nematode, Photorhabdus, Xenorhabdus, Symbiotic bacteria, Phylogeny, Molecular evolution, Genetic variation, Insect cadavers

Entomopathogenic nematodes (EPNs) are generally used as commercial biological control agents against susceptible insect pests in an agro-ecosystem (Shapiro-Ilan et al., 2006). A nematode penetrates an insect host, injures the insect gut and enters into the hemocoel where it releases immune depressive factors to protect from a self recognition. A symbiotic bacterium starts to proliferate together with nematodes and kill the insect host within 24-96 hrs (Cichea et al., 2006). *Xenorhabdus* spp. and *Photorhabdus* spp. are symbiotic bacteria that produce numerous metabolites of insecticidal and bactericidal impact inside the gut of nematodes (Ferreira and Malan, 2014). A nematode protects these bacteria from the environment for its continued existence as symbiosis relationship and pathogens for insects (Murfin et al., 2012).

The new isolates or species identification from the gut of nematodes is complex due to strains that exhibit phenotypic characteristic in a similar way in many diagnostic assays. Various molecular diagnostic approaches have been employed to place the taxonomy status of bacterial symbionts of EPNs (Hsieh et al., 2009; Jung et al., 2012). The taxonomic classification of these symbiotic bacteria has been resolved on the basis of phenotypic and genotypic traits (Tailliez

et al., 2010; Jang et al., 2012) and whole-cell fatty acid content (Janse & Smits, 1990). However, accurate taxonomic status of bacterial symbionts with their nematode hosts could not be revealed at species level.

Phylogeny-based on 16s rDNA gene sequences have been significantly supported for the taxonomic classifications of symbiotic and non-symbiotic bacteria of EPNs (Liu et al., 1997, 2001; Tóth & Lakatos, 2008; Razia et al., 2011a; Abdolmaleki et al., 2016; Sangeetha et al., 2016). Several studies have been identified symbiotic bacteria of EPNs based their 16s rDNA sequence similarity, followed by phylogenetic relationships (Emelianoff et al., 2008; Hsieh et al., 2009; Tailliez et al., 2012; Kuwata et al., 2013). *X. bovienii* and *X. ishibashii* are common symbiotic bacteria of *Xenorhadus* genus which are associated with EPN *Steinernema* spp (Inman et al., 2012). *P. luminescens* subsp. Kayaii, *P. luminescens* subsp. laumondii and *P. luminescens* subsp. Akhurstii are symbiotic bacteria usually associated with EPN *Heterorhabditis* spp (Sangeetha et al., 2016). Phylogenetic relationships of these bacteria have also been studied to know their symbiotic associations with nematode host and find out the phylogenetic neighbors (Zhao et al., 2012; Ferreira et al., 2013; Ferreira & Malan, 2014). Apart from 16s rDNA-based phylogeny, similarity analysis of RNA secondary structures was a great measure to infer the phylogeographic origin of symbiotic bacteria of EPNs (Razia et al., 2011a). Hence, we combined the sequence and secondary structural similarities of 16s rDNA genes with phylogenetically variable sites for the taxonomic classification of symbiotic bacteria isolated from nematodes infecting the insect *Galleria mellonella* in the Western Ghats of Tamil Nadu, India.

MATERIALS AND METHODS

Insect and nematode culture.

The greater wax moth larvae *G. mellonella* (Pyralidae, Lepidoptera) was used for nematode baiting and the multiplication of *H. indica* and *S. glaseri* nematodes isolated from Western Ghats of Tamil Nadu, India. Insect eggs were obtained, kept in rearing plastic boxes with artificial diet and maintained in aerated plastic containers (32.5 x 17.6 x 10 cm) at 25±2°C. The nematodes were cultured in the late instar insect larvae according to the procedure described by Woodring and Kaya (1988). At a concentration of approximately 1000 - 4000 per ml in distilled water with 0.1% formalin, infective juveniles were stored in the tissue culture flask at 19-20°C in BOD incubator.

Isolation of symbiotic bacteria.

