

Faunistic and Molecular Surveys on the Pistachio Hemiptera of Rafsanjan Region and Vicinity, South East Iran

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ABSTRACT

Pistachio is considered to be a strategic crop of Iran. Some groups of true bugs are among key pests of pistachio orchards throughout the country. Here, a comprehensive collecting of true bugs in pistachio orchards and surrounding weeds in Rafsanjan region (Kerman province) and its suburbs since March 2009 up to November 2011, resulted in finding 46 species of 37 genera, 17 subfamilies and 10 families. Among the collected specimens, two species of *Riptortus oxianus* Kiritshenko, 1914 and *Parapiesma rotundatum* Horvath, 1906 from Alydidae and Piesmatidae have been reported as new records for Iran. In order to study the genetic population, a partial segment of COI gene of samples was sequenced. The sequences were compared with those retrieved from the BOLD system and the GenBank through nBLAST. The results showed the COI sequences in the studied specimens can facilitate the separation of the species. Based on the relationships between these species, 10 tribes were clearly distinguished from each other as well as two infraorders, Cimicomorpha and Pentatomomorpha. This is the first effort to molecular study on the true bugs in Iran's pistachio orchards.

Key words: Pistachio, Hemiptera, fauna, molecular study, COI, Kerman, Iran.

INTRODUCTION

Pistachio (*Pistacia vera* L.) is native to North Afghanistan, Iran and Central Asian republics (Kafkas, 2006). Iran is the main world producer of pistachio with more than 400,000 tons followed by Turkey, USA and Syria (Faostat, 2004).

The pistachio bugs are among the main injurious pests of pistachio orchards mainly from families Pentatomidae, Lygaeidae and Miridae (Samih *et al.*, 2005). The stink bugs, *e.g.*, *Acrosternum heegeri*, *Acrosternum millieri*, *Apodiphus amygdali*, *Brachynema germari* and *Brachynema segetum*, which all belong to the family Pentatomidae, are abundant and serious pests of pistachio nuts in the pistachio plantation areas of Iran (Mehrnejad, 2002). Moreover, the seed bug, *Lygaeus pandurus* (Lygaeidae) has also been reported (Samet and Akbary, 1974). Apart from the above true bugs, several species of pentatomids bugs, *e.g.*, *Carpocoris* sp. and *Dolycoris* sp.

attack pistachio nuts, furthermore two mirid bugs, e.g., *Campylomma* sp., *Creontiades* sp. and *Megacoelum* sp. have been reported as pests of pistachio fruits in spring (Hashemi-Rad and Safavi, 1996; Hashemi-Rad, 2005). These insects are known as the important pests of pistachio throughout the pistachio-growing regions of Iran and cause severe damage to pistachio nuts throughout the season, from early spring to the time of harvest (Mehrnejad, 2002).

There are limitations to relying solely or largely on morphology in identifying and classifying life's diversity, often effective only for a particular life stage or gender. As well, finding appropriate experts and distributing specimens can be a time-consuming and expensive process while the number of taxonomic experts has dramatically decreased worldwide (Jinbo *et al.*, 2011). Hence, modern taxonomic work includes analysis other traits of a host, including genes, isoenzymes, physiology, behaviour, population biology and geography. Microgenomic identification systems, which permit life's discrimination through the analysis of a small segment of the genome, represent an extremely promising approach to the diagnosis of biological diversity (Hebert *et al.*, 2003). Advances in DNA-sequencing technologies have enabled researchers studying biodiversity to conduct simple, cost-effective and rapid DNA analyses (Jinbo *et al.*, 2011).

Our understanding of true bugs in the pistachio orchards is still incomplete because of their wide host-range, adult dispersal habits and diverse habitats (Mehrnejad, 2002) while the species determination is the first step in effective programs for true bugs management in pistachio orchards. The objective of this paper is first to introduce different species of pistachio true bugs in Rafsanjan region of Kerman province. Meanwhile two new true bugs species for Iran are introduced for the first time in this paper and finally we will try to study on COI gene sequences divergence among the dominant species of pistachio true bugs in the region.

