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Phylogenetic Analysis on Some Iranian White Grubs with New Data about Natural Pathogen of *Polyphylla adspersa*

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ABSTRACT

Three species of white grubs, *Polyphylla olivieri*, *P. adspersa* and *Melolontha melolontha* (Scarabaeidae: Melolonthinae) are among the serious economically important pests in Iran. Larval identification of those species is a difficult issue. Here, we provided DNA barcodes for these species of scarabids using mitochondrial DNA cytochrome oxidase I (COI). The relationship between these three species of Iranian white grubs and related species was addressed. Among these species, *P. adspersa* is the predominant species in North-East of Iran, Khorasan provinces.The isolation of an unknown pathogen indicated the incidence of a natural infection by a species of entomopathogenic nematode from *Heterorhabditis* genus. Classic data as well as molecular characterization of this insect pathogen using ITS sequences confirmed the occurrence of *H. bacteriophora* in natural populations of *P. adspersa*. This is the first data about DNA barcodes of Iranian white grubs as well as new record for natural entomopathogen of *P. adspersa* larvae.

Keywords: Polyphylla, *Melolontha*, *Heterorhabditis*, Natural Entomopathogen, DNA Barcode, Khorasan

INTRODUCTION

The family Scarabaeidae with includes approximately 27800 species composed of 91% of all Scarabaeoids worldwide. Melolonthinae is the largest Scarabaeidae subfamily containing one-third of the species of this family (Ratcliffe and Jameson, 2004). *Polyphylla* Harris is made up of 28 species (Young, 1988; Skelly, 2009) including *P. olivieri* Castelnau and *P. adspersa* Motschulsky along with the European cockchafer *Melolontha melolontha* Linnaeus are considered to be the economically important pests of this subfamily. *P. olivieri* and *M. melolontha* are reported from all over the world (Davatchi *et al*., 1959; Lakatos and Toth, 2006; Švestka, 2006) while *P. adspersa* is just restricted to the former USSR, North Turkey and Iran (Davatchi *et al*., 1959). In Iran, *P. olivieri*, the most injurious Scarabid is distributed throughout much of the provinces as well as the central regions, Eastern, Northern and Western provinces, *P. adspersa* is localized in some limited areas of the country in North-East and the Northern provinces (Davatchi *et al*., 1959) and *M. melolontha* is limited to Northern provinces of Iran considered as Poplar plantations pest (Sadeghi *et al*., 2009).

In general, it's said that since morphological identification keys are often effective only for a particular life stage or gender, many individuals cannot be identified (Hebert *et al*., 2003). This is also true for immature forms of white grubs that are generally difficult to identify using morphology-based keys due to the variable morphological differences in some species, while rapid identification is sometimes necessary and inevitable. As well, the use of keys often demands such a high level of expertise that misdiagnoses are common (Hebert *et al*., 2003). In this case, molecular markers can be effective enough for determining and linking the larvae and adults. Among the molecular markers used for analyzing molecular variation, sequencing of mitochondrial DNA (mtDNA) and ITS ribosomal DNA (rDNA) have been recognized as effective markers not only for species identification of different insects but also for phylogenetic relationship analysis (Zhu *et al*., 2000; Hebert *et al*., 2003; Monti *et al*., 2005; Qiu *et al*., 2009). As a DNA barcode, a part of mitochondrial Cytochrome Oxidase I (mtCOI) gene is well suited to identify morphologically similar species and provide numerous sequence differences between closely related species (Avise, 1994; Caterino *et al*., 2000). DNA barcoding is a taxonomic method aiming to identify individuals on the species-level and is generally considered to be as a reliable, cost-effective and easy molecular identification tool with a wide applicability across metazoan taxa (Hebert *et al*., 2004a, b; Smith *et al*., 2008). This can be achieved by comparing a short DNA sequence from a specific part of the genome of the individual in question to a reference library of barcode sequences of known identity (Hajibabaei *et al*., 2007; Hebert *et al*., 2003; Ratnasingham and Hebert, 2007). It is "based on the premise that a short standardized sequence can distinguish individuals of a species because genetic variation between species exceeds that within species" (Hajibabaei *et al*., 2007). For the DNA sequence to be assigned, a 648-base fragment of the 5' end of the COI gene the "Folmer region" (Folmer *et al*., 1994) has been proposed by Hebert *et al*., (2003) and has become the designated default barcode region for vertebrates and insects (Consortium for the Barcode of Life, s. a.; Hajibabaei *et al*., 2007). The COI gene was chosen because it evolves rapidly enough to allow the distinction between species (Cox and Hebert, 2001), is highly conserved within species and the desired DNA fragment can be amplified from a broad range of eukaryotic taxa by using universal primers (Folmer *et al*., 1994; Hebert *et al*., 2003) due to its highly conserved flanking regions (Simon *et al*., 1994). COI gene has also been used for phylogenetic studies of a wide range of insects so far (Funk, 1999; Frati *et al*., 1997; Brower, 1996; Howland and Hewitt, 1995; Sperling and Hickey, 1994). Unfortunately information about Iranian scarabids is limited to only a few faunistic surveys (Modarres Awal, 2006) and there is no data with regard to the genetic structure of Iranian white grubs till now. So, this is the necessity of focusing on molecular researches as well as DNA barcoding for Scarabids identification and their phylogenetic analysis based on sequencing.

