MOLECULAR IDENTIFICATION OF IRANIAN ISOLATES OF THE GENUS *PHOTORHABDUS* AND *XENORHABDUS* (ENTEROBACTERIACEAE) BASED ON 16S rRNA

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ABSTRACT: Five bacterial strains of *Photorhabdus* and six strains of *Xenorhabdus* originating from several geographical isolates of entomopathogenic nematodes have been studied moleculary to distinguish their position among the well defined species. Constructed phylogenetic trees based on the sequences of 16S-rRNA showed all of the studied *Photorhabdus* belong to the *P. luminescens* subsp. lumondii and *Xenorhabdus* were identified as *X. bovienii*, *X. nematophilus* and *X. budapestensis.* Among the *Steinernema feltiae* symbiont's one isolate named *Xenorhabdus* sp. IRA22 made a nearly distinct branch in the group and therefore it is possible to represent new species.

KEY WORDS: Entomopathogenic nematodes, *Photorhabdus, P. luminescens* subsp. lumondii, *Xenorhabdus, X. bovienii*, *X. budapestensis, X. nematophilus.*

Bacterial symbionts of entomopathogenic nematodes in the family Heterorhabditidae and Steinernematidaeare are members of the family Enterobacteriaceae and belong to the genera *Photorhabdus* and *Xenorhabdus* respectively (Thomas et al., 1979). The nematodes invade the larvae of susceptible insects and penetrate to the hemocoel, where they release their symbiotic bacteria (Kaya & Gaugler, 1993). In the hemocoel bacteria replicate within and kill the insect host (ffrench-Constant et al., 2003). Strains of *Photorhabdus* and Xenorhabdus species occur in two forms. The first is the bacterium isolated from IJ and was named the phase I variant. The second variant form, named phase II, appears spontaneously during period of an in vitro culture or during nematode rearing on an artificial diet (Boemare et al., 1988.). Different species and strains differ in virulence (Aguillera et al., 1993; Han et al., 1991) and therefore correct identification or grouping of the strains is essential in relation to experimental work.

16s rRNA gene has been revealed as a good marker to determine diversity among bacteria and currently all species description should now include the 16s rRNA sequence species. However, 16S rRNA evolves so slowly and may not always be high enough to distinguish closely related strains (Adams et al., 2006).

Three and nineteen species have been described for *Photorhabdus* and *Xenorhabdus* respectively: *P. luminescens* ssp. *luminescens* (type species), *P. luminescens* ssp. *akhurstii*, *P. luminescens* ssp. *laumondii*, *P. luminescens* ssp. *kayaii*, *P. luminescens* ssp. *thracensis*, *P. temperata*, *P. temperata* ssp. *temperata*, and *P. asymbiotica* ssp. asymbiotica and *P. asymbiotica* ssp. americana (Akhurst et al., 2004), *X. nematophilus*; *X. bovienii*; *X. poinarii*; *X. beddingii* (Akhurst & Boemare, 1988); *X. japonica* (Nishimura, 1994); *X. budapestensis*; *X. innexii*; *X. szentirmaii*; *X. ehlersii* (Lengyel et al., 2005); *X.*

doucetiae; *X. griffiniae*; *X. cabanillassii*; *X. mauleonii*; *X. kozodoii*; *X. hominickii*; *X. koppenhoeferii*; *X. miraniensis*; *X. romanii; X. stokiae* (Tailliez et al., 2006).

In the current study we characterize molecularly the isolates of *Photorhabdus* and *Xenorhabdus* that are symbiotically associated with different isolates of entomopathogenic nematodes, *H. bacteriophora*, *S. bicornutum*, *S. carpocapsae* and *S. feltiae* which were recently isolated from north-west of Iran (Eivazian et al., 2009).

MATERIAL AND METHODS

Bacteria. Symbiotic isolates were provided by the Insect Pathology Lab. Of the Science Department of the Azarbaijan University of Tarbiat Moallem.

Extraction of DNA. Qiagen DNeasy kit was used to extract DNA from cell harvested from 24 h nutrient broth cultures.

Amplification of the 16s rDNAs. The prokaryote-specific primers of Fischer-Le Saux et al. (1999) were used in PCR amplification of the 16S rRNA gene. PCR was performed in 50 µl reaction that contained: 200 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.01% gelatin, 0.1 mM each primer, template DNA (5 µl), and 2 U of *Taq* polymerase. In all cases negative controls contained all components for the PCR except the template DNA. Reactions were run on a Biometra thermocycler, with 35 cycle of denaturation at 96 ^oC for 1 min, annealing at 55 ^oC for min and extension at 72 ^oC for 2.5 min, followed by a final extension at 72 ^oC for 4 min and a renaturation step at 25 ^oC for 10 min.

