TEMPORAL SYMBIOTIC RELATIONSHIPS OF ENTOMOPATHOGENIC NEMATODES (HETERORHABDITIDAE AND STEINERNEMATIDAE) WITH *PROVIDENCIA RETTGERI* AND *PSEUDOCHROBACTRUM* SP.

Reza Sharifi and Naser Eivazian Kary*

* Department of Plant Protection, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, IRAN. E-mail: eivazian@azaruniv.ac.ir

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ABSTRACT: Phylogenetic analysis of 16S-rDNA sequences of isolated bacteria from hemolymph of infected *Galleria mellonella* cadavers with entomopathogenic nematodes revealed different strains of non-symbiont bacteria. Biochemical tests and phylogenetic analysis using Maximum Parsimony, Maximum Likelihood and Neighbor Joining methods were done and two species including three strain of *Providencia rettgeri* and four strains of *Pseudochrobactrum* sp. were identified to be associated with *H. bacteriophora* and *S. carpocapsae*, respectively. As a supplementary tool, RNA secondary structure and minimum free energy was involved in analysis for more confirmation.

KEY WORDS: Entomopathogenic nematodes, *Providencia rettgeri, Pseudochrobactrum*

Entomopathogenic nematodes from the families Heterorhabditidae Poinar, 1976 and Steinernematidae Travassos, 1927 families nematodes have proven to be the most effective biological control organisms. They are soil-inhabiting organisms and can be used effectively to control soil-borne insect pests (Kaya & Gaugler, 1993). These nematodes are symbiotically associated with entomopathogenic bacteria *Photorhabdus* (Boemare et al., 1993) and *Xenorhabdus* (Thomas & Poinar, 1979). The bacterial symbionts are carried monoxenically in a special vesicle in the infective stage of members of the Steinernematidae and throughout the whole intestine of infective juveniles. Both nematodes and bacteria are pathogenic for most insects when they are released into the hemolymph. The bacterial symbionts contribute to the symbiotic relationship by establishing and maintaining suitable conditions for nematode reproduction, providing nutrients and antimicrobial substances that inhibit the growth of a wide range of microorganisms (Boemare et al., 1993).

Until now several non-symbiotic bacteria have been reported from EPNs infected cadaver, for example, *Flavobacterium* sp. was isolated from sawfly larvae infected with *S. kraussei* (Mracek, 1977), *Ochrobacterum cytisi* and *Schineria larvae* associating with *Steinernema siamkayai* and *O. anthropic* with *H. indica* (Razia et al., 2011). For *Steinernema carpocapsae* several non-symbiotic bacteria including *Alcaligenes* sp., *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Serratia liquefaciens* and *Acinetobacter* sp. have been reported as temporal associated bacteria (Gouge & Snyder, 2006a; Lysenko & Weiser, 1974). Similarly, *Ochrobactrum anthropi*, *Paracoccus denitrificans* and *Pseudomonas maltophilia* have been found to be associated in *Steinernema scapterisci* (Aguillera, 1993; Aguillera & Smart, 1993), *H. indica* and *H. bacteriophora* (Babic et al., 2000). Recently, the bacteria *Flavobacterium* sp., *Providencia vermicola* and *Alcaligenes faecalis* were isolated from the nematode *Rhabditis blumi* (Park et al., 2011). Genes that encode proteins and enzymes that are related to pathogenicity, toxicity, and host/environment interactions have been recently reported in *A. faecalis* (Quiroz-Castaneda et al., 2015).

The current investigation was done to identify the bacteria from cadavers of *G. mellonella* infected with different species of EPNs. Furthermore, we conducted the phylogenetic analysis within the genus and species level and compared their RNA secondary structure and minimum free energy of aligned16s-rDNA sequences with the related ones.

MATERIAL AND METHODS

EPN species

During a survey of entomopathogenic nematodes throughout north-west of Iran in 2013, several entomopathogenic nematodes were isolated using Galleria baiting method from soil samples and identified as *H. bacteriophora* and *S. carpocapsae* based on morphology and morphometric characters, cross breeding test, as well as molecular data (Eivazian Kary et al., 2009).