Although application of chemical insecticides, trichlorofon, chlorpyrifos, carbaryl and diazinon, has been the main method for control white grubs (Grewal *et al*., 2005), effective management of these pests often is difficult because larval infestations within the soil environment would not be obvious enough until above ground symptoms

are apparent (Koppenhöfer and Kaya, 1998). On the other hand, these species have more than one year developmental cycle and therefore, spend long terms of developing time within the soil as larva. Hence, alternative solution must be used for controlling the white grubs. Soil-dwelling entomopathogenic nematodes (EPNs) are supposed to be environmentally safe and can be applied as suitable candidates for potential biocontrol agent against the larval stages of white grubs (Koppenhöfer *et al*., 2002). Some species of EPNs have been reported to infect white grub species in the laboratory and field (Converse and Grewal, 1998). Susceptibility of different species of white grubs has evaluated to EPNs, but no studies have been conducted on natural entomopathogens of *P. adspersa* so far. So, we addressed for a natural entomopathogen of this grub in North-East of Iran. This is the first research towards finding an insect pathogenic nematode in larval population of *P. adspersa*, promising the use of these entomopathogens as biological control agents against this pest in Iran. Also, we examined individuals collected from Razavi Khorasan using mtCOI for determining phylogenetic relationship of these economically important white grubs.

MATERIALS AND METHODS

The Study Insect

A total of 25 white grub specimens including *P. olivieri*, *P. adspersa* and *Melolontha melolontha* were obtained from the insect collection, department of plant protection, Ferdowsi University of Mashhad, Iran. In order to isolate and characterize the natural entomopathogenic nematode of *P. adspersa,* different larval stages this species were collected from different orchards of Mashhad, Razavi Khorasan province. Larvae were kept individually in a mixture of soil and organic material in plastic containers at room temperature with pieces of carrot provided as food.