Sequencing and Phylogenetic analysis. Multiple-sequence alignments were created using CLUSTAL X version 2 (Thompson et al., 1997). Sequence data were analyzed by unweighted maximum parsimony (MP) using PAUP* version 4.0b (Swofford, 1998). All data were assumed to be unordered. Tree searches of the ITS datasets were performed using heuristic methods with TBR (tree-bisection reconnection) branch swapping, and a minimum of 1000 replicates of random stepwise addition. Sequences used as outgroups were the 16S rRNA gene sequences of *E. coli* (GenBank accession no. J01695). Trees were represented graphically with the software TREEVIEW version 1.6.6 (Page, 2001).

RESULTS AND DISCUSSION

Molecular methods can be employed to determine diversity among bacteria or used for rapid Identifcation of a bacterium in question so as to avoid laborious phenotypic characterization (Adams et al., 2006). Phylogenies based on 16S rRNA gene sequences have previously been used to distinguish several groups within the genus *Photorhabdus* and *Xenorhabdus* (Szállás et al., 1997; Fischer-Le Saux et al., 1999). The almost complete 16S rRNA gene sequences of the isolates were aligned to the homologous sequences of *Xenorhabdus* and *Photorhabdus* (GenBank). In the case of studied *Photorabdus* isolates phylogenetic dendrogram grouped them together with the EU513181, AY278650 and AB355874 sequences, while isolates IRA10 and EU513181 and IRA3 and IRA4 were in separate sub-groups. Although phylogenetic analysis of 16S rRNA gene sequences supported the grouping of isolates into the subspecies groupings identified by Fischer-Le

Saux et al. (1999), these subspecies groupings did not form consistent groups at the species level. Isolates of *Photorhabdus luminescens* ssp. luminescens did not cluster closely with the other subspecies of *P. luminescens* (Fig. 1). In the constructed tree, all studied 16s-rRNA sequences of different species or subspecies made separate groups. The inconsistency of the species-level groupings in this analyses suggests that 16S rRNA gene sequence data are not suitable for the definition of species within the genus *Photorhabdus*, although they are useful at the subspecies level. This is because sequence differences are restricted to a few variable regions, and so the information content is not sufficient to provide reliable species-level groupings (Akhurst et al., 2004). Similar results were obtained earlier by Akhurst et al., 2004. The majority of branching points in Fig. 1 were supported by relatively high bootstrap values on terminal and low values for interior nods, this indicates that the branching order of the studied *Photorhabdus* isolates isn't settled using the database`(16S-rRNA) that was used to generate (Fig. 1) but although branch support is weak for the majority of nodes in the tree, the terminal taxa correspond closely with DNA homology groups (Fischer-Le Saux et al., 1999). Use of ribosomal subunit sequences for determining phylogenetic relationships has limitations. These genes can undergo lateral gene transfer across taxonomic groups or can be recombined, which could provide false evolutionary data (Yap et al., 1999). Therefore, bacterial phylogeny based solely on 16S rDNA sequence should be regarded as preliminary (Lerat et al., 2003). Dauga (2002) demonstrated that phylogeny based on the *gyr*B gene provided a more robust tree for determining intrageneric relationships among *Serratia* spp., whereas the 16S rDNA gene was effective for determining phylogenetic relationships among more distantly related enteric bacteria. Akhurst et al., 2004 in the study of different isolates of *P. asymbiotica* showed that although symbiotic isolate clustered consistently with *P. asymbiotica* in *gyr*B phylogenetic analyses, DNA–DNA hybridization indicated that this isolate does not belong to the species *P. asymbiotica* and that there is a clear distinction between symbiotic and clinical species of *Photorhabdus*. Therefore using a polyphasic approach that involved DNA–DNA hybridization, phylogenetic analyses of 16S rRNA and gyrB gene sequences and phenotypic characterization are necessary to define species but for sub-species identification one of the mentioned molecular markers are enough. Indeed, many molecular biologists and taxonomists believe that bacterial systematics will one day be based solely on the recognition of molecular patterns. However, the time has not yet come to discard morphology, metabolic properties, and other traditional approaches that have served systematists well in the last decades (Adams et al., 2006).

The interesting result was obtained in the case of two isolates including *P. luminescence* lumondii IRA3 and *P. luminescence* lumondii IRA2 which grouped together in separate sub-group within *P. luminescence* lumondi clade (Fig. 1). Both of these isolates are symbionts of *H. bacteriophora* isolates which belong to the nearest geographical regions with the similar soil type and vegetation compared other ones. There is a high degree of specificity imposed on the symbiotic relationship between the bacteria and the nematode. This specificity is generally more restrictive for the *Heterorhabditis–Photorhabdus* pair where one species of nematode retains one species of bacteria.