Isolation of bacterial non-symbionts from insect hemolymph

Surface sterilized infective juveniles were used to infect last instar great wax moth larvae which have been already immersed in 70% alcohol to remove putative bacterial contaminations. In each sealed Petri dishes 10 larvae exposed to 500 IJs for 24 hours at room temperature then transferred to sterile Petri dishes for another 24 h. Hemolymph from infected cadavers with typical signs of EPNs infection was chosen for bacterial isolation. Hemolymph were extracted by dissecting larvae ventrally between the 5th and 6th abdomen segments and was collected with a sterile loop and streaked on both MacConkey and NBTA agar. Bergey's manual was followed for primarily biochemical characteristics of the strains (data not presented) (Krieg & Holt, 1984).

DNA extraction and PCR

Genomic DNA was purified from isolates in culture using the DNeasy tissue kit (QIAgen) as per the manufacturer's protocol. DNA was eluted from the column into 20 µl of TE buffer and stored at -20° C.16s-rDNA gene amplification was carried out by a standard PCR reaction mixture that included 10X Taq buffer, 1.25 mM of $Mgcl_2$, 0.25 mM dNTPs, 1 mM of each primer and 1 µl of Taq polymerase using forward primer 5´-GAAGAGTTTGATCATGGCTC and reverse primer 5′-AAGGAGGTGATCCAGCCGCA-3′. All amplifications were performed with an initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. PCR products were purified using QIAquick PCR purification kit (Qiagen) in order to remove the salts, primers and unincorporated dNTPs then subjected to direct sequencing. DNA sequences were analyzed and assembled using the SeqMan program of the DNAstar Lasergene software. Sequence data generated for 16s-rDNA (accession numbers KR091943, KR091944, KR091945, KR091946, KR091947, KR091948 and KR091949) have been deposited in GeneBank.

Molecular characterization and phylogenetic analysis

The entire 16S rRNA gene sequences from type strains of the genus were obtained from NCBI databases linked in LPSN (List of Prokaryotic names with Standing in Nomenclature) (http://www.bacterio.net). BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used to find the most closely related sequences (Table 1). Two separate phylogenetic analyses were conducted to show the genealogic relationships of studied strains within the genus and phylogeographic relationships with other strains of species. Clustal X 2.0.11 (Thompson et al., 1997) with the default parameters (gap opening penalty 10 and gap extension penalty 5) was used to align 16s-rRNA sequences strains along with other homologous sequences obtained from GeneBank. MEGA6 with the

following settings were used for evolutionary analysis. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). For ML analysis, Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. In the case of NJ, The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Pairwise distances were conducted using the Maximum Composite Likelihood model. All positions containing gaps and missing data were eliminated. GeneBee-Net program (Brodsky et al. 1995; Brodsky et al. 1992) using greedy algorithm was used to RNA secondary structure prediction and minimum energy calculations of aligned sequences. The parameters were set as: energy threshold= -4.0; cluster factor=2; conserved factor=2; compensated factor=4 and conservativity=0.8.

RESULTS

Seven strains of non-symbiotic bacteria including two species were isolated from the infected cadavers of *G. mellonella*. According to biochemical test, these strains were identified as *Providencia rettgeri* strains IR14, IR16 and IR21 (non-symbiont of *H. bacteriophora*) and *Pseudochrobactrum* sp. Isolates IR5, IR7, IR13 and IR17 (non-symbiont of *S. carpocapsae*). Furthermore we conducted a throughout 16S-rDNA based phylogenetic analysis within the genus with a complete set of well-defined species in reconstructed MP, ML and NJ phylogenetic trees.

Phylogenetic analysis of the genus *Providencia*

All constructed phylogenetic trees (MP, ML and NJ) yielded same hypothesis of relationships between *Providencia* spp. Minor differences (not conflict) were observed between ML (Fig. 1) and MP (Fig. 2) trees in depicting relationship between *P. burthodogranariea* and *P. heimbachae*. In MP tree these species fall into one monophyletic group albeit bootstrap MP analysis of the 16S-rDNA dataset revealed moderate support for this clade. In all trees, *P. rettgeri* isolates IR14, IR16, IR21 and *P. rettgeri* type strain appeared as members of monophyletic group with relatively high support in which isolates IR16 and IR21 appeared as a closest relatives (100% bootstrap value). Reconstructed phylogenetic trees on the basis of 16S-rDNA sequences of 10 strains with different sources or geographic origins yielded different topologies. Remarkable result was found for *P. rettgeri* strain IR21 and *P. rettgeri* IR16, despite having EPN symbionts with different geographic origins, these strains appeared as closest relatives in trees. *P. rettgeri* strain IR14 appeared as different clade in all trees.