DNA Extraction, Amplification and Sequencing Analysis for DNA Barcoding

DNA of 25 individuals of the three species were extracted from musculature of the legs of adult or larval stages using Qiagen DNA Easy blood and Tissue Kit following the manufacturer's instructions (www.qiaqen.com) and the mitochondrial DNA was amplified by PCR using universal COI primers, LCO1490 and HCO2198 (Folmer *et al*., 1994) in standard 25 µl reactions containing 1 µl DNA template, 2.5 µl (10X) buffer, 1µl $MgCl_2$, 0.5 µl dNTPs, 1 µl forward and reverse primer (10 picomoles/µl), 0.3 µl *Taq* polymerase (5U). Temperature conditions for COI amplification were denaturation at 94°^{C} for 60 sec; annealing at 54°^{C} for 90 sec and extension at 72°^{C} for 90 sec (30 cycles, plus an initial denaturation at 94°^{C} for 1 min and a final extension at 72°^{C} for 8 min). PCR-amplified products were purified using Bioneer's PCR purification Kit (www.Bioneer.com). Several individuals from each species were sequenced and chromatograms were subjected to VSQual (Binneck *et al.,* 2004) to evaluate the reliability of the data, and good quality fragments were used to construct a consensus sequence for each sample. Consensus sequences of COI partial gene were multiple aligned using Clustal W (ver. 1.83) (Thompson *et al.,* 1994) with default

parameters. BLAST (http://www.ncbi.nlm.nih.gov/blast/) and BOLD system (http://www.boldsystems.org) were employed to identify similarities between our sequences and those of GenBank. The MEGA5 program (Tamura *et al.,* 2011) was used to calculate the number of variable sites among species studied in this work and related species retrieved from the GenBank (Table 1). Gap barcoding between interspecific and intraspecific distances in COI gene of 155 taxons of subfamily Melolonthinae was addressed. COI sequences were retrieved from the BOLD system (www.boldsystems.org). The histogram constructed after measuring the distances and calculation of pairwise sequence divergence on the basis of Kimura 2-parameter distances model (Kimura, 1980) using TAXONDNA (Meier *et al*., 2006). To visualize these patterns of divergence, the trees were constructed using neighbor-joining (NJ) method (Saitou and Nei, 1987), maximum likelihood (ML) and maximum parsimony (MP) via the MEGA5 program (Tamura *et al.,* 2011). Bootstrap analysis with 10000 replicates was performed to estimate support for nodes in groups (Felsenstein, 1985).

Table 1. List of species and their GenBank accession numbers used in studying *Polyphylla* phylogeny based on COI sequence.

Species	Accession numbers	Species	Accession numbers
Phyllophaga prunina	EU156723	*Phyllophaga rubiginosa*	EU156725
Phyllophaga corrosa	EU156650	*Phyllophaga torta*	EU156747
Phyllophaga profunda	EU156717	*Phyllophaga glabricula*	EU156691
Phyllophaga ilicis	EU156595	*Phyllophaga submucida*	EU156737
Phyllophaga bipartite	EU156625	*Hoplia argentea*	DQ295287
Phyllophaga calceata	EU156632	*Hoplia argentea*	DQ295286
Phyllophaga implicita	EU156616	*Hoplia argentea*	DQ295287
Phyllophaga futilis	EU156599	*Melolontha melolontha*	DQ295258
Phyllophaga hirtiventris	EU156701	*Polyphylla olivieri*	GQ402094
Phyllophaga congrua	EU156644	*Maladera holosericea*	DQ295298
Phyllophaga congrua	EU156647	*Onthophagus sugillatus*	EU162475
Phyllophaga crassissima	EU156656	*Onthophagus australis*	EU162441
Phyllophaga praetermissa	EU156713	*Onthophagus alcyonides*	EU162439
Phyllophaga fusca	EU156615	*Onthophagus vermiculatus*	EU162477
Phyllophaga affabilis	EU156618	*Onthophagus asperulus*	EU162440
Phyllophaga ephilida	EU156676	*Onthophagus stockwelli*	EU162474
Phyllophaga longitarsa	EU156606	*Onthophagus acuminatus*	EU162437
Phyllophaga longitarsa	EU156609	*Onthophagus aeruginosus*	EU162438
Phyllophaga inepta	EU156604	*Onthophagus praecellens*	EU162464
Phyllophaga inepta	EU156603		

The Isolation and Characterization of Natural Entomopathogenic Nematode from *P. adspersa*