Symbiotic bacteria were isolated from insect hemolymph within 48 hrs of infected nematodes. Late instar insects were placed on the surface of a filter paper in 35mm Petri dishes. Nematodes were separately transferred onto a filter paper surface at a dose rate of 400 per petri dish. All the dishes were sealed with parafilm and then incubated at 25°C for 24 hrs. Then, the larvae were removed, rinsed in distilled water and surface sterilized with 70% ethanol, and left to drying in a laminar flow cabinet. Hemolymph was obtained by dissecting dorsally between the 5th and 6th segments. A loopful of hemolymph was streaked on NBTA agar (nutrient agar supplemented with 25mg bromothymol blue and 40mg triphenyltetrazolium chloride per liter) plates (Woodring & Kaya, 1988) and then incubated for 48 hrs at 28°C. The dark blue or green colonies were selected and subculture onto the fresh NBTA plates. The isolated bacterial colonies were preserved at -80°C in nutrient broth containing 15% (v/v) glycerol. Cell morphology and motility of the isolated bacterial colonies were studied by direct

contact and phase-contrast microscopy. Gram staining and biochemical characteristics of these isolates were carried out according to the methods described in Bergey's manual (Krieg & Hart, 1984).

DNA extraction, PCR amplification and sequencing.

Genomic DNA was extracted from pure bacterial culture broth. The bacterial cells were washed-out in TE buffer and centrifuged for 5 min. Total DNA from each culture was extracted by using the DNA extraction Kit (Genei, India). The quality and yield of genomic DNA were checked by running 5µl samples on a 1% agarose gel. Each DNA sample was diluted in 50- to 100-fold with ultrapure water and then used as a template for PCR amplification (Liu et al., 1997, 2001). The 16s rDNA gene sequence (approximately 1.4 kb) of each isolate was amplified using the following specific oligonucleotide primers; Forward primer: 5'-AGAGTTTGATTCTGGCTCAG-3' and Reverse primer: 5'-GACGGGGGGTGTGTACAA-3'. The total volume of a PCR mixture was 50µl, containing 5µl of 10x PCR buffer, 1µl of dNTP mixture, 0.4µl of Taq DNA polymerase, 0.4µl of each primer, 5µl of template DNA and finally make up with sterile distilled water. The PCR reaction condition was 94°C for 5 min for initial denaturation, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 15 min. All samples were amplified by a gradient Thermocycler (Eppendorf, Nexus). The PCR products were purified using the PCR purification Kit (Chromous, India), separated in a 2% agarose gel (containing 0.5 mg/ml ethidium bromide) electrophoresis and bands visualized under a gel documentation system. The DNA sequencing was performed by ABI 3130 Genetic Analyzer.

Phylogenetic analysis.

Sequence similarity analysis was conducted with NCBI-BLASTn (Altschul et al., 1997) against GenBank of NCBI (http://www.ncbi.nlm.nih.gov) and RDP-II (Ribosomal database project) (Wang et al., 2007) in searching of highly identical hits for our PCR products (16s rDNA sequences). Multiple sequence alignment was carried out for the selected sequence hits using the ClustalX 2.0 software (Thompson et al., 2007). Sequence alignment was inspected manually, unreliable sequences deleted and then the final dataset for phylogenetic analyses prepared as presented in Table 1. The Neighbor joining algorithm was employed to reconstruct the phylogenetic trees by using the MEGA 5.03 software (Tamura et al., 2011) with Jukes-Cantor-Kimura evolutionary model. Bootstrap analyses with 1000 resamplings were performed to obtain estimates of phylogenetic tree topologies.

RNA Secondary structure analysis.

RNA secondary structures of our strains and their phylogenetic neighbors were modeled from 16s rDNA sequences using the GeneBee-NET program (Brodsky et al., 1992; Brodskii et al., 1995). The parameter was energy threshold -4.0, cluster factor 2, conserved factor 2, compensated factor 4 and conservativity 0.8. Free energy of structure for each strain was calculated with a greedy algorithm (Zuker and Stiegler, 1981) and compared together to detect the variations in energy of loop structures.