Maximum Parsimony constructed tree based on the 16s-rRNA showed that studied *Xenorhabdus* isolates fall in three different groups including *X. nematophilus* symbiont of *S. carpocapsae* IRA18, *Xennorhabdus bovienii* symbiont of different geographical isolates of *S. feltiae* and *X. budapestensis*

symbiont of *S. bicornutum* but among *X. bovienii* group, symbiotic bacteria of *S. feltiae* IRA22 named here *Xenorhabdus* sp. IRA22 made a nearly distinct branch in the group and therefore it is possible to represent new species (Fig. 2; Table 2) although the recognition of a phylogenetic substructure does not immediately imply that the description of new species is a straight forward process (Adams et al., 2006) and more studies should be carried out.

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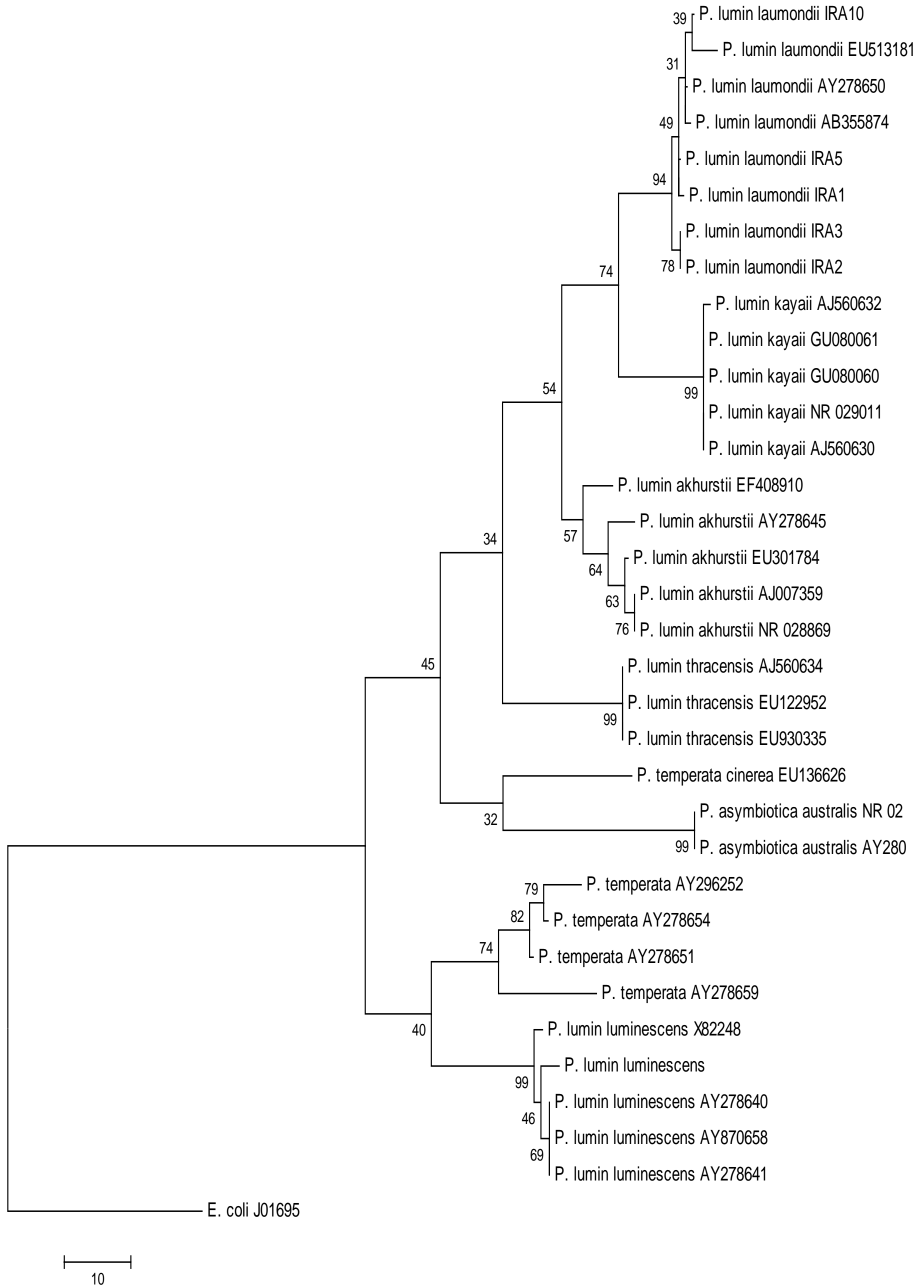

Figure 1. Phylogenetic relationship of studied isolates with other *Photorhabdus* species based on 16S-rRNA.