MATERIALS AND METHODS

Collection and Preparation of the Specimens

Samplings were carried out during 2009-2011 to determine the true bugs in several pistachio orchards of Rafsanjan region (Kerman province, South-Eastern Iran) including East and West vicinity, Kashkoiye, Ahmad Abad, Kabotar Khan and Nogh. Different sampling equipments including sweeping net, aspirator, light trap and manual method were used.

Male Genitalia Organ

The sexual organs of the males were investigated as an important trait in the separation of the bug species. Hence, a binocular was used and separation was done in a concave slide containing glycerin. A section of the abdomen was cut using a blade and then placed in a 10% solution of KOH. The duration of soaking differed according to the species and required organs. To eliminate the remaining KOH residue, the specimens were placed in a petri dish containing distilled water for five minutes.

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The specimens were then placed in containers containing 50, 70 and 90% ethanol respectively, for a few minutes. The male sexual organs were put inside microtubes containing diluted glycerin (Lequesne *et al.*, 1981). The specimen's pictures were taken using a dinocapture (Model AM413T) and a digital camera. To illustrate the pygophore and paramere diagrams, a 0.1 rapid pen was used. All the identified specimens are placed in the insect collection of the Ferdowsi University of Mashhad.

Classic Identification of the Specimens

To identify the specimens, various identification keys including, China *et al.* (1959), Heiss *et al.* (1983), Slater (1998), Triplehorn *et al.* (2005), Akingbohunge (2007) and Modarres Awal (2009) were used. For the identification of the specimens, various traits including the thorax structure, front legs, pronotum, paramere and pygophore, abdomen rings, scent emitting gland structure and so on were used. The identified specimens and also those, whose identification was not possible at species level, were sent to Dr. Rauno E. Linnavuori in Finland for confirmation.

DNA Extraction, Amplification and Sequencing

Adults true bugs were killed directly in 96% ethanol. DNA was extracted from single specimens using the Bioneer genomic DNA extraction kit (AccuPrep®) with catalog number K-3032. First, legs of individual insects were ground in liquid Nitrogen in a 1.5 ml microcentrifuge tube. Then extraction was done based on the kit instruction. After extraction, PCR was used for amplification COI gene.

The primer set LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') was subsequently used in PCR (Folmer *et al.*, 1994). Each PCR contained 2.5 µl of 10X PCR buffer (20 mM of Tris-HCl; pH 8.0, 100 mM of KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5% tween 20, 0.5% NP-40, 50% Glycerol), 15.7 µl of distilled water, 0.5 µl of dNTPs, 0.3 µl of *Taq* polymerase, 1 µl of each primer and 3 µl of DNA template. The PCR thermal regime consisted of one cycle of 1 min at 94 °C, 35 cycles of 30s at 94 °C, 1.5 min at 52 °C and 1.5 min at 72 °C and a final cycle of 8 min at 72 °C. Amplicons were checked on a 1% Agarose gel stained with Ethidium Bromide. Sequencing reactions were performed in 3730XL DNA analyzer in MacroGen Co. (Korea) (<http://dna.macrogen.com>) following purification. Primers for the sequencing reaction were those used in the amplification step. All sequences were confirmed in both directions and repeated.

Analysis of Molecular Data

Sequences were aligned using the ClustalX multiple sequence alignment program (Larkin *et al.*, 2007) and subsequently edited manually using BioEdit (Hall, 1999). Specimen identification were made by inputting the sequence in nblast tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The MEGA5 was used to estimate evolutionary distances and to compute the basic statistical analysis (Tamura *et al.*, 2011). Phylogenetic analyses were done using neighbour joining (Saitou and Nei, 1987) for COI with 1000 replications of bootstrap (Felsenstein, 1985). COI sequence of *Cimex lectularius* with accession number HQ105554 was used as an outgroup.