Nucleotide sequence accession numbers.

The partial 16s rDNA gene sequences studied herein has been deposited in the GenBank of NCBI database under accession numbers FJ006727 to FJ006729 and EU513180 to EU513181.

RESULTS

Analysis of PCR amplified products.

In our study, three *Photorhabdus* spp. (SRK2, SRK6 and SRK8) and two *Xenorhabdus* spp. (SRK1 and SRK7) were isolated from the intestine of insect juvenile stage of *H. indica* and from vesicle of insect juvenile stage of *S. glaseri*, respectively. These symbiotic bacterial isolates were confirmed from PCR amplified products. A single band was produced of about 1,453bp for SRK2 strain, 1460bp for SRK6 and SRK8 strains, 1540bp for SRK1 and 1444bp for SRK7. The alignments of partial 16s rDNA gene sequences were comprised approximately 1500bp, indicating of sequencing accuracy of 16s rDNA genes.

Sequence similarity analysis.

Sequence similarity analysis indicated that 16s rDNA gene sequences of these bacteria have shown 98-99% sequence identity to organisms which are related to respective genera. The sequence-specific similarities exhibited to identify all of these isolates at species level. We have selected 23 numbers of *Photorhabdus* spp. and 25 numbers of *Xenorhabdus* spp. isolated from different nematode hosts of various countries (Table 1). Many *Photorhabdus* spp. associated with *H. bacteriophora* nematode whereas several *Xenorhabdus* spp. survived in *S. feltiae* and *S. carpocapsae* nematodes. Even if different out-group combinations used, *Proteus vulgaris* alone represented an optimal tree construction for the phylogenetic classification of these isolates associated with EPNs.

Phylogenetic analysis of *Photorhabdus* spp.

As represented in Fig. 1, the different strains of *P. luminescens* formed three separate major groups including subspecies of laumondii, kayaii and luminescenes. In the first group, strains of Hm, Hb, ATCC29999 and DSM3368 clustered together belonging to subsp. luminescens and the strain SRK2 grouped to the *P. luminescens*. It has highly grouped with six strains of ARG, Iran8, FR42, Az36, DSM15195 and TT01 and then clustered with strains of Brecon and E21. The strains of ITH-LA3, FR33, DSM15198, DSM15197 and DSM15194 grouped under subsp. kayaii. The strains of SRK6, SRK8 and FR32 show a strong phylogentic relatedness wherein SRK6 and SRK8 strains belonged to the *P. luminescens* subsp. thracensis. The strains of aqaba, balqa-arida2 and kfr-anja were further related to each other and then clustered with the above groups. SRK2 strain was phylogeographically related to the strains of UK (Az36, ARG and TT01), France (FR42) and Iran (Iran8) whereas SRK6 and SRK8 strains were similar to the strain of France (FR32).

Phylogenetic analysis of *Xenorhabdus* spp.

As shown in Fig. 2, strain SRK1 was phylogenetically related to the *X. poinarii* strain Iran2 and adjoined with FR44 and AiAt199 strains of *X. bovienii*. Malka and Bdr-2 strains of *X. bovienii* were highly diverged and distantly related with FR44 and AiAt199 strains, showing that there was a strain-specific variation for the speciation of *X. bovienii*. Nonetheless, all of the strains have sequence-specific variations in the different sites of aligned sequences. A cluster comprising *X. budapestensis* CN03, *X. ehlersii* KR02 and *X. innexi* UY61 showed to have a phylogenetic resemblance to the SRK7 strain, but its 16s rDNA sequence distinctly matched with *X. stockiae*. It clearly indicated that strain SRK1 was equivalent to *X. poinarii* and strain SRK7 belonged to *X. stockiae*. Phylogeographic analysis implied that as similar to SRK2 strain, SRK1 strain might be equivalent to the strain of Iran2 (Iran). SRK7 strain has shown a phylogeographic connection with the strains of China (CN03), South Korea (KR02) and France (UY61).