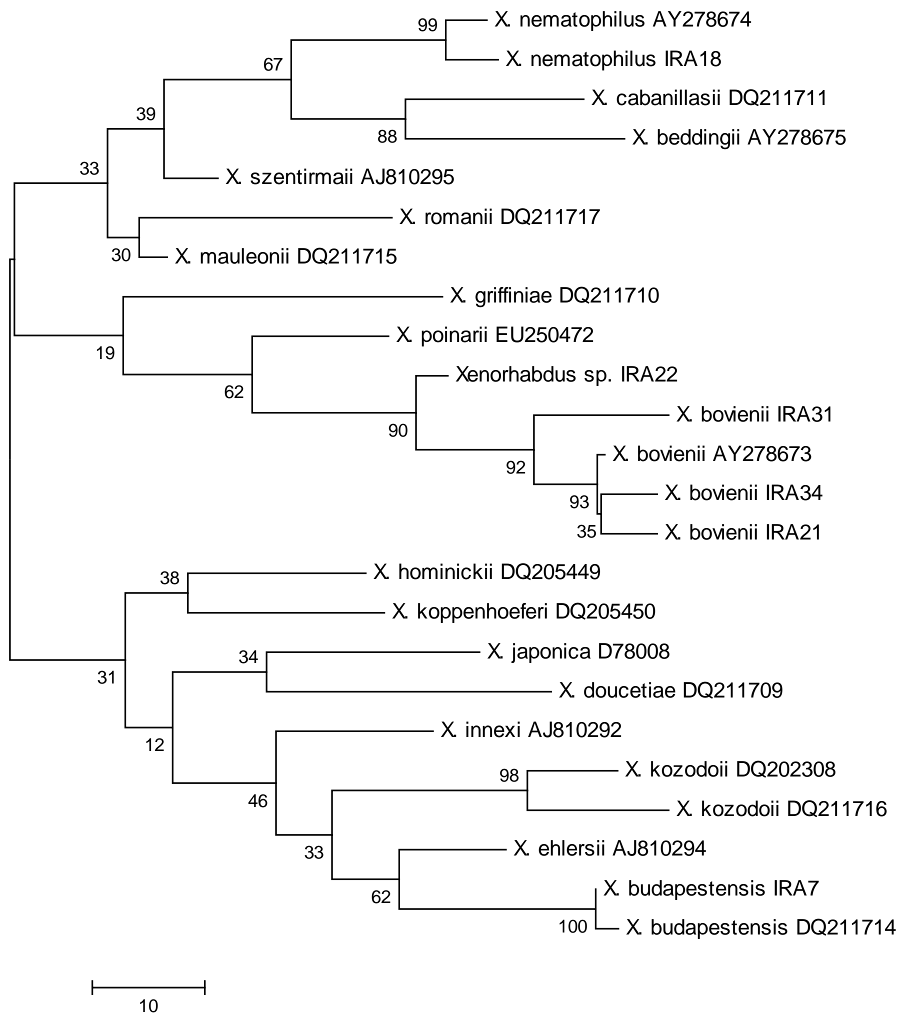

Figure 2. Phylogenetic relationship of studied isolates with other ***Xenorhabdus*** species based on 16S-rRNA.

Table 1. Pairwise distances between *Photorhabdus* species / subspecies.

	1	2	3	4	5	6
P. luminescens ssp. *kayaii*						
P. luminescens ssp. *thracensis*	0.030					
P. luminescens ssp. *akhurstii*	0.021	0.022				
P. luminescens ssp. *laumondii*	0.015	0.027	0.017			
P. temperate	0.040	0.026	0.038	0.040		
P. luminescens ssp. *luminescens*	0.029	0.037	0.028	0.029	0.024	
P. asymbiotica	0.035	0.044	0.035	0.034	0.042	0.036

Table 2. Pairwise distances between *Xenorhabdus* species.

	1	2	3	4	5	6	7	8	9	10
X. nematophilus AY278674										
X. nematophilus IRA18	0.006									
Xenorhabdus sp. IRA22	0.041	0.041								
X. budapestensis DQ211714	0.051	0.051	0.046							
X. bovienii AY278673	0.041	0.041	0.012	0.041						
X. bovienii IRA31	0.046	0.044	0.018	0.045	0.013					
X. budapestensis IRA7	0.049	0.050	0.044	0.002	0.040	0.044				
X. bovienii IRA34	0.046	0.047	0.018	0.044	0.006	0.017	0.044			
X. bovienii IRA21	0.046	0.046	0.017	0.044	0.005	0.016	0.043	0.008		
X. poinarii EU250472	0.039	0.039	0.021	0.043	0.032	0.034	0.041	0.036	0.035	
X. griffiniae DQ211710	0.049	0.054	0.035	0.047	0.045	0.050	0.044	0.050	0.050	0.030