Larvae of *P. adspera* with symptoms of infection by EPNs were transferred to the white trap. Isolated infective juveniles were used for morphological and molecular characterization. Key criteria of the infective juvenile, hermaphrodite, males and females were determined and compared with available data (Stock and Hunt, 2007). For molecular studies, single a female was used for DNA extraction using Bioneer Kit (Bioneer Inc, Korea). The ITS region was amplified in a sterile 0.5 ml tube using the primers described by Joyce *et al.* (1994). The PCR conditions were as described by Hominick *et al*. (1997). PCR product was purified and sequences of both strands of DNA were determined, edited and verified by DNASTAR (Lasergene, USA). In order to determine the phylogenetic status of the isolated nematode, ITS sequences of *Heterorhabditis* were compared to those of obtained from Genbank (Table 2).

Phylogenetic and molecular analyses were conducted based on NJ method of the ITS region using the software ClustalX ver. 1.83 (Thompson *et al*., 1994) and MEGA5 (Tamura *et al*., 2011). Following morphological and molecular characterization, it was attempted to identify the new isolate of EPN via cross-breeding test. Following Susurluk *et al*. (2001), the isolated nematode from a commercial formulation based on H. bacteriophora (Larvanem from Koppert Inc.) was used. The fertility of the offsprings were checked by infestating of *Galleria melonella* larvae and propagation potential was recorded after two weeks.

RESULTS

Identification and Phylogenetic Relationships

Partial fragment of COI gene were amplified successfully for 25 individuals. The mtDNA sequence corresponds to positions between 1.490 and 2.199 of the *Drosophila melanogaster* sequence encoding the cytochrome oxidase I gene, partial cds (GenBank accession U37541, NC001709). The COI gene had 658 bp length, among them 344 characters were constant, 9 characters were parsimony-uninformative and 238 sites were parsimony-informative characters. Obtained sequences for *P. olivieri*, *P. adspersa* and *M. melolontha* (as representative of all samples) were submitted in GenBank with accession numbers of GQ402094, JN033794 and JN033793, respectively.

Based on NJ method, two subfamilies of Scarabaeidae (Melolontinae and Scarabaeinae) distinctly categorized (Fig. 1). Maximum likelihood and maximum parsimony are also shown the same result (Not shown here), *P. olivieri* and *P. adspersa* made a clade with high bootstrap. In all cladograms (NJ, ML and MP), these separated clearly with high support of bootstrap. Due to similarity of resulted clades from three methods, we presented here only NJ cladogram. The result showed that Polyphylla species are a monophyletic group. This is supported with bootstrap values resulted from NJ tree, ML and MP trees. Analyses showed a well-defined clade comprised of *P. olivieri* and *P. adspersa*. Also, the result confirmed the occurrence of *M. melolontha*

in Iran by molecular data.

Table 2. List of species and their GenBank accession numbers used in studying *Heterorhabditis* phylogeny based on ITS sequence.