Analysis of species-specific variations.

Multiple sequence alignment of this study showed that SRK1 and SRK7 (*Xenorhabdus* spp.) strains have more sequence variations than the strains of SRK2, SRK6 and SRK8 (*P. luminescens*) (Fig. 3). We found four major sequences-specific variations in the sequences of SRK2, SRK6 and SRK8 strains. The frequent sequence variability noted at 'G' base and rarely at 'A' base in these sites. A maximum sequence variation observed to be occurring in the strain SRK8, which was rather than that appeared in the strains SRK2 and SRK6. We found six major regions showing sequence-specific variations in the SRK1 strain.

Analysis of RNA secondary structures.

Apart from phylogenetic inference, we analyzed the RNA secondary structures generated from 16s rDNA gene sequences of our isolates (Fig. 4 & 5). We found SRK2 strain showing energetically favorable RNA secondary structure as similar to FR42 strain (France). The free energy of structures in strains SRK6 and SRK8 closely related to the strain of France (FR32). Both SRK1 and Iran2 strains phylogeographically related together. Even if SRK7 strain had many phylogeographic neighbors, it has shown a significant relatedness to strain CN03 from China.

DISCUSSION

Nematodes *H. indica* and *S. glaseri* are commonly survived in an insect *G. mellonella* predominately existing at the geographical location of Western Ghats of Tamil Nadu, India (Ciche & Ensign, 2003). Nematode infected insect cadaver reported to be a good source for the isolation of symbiotic and non-symbiotic bacteria (Maneesakorn et al., 2011; Razia et al., 2011a). Symbiotic bacteria of EPNs are considered as host-specific species/strains, as described in many reports (Kuwata et al., 2008; An & Grewal, 2011; Ferreira et al., 2013; Ferreira & Malan, 2014). As similar to that we have isolated SRK2, SRK6 and SRK8 strains belonging to the *Photorhabdus* genus from the intestine of *H. indica* and SRK1 and SRK7 strains belonging to the *Xenorhabdus* genus from vesicle of *S. glaseri*. Morphological and biochemical studies pinpointed that these organisms closely related to the Enterobacteriaceae family in many phenotypic characteristics, as suggested earlier (Woodring & Kaya, 1988; Tóth & Lakatos, 2008; Ferreira et al., 2013).

Many of the species and subspecies of these symbiotic bacteria showed to form a separate monophyletic clade in the phylogenetic trees. Phylogenetic analyses implied that intra-generic relationship of *Xenorhabdus* spp. and the taxonomic positions of *P. luminescens* and related species changed radically. It suggested optimal phylogenetic placement of these isolates may be determined by sequence conserved informative sites in accordance with previous works on γ-Proteobacteria (Wang et al., 2007; Tóth & Lakatos, 2008). However, the topology of such phylogeny can be manifested by using multiple candidate out-groups (Jang et al., 2012; Tailliez et al., 2010, 2012).

We performed optimal out-group analysis to render the most accurate estimate of the ancestral states assessed for the in-group node, as described in earlier works (Brodsky et al., 1992; Wang et al., 2007; Hsieh et al., 2009). In our work, closely related members of both genera selected to reconstruct the phylogenetic tree in which *P. vulgaris* CIP103181T found as the nearest phylogenetic neighbor to place the taxonomic position of these strains. This out-group organism also considered as a sister group of these genera since other species of these genera do not share a common ancestor with any other group

(Hsieh et al., 2009). Moreover, we found sequence-specific informative sites that can impose on the taxonomic discrimination among these strains. Zhao et al., (2012) have already used variable and parsimony-informative sites of 23S rDNA sequences for classification and identification of *Xenorhabdus* from EPNs at species level. Based on the conserved sequence-structure informative sites, the strains SRK2, SRK6 and SRK8 strain are placed in the phylogenetic tree as *P. luminscens*. We proposed to place the strains SRK1 and SRK7 as equivalent to *X. poinarii* and *X. stockiae*, respectively.