RESULTS

Traditional Identification

Totally 46 species belonging to 37 groups out of 17 subfamilies from 10 families including Alydidae, Anthocoridae, Coreidae, Lygaeidae, Miridae, Nabidae, Pentatomidae, Piesmatidae, Rhopalidae and Pyrrhocoridae have been identified. Two species including, *Riptortus oxianus* Kiritshenko, 1914 and *Parapiesma rotundatum* Horvath, 1906 are newly recorded from Iran. The list of identified species is given in Table 1.

The important characteristics of the two new country records are given below.

Riptortus oxianus Kiritshenko, 1914

Material examined: Kerman province, Rafsanjan (Kashkoiye and Ahmad Abad), 2♀♀, May 2010.

Description. The adult insect has a long and slender body measuring 11.8 mm long and 2.8 mm wide (Fig. 1) being reddish brown in color. The yellow connexivum with a dark spot at both corners of the base of the tergum. A yellow line at the mid section of the bottom abdominal sternum. The shield's ending light yellow and its front margin relatively concave and the edges of the rear sides of the pronotum spiked and dark in color. The pronotum trapezoidal; the mid section of the rear margin with a yellow spot and the two edges of the rear margin of pronotum with black spiked burs. The head triangular and its length longer than its width. Compound eyes large, convex, brown with yellow internal margins and an internal irregular black spot. The simple eyes very large, yellow and situated on a black protrusion. The antennae long slender and with four segments; the fourth segment curved and longer than the third one. The openings of the odor emitting glands oval, raised and placed between the middle coxa and the rear coxa (Modarres Awal 2009). Proboscis long, slender arrived between the middle coxa. The legs long and slender and the hind leg femur very wide and posses black spikes in the inner margin.

Identification key of studied *Riptortus* species (with some modifications on Akingbohungebe 2007)

- 1- pronotum of adult insect spotted.....2
- 1'- pronotum of adult insect not spotted, average body length 11.2mm and width 2.7 mm, reddish brown color, the connexivum yellowish.....*Riptortus oxianus* Kiritshenko
- 2- Adult insect body length 15.69mm, width 3.65mm, frontal sternum wide and 3.02mm usually light brown, mid sternum and rear sternum usually spotted.....*Riptortus dentipes* Fabricius
- 2'- Adult insect body length 18.6mm and width 4.17mm, frontal sternum 3.38mm wide usually dark brown to black with two black spots.....*Riptortus acantharis* Dallas

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Table 1. List of true bug species, hosts and areas of their distribution in Rafsanjan.