Species	Accession numbers	Species	Accession numbers
Heterorhabditis bacteriophora	EU074157	*Heterorhabditis indica*	GU174006
Heterorhabditis bacteriophora	FJ217351	*Heterorhabditis indica*	JN157774
Heterorhabditis bacteriophora	EU850799	*Heterorhabditis indica*	HQ225858
Heterorhabditis bacteriophora	EU796074	*Heterorhabditis indica*	JN157773
Heterorhabditis bacteriophora	EU860183	*Heterorhabditis indica*	HQ225902
Heterorhabditis bacteriophora	EU715293	*Heterorhabditis indica*	HQ225903
Heterorhabditis bacteriophora	EU699435	*Heterorhabditis zealandica*	EU860184
Heterorhabditis bacteriophora	EU848594	*Heterorhabditis zealandica*	GU174010
Heterorhabditis bacteriophora	FJ217350	*Heterorhabditis zealandica*	EU718483
Heterorhabditis bacteriophora	EU848595	*Heterorhabditis zealandica*	FJ455845
Heterorhabditis bacteriophora	EU850800	*Heterorhabditis zealandica*	GU174009
Heterorhabditis bacteriophora	EU715292	*Heterorhabditis zealandica*	EU727166
Heterorhabditis bacteriophora	FJ360729	*Heterorhabditis downesi*	EF043442
Heterorhabditis bacteriophora	EU740971	*Heterorhabditis downesi*	EU921443
Heterorhabditis bacteriophora	FJ360728	*Heterorhabditis downesi*	EU921444
Heterorhabditis bacteriophora	EU860186	*Heterorhabditis megidis*	AY293284
Heterorhabditis bacteriophora	EU860187	*Heterorhabditis megidis*	EF043439
Heterorhabditis bacteriophora	FJ360727	*Heterorhabditis megidis*	AY321480
Heterorhabditis bacteriophora	FJ217349	*Heterorhabditis megidis*	EU921442
Heterorhabditis bacteriophora	EU796073	*Heterorhabditis megidis*	EU921441
Heterorhabditis baujardi	AF548768	*Heterorhabditis megidis*	HQ225905
Heterorhabditis baujardi	EU363039	*Steinernema carpocapsae*	AY171282

In this study, the sequence variability of COI in the species of subfamily Melolonthinae were examined. In order to evaluate the effectiveness of barcodes in the species determination of Melolonthinae subfamily of white grubs, we used our data and all COI sequences of this subfamily from BOLD system. These barcodes can contribute to species identification. Our results confirmed the existence of a wide gap between intra- and -interspecies divergences's for COI gene without any overlap (Fig. 2). This gap between the intra-species and inter-species variations of COI sequences enables these specimens to be distinguished by DNA barcoding.