The adaptation of a nematode strain to the environment is probably related to its association with specific bacterial strain (Rainey et al., 1995; Ferreira et al., 2013; Ferreira & Malan, 2014). Apart from *P. luminescens* and *P. temperata*, an opportunistic human pathogen *P. asymbiotica* was associated with Japanese nematodes *H. indica* CbKj163 and OnIr40 (Kuwata et al., 2008, 2013; Razia et al., 2011a,b). It proved that some clinical isolates of *P. asymbiotica* could have originated from bacteria associated with EPNs. Phylogeographic analysis of this study indicated that SRK1 could be originated from the strain of Iran. Symbiotic bacteria of EPNs isolated from France (FR32, FR42) may be phylogeographic origin for SRK2, SRK6 and SRK8 strains. Since SRK7 strain was highly diverged from other closely related strains, it may be originated from CN03 strain of China. It may be resulted due to the attribution of genetic variation, evolution process, ecological and host definitions on the respective strains (Boemare & Akhurst, 1988; Liu et al., 1997, 2001; Maneesakorn et al., 2011; Razia et al., 2011a,b). Thus, a proper identification and characterization of EPNs associated symbiotic bacteria are intended to be carried out prior to develop an EPN-based bio-control agent.

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Transparency declaration

The authors confirm that this article's content has no conflicts of interest.

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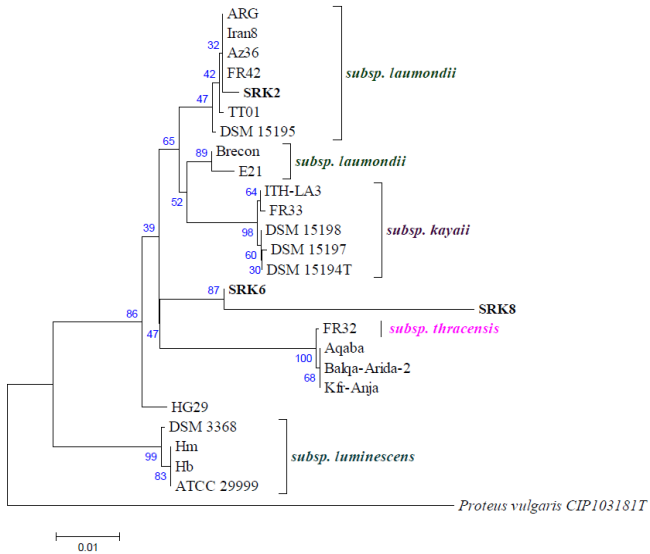

Figure 1. 16s rDNA gene sequence-based phylogenetic tree of *P. luminescens* associating with EPNs isolated from different geographic origins. Branch lengths are proportional to evolutionary distances. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates and taxa with same evolutionary distances are collapsed. The sequences with more than 90% sequence identity are included in the tree. Bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. *Proteus vulgaris* CIP103181T was served as an out group organism for optimized tree.

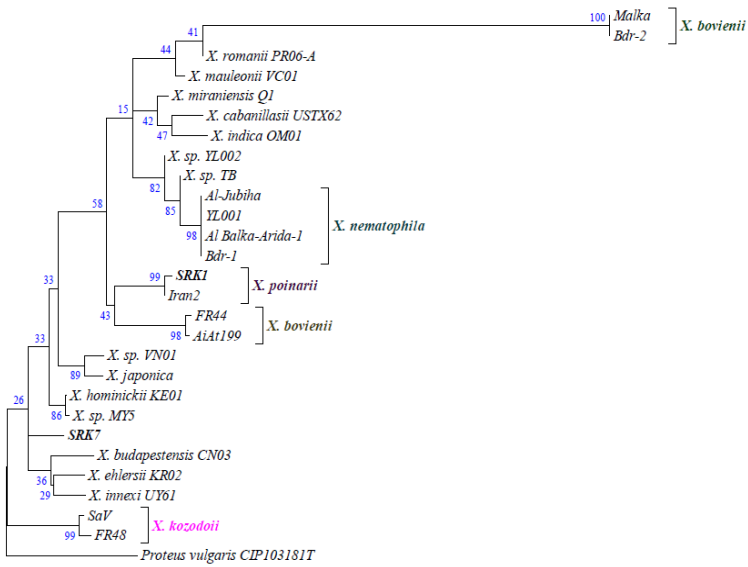

Figure 2. 16s rDNA gene sequence-based phylogenetic tree of *Xenorhabdus* sp. associating with EPNs isolated from different geographic origins. *Proteus vulgaris* CIP103181T was served as an out group organism for optimized tree.