Free energy of secondary structures of ribosomal RNA offers an additional source of information for genealogic study (Fig. 6). Compared to type strain (-287.4 kcal/mol), free energy of secondary structures of ribosomal RNA of strains IR14, IR16 and IR21 (-303.6, -299.4 and -298.9 kcal/mol respectively) were significantly related, and correspond to intraspecific phylogenetic trees, IR16 and IR21 strains were the closest ones.

Phylogenetic analysis of the genus *Pseudochrobactrum*

In MP, ML and NJ trees *Pseudochrobactrum* sp. strains IR5, IR13, IR7 and IR17 grouped together with high bootstrap value (~100%) to make high supported monophyletic group but in depicting relationship with sister group different patterns were observed. In ML and NJ trees *P. asaccharolyticum* type strain appeared as a most relative clade to mentioned monophyletic group, but in MP tree polytomy were observed. In MP tree, terminal nodes received high bootstrap supports: 100% (*Pseudochrobactrum* sp. strains IR5*, Pseudochrobactrum* sp. strains IR13); 100% (*Pseudochrobactrum* sp. strains IR7*, Pseudochrobactrum* sp. strains IR17); 80% (*P. kiredjianiae, P. glaciei*); 68% (*P. saccharolyticum, P. lubricantis*) but polytomies were observed in deeper nodes. In depicting intraspecific phylogenetic relationships (Fig. 10), similar hypothesis were observed by ML and NJ trees. Two major monophyletic groups observed for homologous sequences. *Pseudochrobactrum* sp. strains IR5, IR13, IR7 and IR17 grouped together with high bootstrap value (~100%) to make high supported monophyletic group and other isolates made another clade in which the relationships between lineages were unresolved. Predicted hypothesis by MP tree was somewhat different from others but similar topology, ((strain IR15, strain IR13)(strain IR7, strain IR17)), was observed (Fig. 11).

The free energy of secondary structures of ribosomal RNA in studied isolates were too close to each other, among them strain IR13 with -302.7 kcal/mol was different from strain IR5 (-295.7 kcal/mol), strain IR7 (-292.3 kcal/mol) and strain IR17 (-295.7 kcal/mol) (Fig.12).

DISCUSSION

Xenorhabdus and *Photorhabdus* species are symbiotically associated with entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* respectively. The symbiotic bacteria are released in the host hemolymph by infective juvenile where they proliferate and produce a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and bioconversion of the insect larva into a nutrient soup that is ideal for nematode growth and reproduction (Fodor et al., 1997).

In this study, seven isolates of non-symbiotic bacteria are reported from hemolymph of insect *G. mellonella* infected by indigenous species of EPNs, *H. bacteriophora* and *S. carpocapsae*. Although the isolation of non-symbiotic bacteria from IJs may be the results of inadequate surface sterilization procedures, a mechanism for the transmission of non-symbiotic bacteria into the host insect has been detected. Bonifassi et al. (1999) proposed that the cuticular space between J2 and J3 is the main penetration route for bacteria in the case of *S. scapterisci* (Bedding & Molyneux, 1982). Gouge & Snyder (2006b) showed that there was no difference in bacterial species identified from non-sterile or surface sterilized nematodes, suggesting that the bacteria identified originated from either inside the nematode or between the second and third stage juvenile cuticles.

Providencia rettgeri is a Gram negative bacterium that is commonly found in both water and land environments. It is a faculative anerobe, and is fairly ubiquitous across a wide range of environments. *P. rettgeri* is known mainly for its association in the gut microbiome with humans and insects, and can potentially be the cause of oppurtunistic infections among these species. *P. rettgeri* has been known to interact with a variety of organisms, including loggerhead sea turtles, humans, insects (Galac & Lazzaro, 2011), nematodes (Jackson et al., 1995), and frogs (Penner & Hennessy, 1979). Depending on context, it can either function as a pathogen or a non-pathogenic symbiont. Isolates of *P. rettgeri* have been prepared from a variety of insect types, such as

the oil fly *Helaeomyia petrolei* (Kadavy et al., 2000), the great wax moth *Galleria mellonella* (Jackson et al., 1995), however, *P. rettgeri* may play a non-pathogenic role, such as in Australian tropical fruit flies *Bactrocera cacuminata* and *B. tryoni* (Thaochan et al., 2010). In these flies, *P. rettgeri* were found to occupy the midgut region of the digestive system, along with a variety of other bacteria. While the role of each member within this system is unknown, it is possible that in this context, *P. rettgeri* might play a mutualistic role.