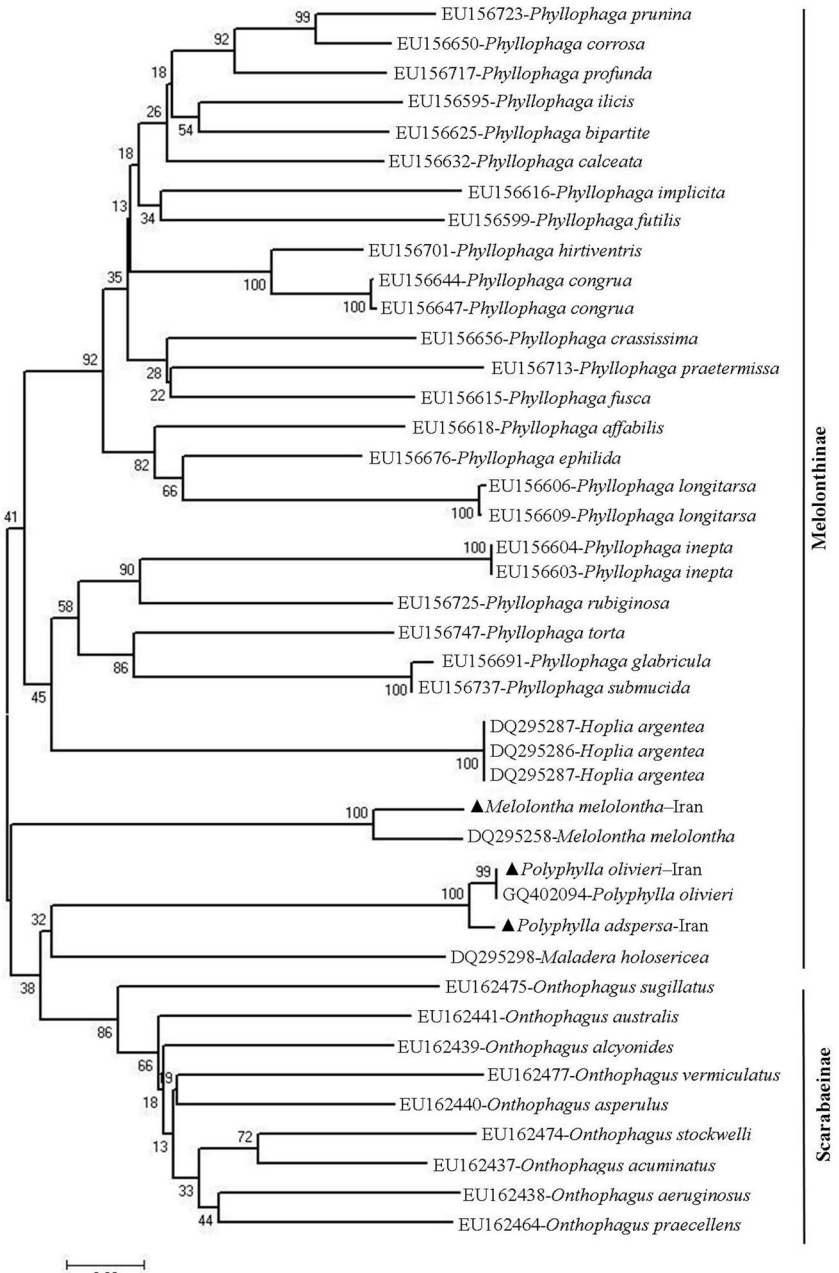

Fig. 1. Phylogenetic relationship among *Polyphylla* and related white grubs species based on COI gene using neighbor joining method (NJ) and uncorrected p-distances model with 10000 replicates of bootstrap.

Identification and Phylogenetic Relationship of Natural Entomopathogen of *P. adspersa*

A single isolate of entomopathogenic nematode (assigned as Mashhad 2) was isolated from second larvae of *P. adspersa*. The infected grubs showed typical symptoms of infection by *Heterorhabditis* species. This strain had the specific developmental characters of *Heterorhabditis.* Morphological identification indicated that most of the characters of the isolate resemble to those of *Heterorhabditis*. Key diagnostic features of the third-stage infective juveniles (IJs) and males were identical to those of "*bacteriophora*" group. Also, phylogenetic analysis of ITS rDNA sequence data placed this species in a clade with other isolates of *H. bacteriophora.* As showed in figure 3, all species-group of Heterorhabditis including "bacteriophora", "indica" and "megidis" were separated clearly. The isolated *Heterorhabditis* clustered with other isolates of *H. bacteriohora* confirmed the previous results by Stock and Hunt (2007) and Phan *et al*. (2003).

Cross-breeding confirmed the species designation for the new strain of EPN. Crossing of Mashhad 2 with a strain of *H. bacteriophora* resulted in fertile offspring. ITS sequences implied to assign the new EPN strain to the species *H. bacteriophora*. This is the first report of natural infection of the white grub, *P. adspersa* by *H. bacteriophora*. The infection rate of the first and second larval stages of the white grub, *P. adspersa* was moderate.