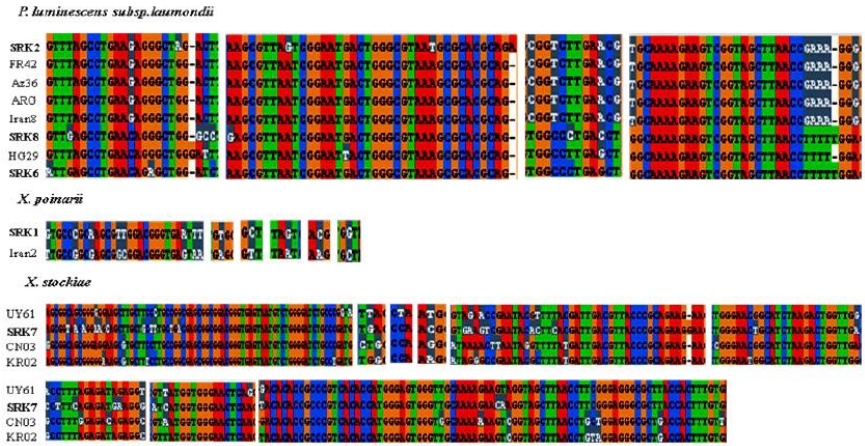

Figure 3. Multiple sequence alignment of 16s rDNA gene sequences for SRK2, SRK6 and SRK8 strains of *P. luminescens* and for *X. poinarii* SRK1 and X. stockiae SRK7 with phylogentically related organisms. Letter (white color) in gray shade showing the sequence variations among the strains.

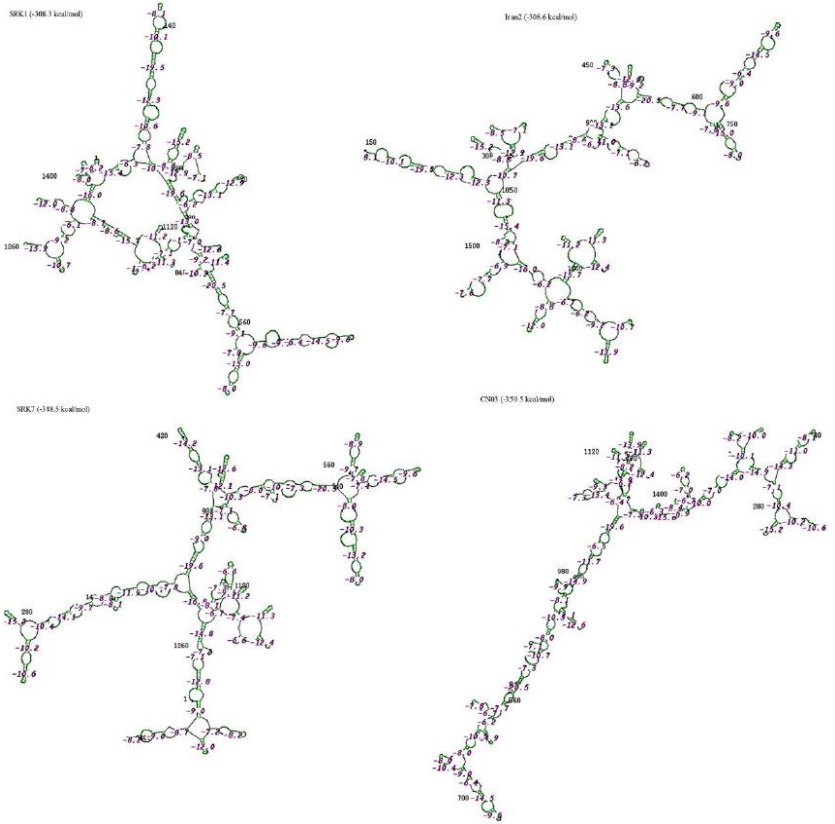

Figure 4. Graphical depiction of the free energy of RNA secondary structures calculated for the sequences of strains SRK2 verses FR42 and SRK6, SRK8 verses FR32.