It is reported that the majority of *Heterorhabditis* spp. strains tested contained a second bacterial species which was identified as *Providencia rettgeri*. Injection of the bacteria into wax moth larvae has showed that *P. rettgeri* was at least as pathogenic as *Photorhabdus* sp. K122. Both had LD50 values of less than one bacterial cell/larva, but *P. rettgeri* killed the insects at a considerably faster rate than K122 at both 28°C and 9°C. Since *Photorhabdus* kills very slowly at low temperatures, it appeared that *P. rettgeri* might be a better pest control agent under these conditions. However, *P. rettgeri* was not pathogenic when carried into insect larvae by the nematode, indicating that the nematode suppressed either its release or pathogenicity (Jackson et al., 1995).

Association of *Pseudochrobactrum* sp. with EPNs is reported here for the first time. Based on the phylogenetic analysis of 16s-rDNA sequences, studied isolates are differed from all other well defined species in the genus and form separate monophyletic group, it is possible that these strains are representative of putative new species. Complementary studies are undertaken for exact characterizations of the isolates.

The evolutionary impact of this temporary tripartite association in Insect-Nematode-Bacteria triangle remains to be elucidated. Phylogenetically two scenarios could be postulated, first, the non-symbiotic bacteria have shared common ancestor and this mode of action is a plesiomorphic character belonged to their ancestor tried to establish a permanent symbiotic association with entomopathogenic nematodes which is obvious in its extreme form in *Xenorhabdus* and *Photorhabdus* as an autapomorphic character. Second, these characters are homoplasies shared by different taxa without having genealogic relationships. Regardless to the evolutionary path, such temporary associations could be assumed as an evolutionary novelty which temporarily enables bacteria to access new host in interaction with phoretic nematodes.

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Table 1. Geographic origins and sources of different isolates of *Providencia* and *Pseudochrobactrum*.

Species	strain	Accession Number	Source	Geographical location
Providencia rettgeri	MSS2	KF923809	rhizobacterial isolate	India
	ALK420	KC456550	soil	China
	ALK421	KC456551	soil	China
	SL1_1A	JN644625	Midgut of *Culex quinquefasciatus*	India
	NCTC	NR_115880	-	Italy
	DSM 4542	NR_042413	entomopathogenic nematode	USA
Pseudochrobactrum asaccharolyticum	CD24	KF263562	Cow dung (Isolate with Nematicidal Activity)	China
	ALK635	KC456600	Soil	China
	CCUG 46016	NR_042474	-	USA
	-	KM488426	Rhizobacteria	Mexico
	LY6	KC618329	Chromium Contaminated Soil	China
	KH-25	JQ612518	-	Pakistan
	TY39OpD	HQ406751	Rhizosphere of cactus	Mexico

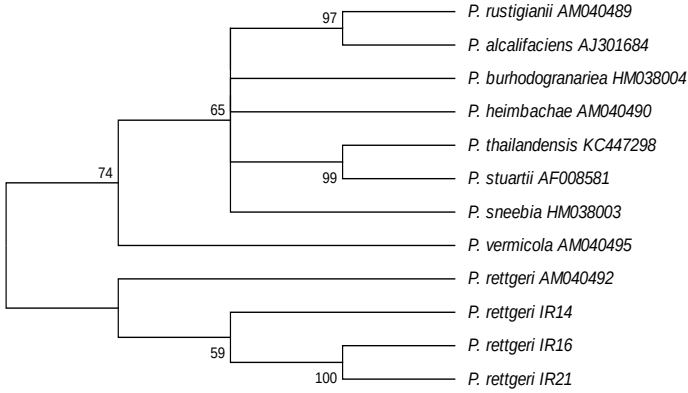

Figure 1. Hypothesis of phylogenetic relationships for *Providencia* based on 16S-rDNA produced by maximum likelihood.