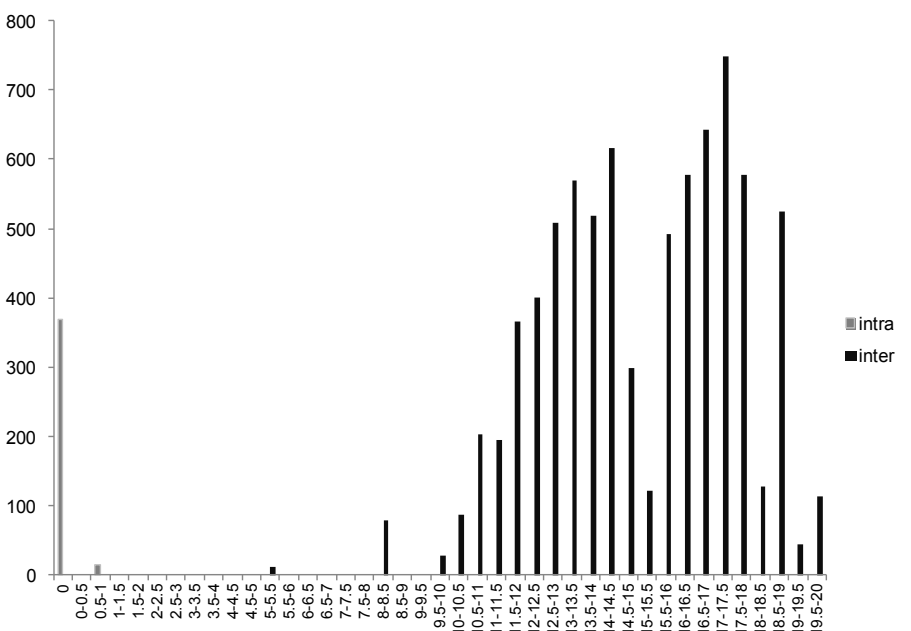

Fig. 2. Intraspecific (gray) and interspecific (black) variation in K2P distances in the COI gene of 155 taxon of Melolonthinae subfamily, showing a clear gap barcoding.