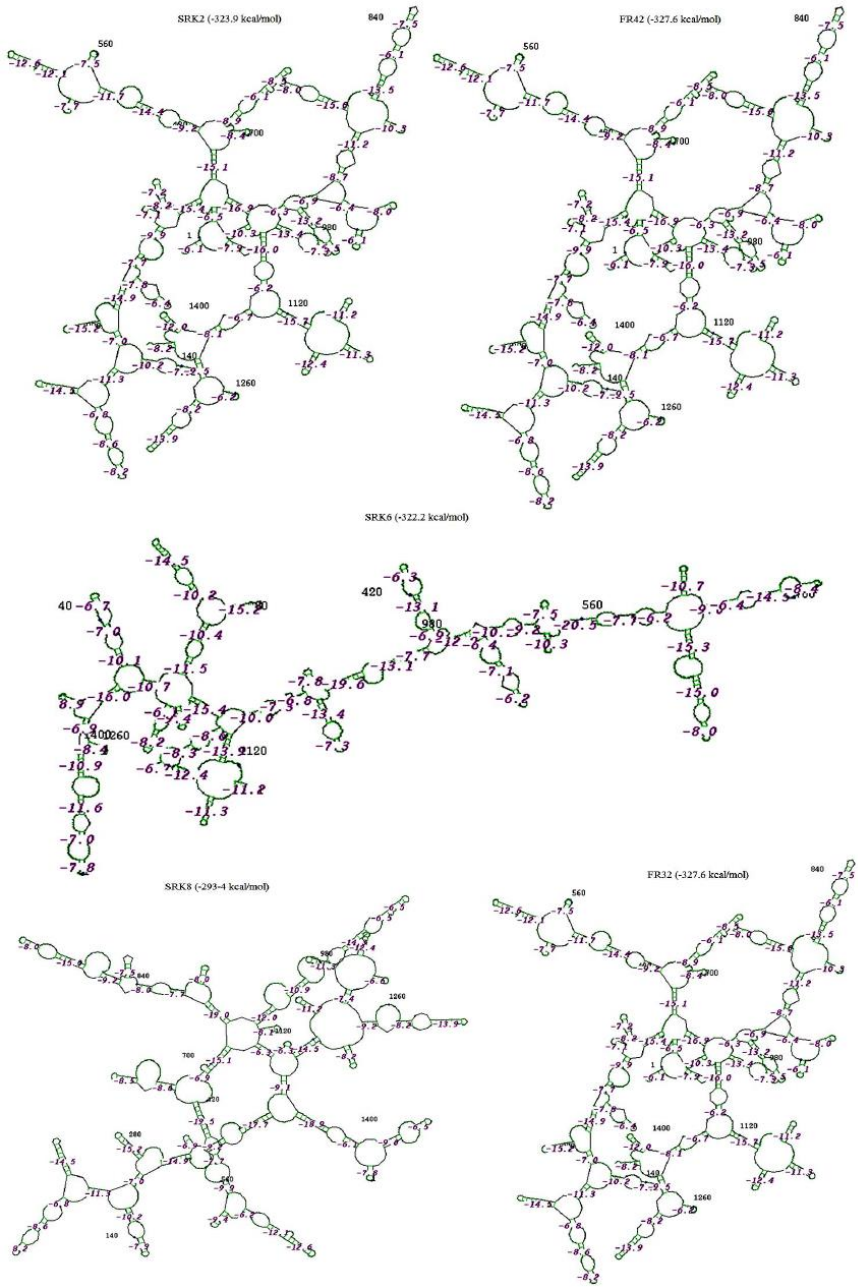

Figure 5. Graphical depiction of the free energy of RNA secondary structures calculated for the sequences of strains SRK1 verses Iran2 and SRK7 verses CN03.