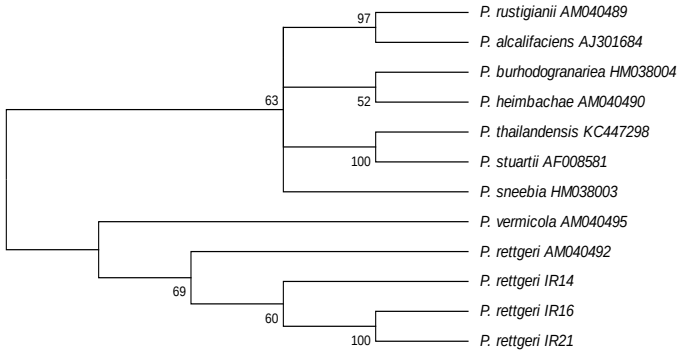

Figure 2. Hypothesis of phylogenetic relationships for *Providencia* based on 16S-rDNA produced by maximum pars.

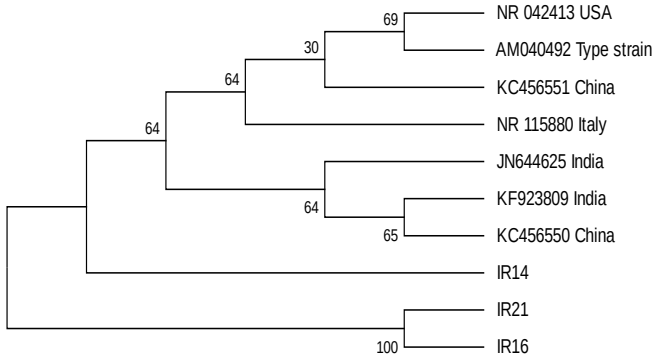

Figure 3. Hypothesis of phylogenetic relationships for *Providencia rettgeri* strains based on 16S-rDNA produced by maximum likelihood.

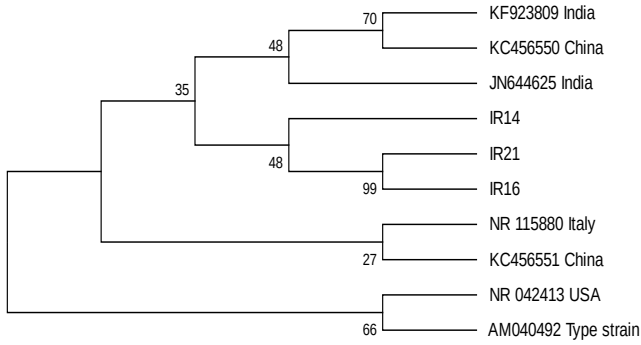

Figure 4. Hypothesis of phylogenetic relationships for *Providencia rettgeri* strains based on 16S-rDNA produced by maximum likelihood.

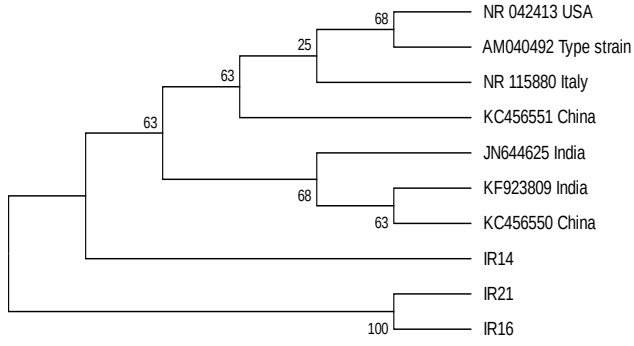

Figure 5. Hypothesis of phylogenetic relationships for *Providencia rettgeri* strains based on 16S-rDNA produced by neighbor-joining.