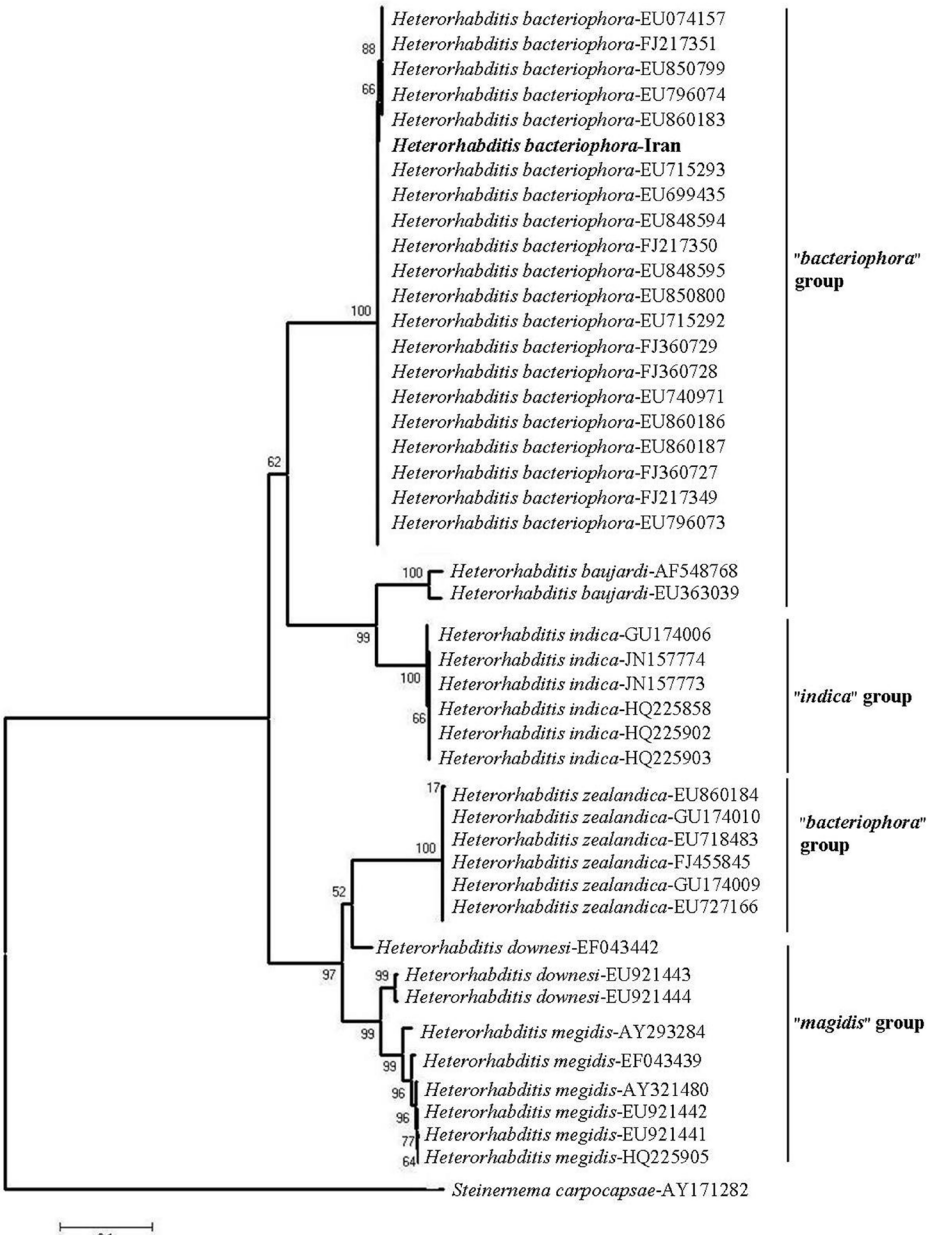

Fig. 3. Phylogenetic analysis of ITS rDNA of *Heterorhabditis bacteriophora* strains and other species of *Heterorhabditis* species groups. The dendrogram was constructed by the neighbor-joining method and Kimura-2 parameter model. *Steinernema carpocapsae* (AY171282) was included as an outgroup. Bootstrap values (10000 resamplings) are included. Bar indicates 1% nucleotide substitutions.

DISCUSSION

Weak phylogenetic foundation of the Scarabaeoidea superfamily is a problem. This problem resulted from issues like rare information about all Scarabaeoid taxa which indicate the necessity for research on the less speciose groups of Scarabaeoid especially on the more speciose groups. All these, remarks the significance of phylogeny of scarabs especially using DNA data. At the family level, classification of the world Scarabaeidae is variably known. The classification of the world Dynastinae is fairly well established due to the work of Endrödi (1985). Most Melolonthinae, Rutelinae, and Cetoniinae remain are poorly known taxonomically worldwide, and many new world genera cannot be reliably identified (Ratcliffe and Jameson, 2004). So, this study is a pioneer towards understanding genetic variation of not only Melolontinae subfamily, but also of Iranian Melolontinae white grubs. Unfortunately, there are no additional DNA sequences present, which could corroborate our present findings. Therefore, more studies including different geographic populations and additional DNA regions are necessary to elucidate the molecular phylogeny of these beetles. Due to the low divergence obtained in these beetles, a larger study of many populations and different genes may help for a better reconstruction of the phylogeny of *Polyphylla* and related genera in Iran.

Furthermore, DNA barcoding; the analysis of sequence divergence in the 5′ region of the COI gene, has been shown to provide an efficient tool to identify white grub species based on this study. This is the first data about DNA barcodes of Iranian white grubs as well a new record for natural entomopathogen of *P. adspersa* larvae. Identification of nematode species by means of taxonomic keys (Hominick *et al*., 1997; Nguyen and Hunt, 2007) is a complicated issue due to the overlap in morphological features. In this work, we used molecular and classic data for characterization of a new isolate of entomopathogenic nematode. Molecular identifications based on sequences of the ITS region were supported by morphometrics, morphological and biological observations.

A critical step towards finding an effective bacto-helminthic complex for pest control is to seek endemic EPN isolates. These types of biocontrol agents are likely to possess physiological traits that are adapted to local climatic and ecological conditions. Some species of EPNs originally were isolated and described from naturally infected white grubs collected from fields. These species are *S. anomali* Kozodoi and Poinar, *S. scarabaei* Stock and Koppenhöfer, *S. glaseri* Steiner, *S. kushidai* Mamiya, and *H megidis* Poinar, Jackson and Klein (Klein, 1990; Poinar, 1992; Koppenhöfer and Fuzy, 2007) due to the difficulty in managing the white grubs like *P. adspersa.*

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