Table 1. Origins of the symbiotic bacteria and their nematode associates used for the phylogenetic analysis of this study.

Accession	Species/Strain	Nematode associates	Country	Accession	Species/Strain	Nematode associates	Country
Photorhabdus luminescens				*Xenorhabdus* genus			
P. luminescens subsp.laumondii				*X. poinarii*			
EU513181	**SRK2**	***Heterorhadus indica***	**India**	**EU513180**	**SRK 1**	***Steinernema glaseri***	**India**
EU190980	FR42	*H.bacteriophora*	France	EU250472	Iran2	-	Iran
AY278649	Az36	-	UK	*X. nematophila*			
AY278646	TT01	-	UK	EU214637	Al-Jubiha	*S.anatoliense*	Jordan
AY278647	Brecon	-	UK	EU214636	Bdr-1	*S.carpocapsae*	Jordan
EU930341	E21	*H.bacteriophora*	Australia	EU214635	Al Balka-Arida-1	*S.carpocapsae*	Jordan
EU250473	Iran8	-	Iran	EU124381	YL001	*S.* sp.	China
AY278650	ARG	-	UK	*X. bovienii*			
AJ560633	DSM 15195	-	Germany	EU214634	Malka	*S.feltiae*	Jordan
P. luminescens subsp. kayaii				EU214633	Bdr-2	*S.feltiae*	Jordan
EU930334	ITH-LA3	EPN	Italy	EU190978	FR44	*S.feltiae*	France
EU930333	FR33	EPN	France	AB243430	AiAt199	*S.litorale*	Japan
AJ560630	DSM 15194	EPN	Germany	*X. budapestensis*			
AJ560631	DSM 15198	-	Germany	DQ211714	CN03	*S. ceratophorum*	China
AJ560632	DSM 15197	-	Germany	*X. mauleonii*			
P.luminescens subsp.luminescens				DQ211715	VC01	*S.* sp.	Saint Vincent
AY278641	Hm	-	UK	*X. kozodoii*			
AY278640	Hb	-	UK	DQ211716	SaV	*S.arenarium*	Russia
AY870658	ATCC 29999	-	China	EU190977	FR48	*S.* sp.	France
X82248	DSM 3368	-	Germany	*X.romanii*			
EU930345	HG29	*H.bacteriophora*	France	DQ211717	PR06-A	*S.puertoricense*	Puerto Rico
P.luminescens subsp. thracensis				*X. indica*			

EU930335	FR32	EPN	France	DQ211718	OM01	*S.abbasi*	Oman
P.luminescens				*X.hominickii*			
EU214638	Kfr-Anja	*H.bacteriophora*	Jordan	DQ211719	KE01	*S.karii*	Kenya
EU214640	Balqa-Arida-2	*H.bacteriophora*	Jordan				
EU214642	Aqaba	*H.bacteriophora*	Jordan				
FJ006727	**SRK6**	***H. indica***	**India**	*X.ehlersii*			
FJ006729	**SRK8**	***H. indica***	**India**	DQ208308	KR02	*S.longicaudum*	South Korea
				X.innexi			
				AY521243	UY61	*S.scapterisci*	France
				X.cabanillasii			
				AY521244	USTX62	*S. riobrave*	France
				X. miraniensis			
				DQ211713	Q1	Steinernematidae	Australia
				Xenorhabdus. sp.			
				DQ205447	VN01T	*S.sangi*	Viet Nam
				AB243432	MY5	*S.* sp.	Japan
				EU124383	YL002	*S.* sp.	China
				EU124382	TB	*S.* sp.	China
				X.japonica			
				AB243426	Japonica	*S.kushidai*	Japan
				X.stockiae			
				FJ006728	**SRK7**	***S. glaseri***	**India**