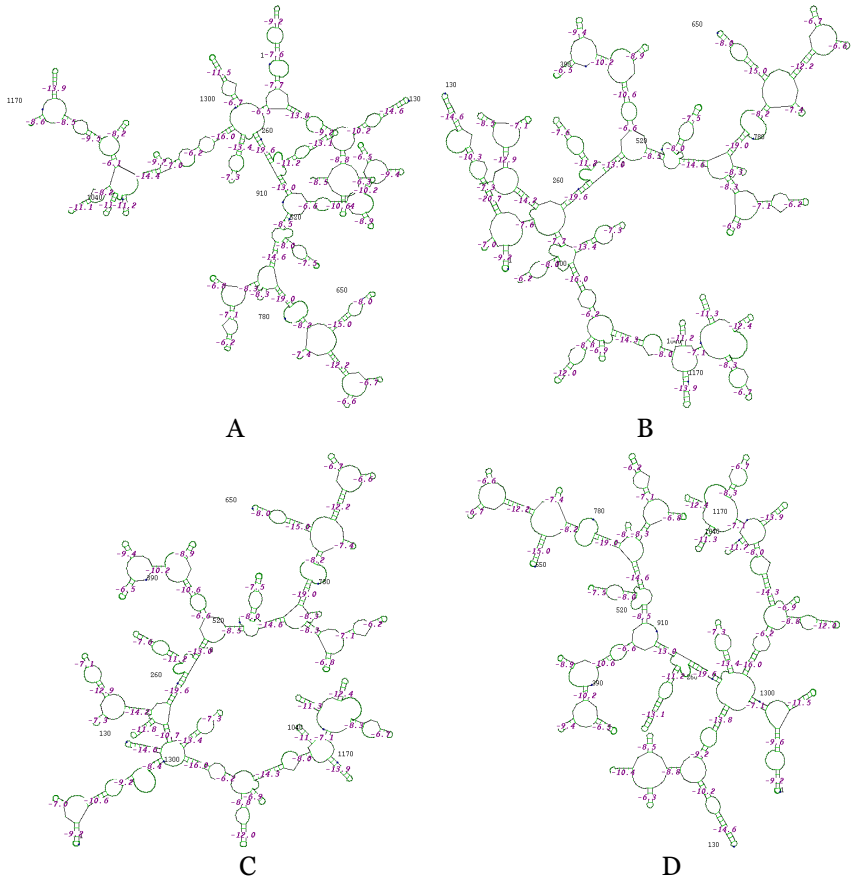

Figure 6. Graphical depiction of the predicted minimum free energy secondary structure for the 16S-rDNA sequences of *Providencia rettgeri* (A) IR14; (B) IR16; (C) IR21 and (D) type strain.

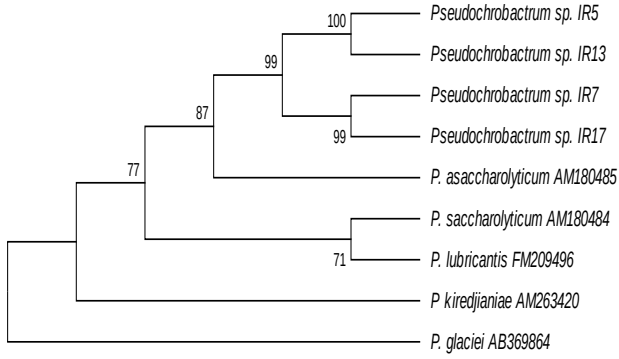

Figure 7. Hypothesis of phylogenetic relationships for *Pseudochrobactrum* based on 16S-rDNA produced by maximum likelihood.

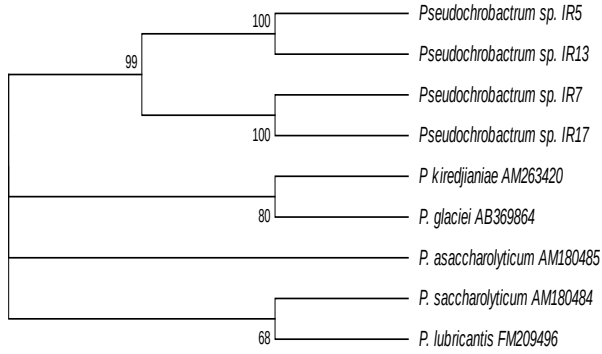

Figure 8. Hypothesis of phylogenetic relationships for *Pseudochrobactrum* sp. strains based on 16S-rDNA produced by maximum parsimony.

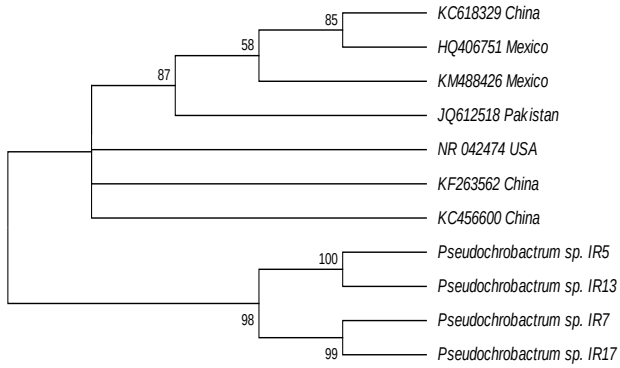

Figure 9. Hypothesis of phylogenetic relationships for *Pseudochrobactrum* strains based on 16S-rDNA produced by maximum likelihood.

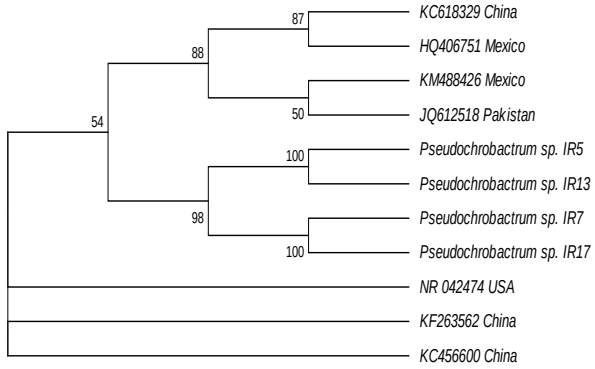

Figure 10. Hypothesis of phylogenetic relationships for *Pseudochrobactrum* sp. strains based on 16S-rDNA produced by maximum parsimony.

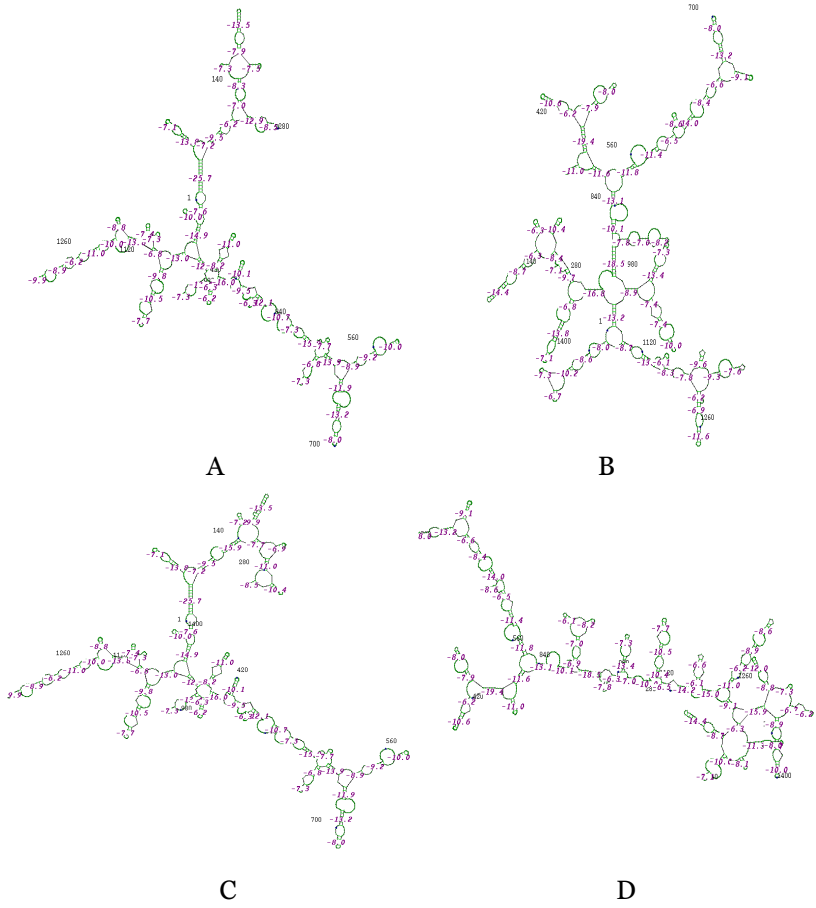

Figure 11. Graphical depiction of the predicted minimum free energy secondary structure for the 16S-rDNA sequences of *Pseudochrobactrum* sp. strains (A) IR5; (B) IR7; (C) IR13 and (D) IR17.