Row	Family	Species	Area	Host
1	Pentatomidae Leach, 1815	<i>Acrosternum heegeri</i> Fieber, 1861	All areas	<i>Pistachio vera</i> , <i>Alhagi pseudalhagi</i> , <i>Peganum harmala</i>
2		<i>Acrosternum milleri</i> Mulsant - Rey, 1866	"	"
3		<i>Apodiphys amygdaly</i> Germar, 1817	Ahmad Abad	"
4		<i>Brachynema germarii</i> Kolenati, 1846	All areas	<i>Pistachio vera</i> , <i>Alhagi pseudalhagi</i> , <i>Peganum harmala</i> , <i>Salsola kali</i> , <i>Artemisia annua</i> , <i>Zygophyllum fabago</i>
5		<i>Brachynema signatum</i> Jakovlev, 1879	"	"
6		<i>Carpocoris fuscispinus</i> Boheman, 1850	"	"
7		<i>Sciocoris luteolus</i> Fieber, 1861	Kashkoiey, Ahmad Abad	<i>Chenopodium alba</i> , <i>Salsola kali</i>
8		<i>Sciocoris orientalis</i> Linnavuori, 1960	"	"
9	Miridae Hahn, 1833	<i>Campylomma diversicornis</i> Reuter, 1878	All areas	† <i>Pistachio vera</i> , <i>Salsola kali</i> , <i>Peganum harmala</i> , <i>Chenopodium alba</i>
10		<i>Campylomma unicolor</i> Poppius, 1919	"	†
11		<i>Creontiades pallidus</i> Rambur, 1839	"	<i>Medicago sativa</i> , <i>Chenopodium alba</i>
12		<i>Eurystylus bellevoeyi</i> Reuter, 1879	Ahmad Abad	<i>Chenopodium alba</i> †
13		<i>Halodapus costae</i> Reuter, 1890	Kashkoiey, Ahmad Abad	"
14		<i>Lygus pratensis</i> Linnaeus, 1758	Ahmad Abad	† <i>Pistachio vera</i>
15		<i>Megacoelum brevistrore</i> Reuter, 1879	All areas	<i>Pistachio vera</i> , <i>Chenopodium alba</i>
16		<i>Megacoelum tricolor</i> Wagner, 1958	"	"
17		<i>Megalocoleus hormozganicus</i> Linnavuori 1981	Ahmad Abad	<i>Pistachio vera</i>
18		<i>Pseudoloxops sangrudanus</i> Linnavuori, 2006	Kashkoiey, Ahmad Abad and Nogh	"
19	<i>Stenodema turanica</i> Reuter, 1904	Ahmad Abad	<i>Chenopodium alba</i> , <i>Medicago sativa</i>	
20	<i>Trigonotylus pulchellus</i> Hahn, 1834	"	<i>Medicago sativa</i>	
21	Pyrrhocoridae Fieber, 1860	<i>Pyrrhocoris apterus</i> Linnaeus, 1758	All areas	<i>Chenopodium alba</i> , <i>Alhagi pseudalhagi</i>
22	Rhopalidae Amyot - Serville, 1843	<i>Agrhopopus lethierryi</i> Stål, 1872	Kabotar khan, West vicinity, Kashkoiey and Ahmad Abad	<i>Medicago sativa</i> , <i>Chenopodium alba</i>
23		<i>Liorhysus hyalinus</i> Fabricius, 1794	Kashkoiey, Ahmad Abad	<i>Pistachio vera</i> , <i>Chenopodium alba</i> , <i>Medicago sativa</i> and <i>Agropyron repense</i>
24		<i>Stictopleurus pictus</i> Fieber, 1861	Ahmad Abad	<i>Chenopodium alba</i> , <i>Agropyron repense</i>
25	Lygaeidae, Schilling, 1829	<i>Cymophyes ochroleucus</i> Fieber, 1870	"	<i>Chenopodium alba</i>
26		<i>Dieuches schmitzi</i> Reuter, 1893	East vicinity, West vicinity, Kashkoiey and Ahmad Abad	"
27		<i>Emblethis dilatocolis</i> Jakovlev, 1874	All areas	"
28		<i>Engistus exsanguis</i> Stål, 1872	Ahmad Abad	<i>Pistachio vera</i> , <i>Chenopodium alba</i> , <i>Medicago sativa</i>
29		<i>Geocoris acuticeps</i> Signoret, 1881	Kashkoiey, Ahmad Abad	<i>Chenopodium alba</i>
30		<i>Geocoris ater</i> var. <i>albipennis</i> Fabricius, 1787	"	"
31		<i>Geocoris luridus</i> Fieber, 1844	Ahmad Abad	<i>Pistachio vera</i> , <i>Chenopodium alba</i>
32		<i>Geocoris pallidipennis</i> Costa, 1843	All areas	<i>Chenopodium alba</i>
33		<i>Hyalocoris pilicornis</i> Jakovlev, 1874	Ahmad Abad	<i>Agropyron repense</i>
34		<i>Lamprodema maurum</i> Fabricius, 1803	All areas	<i>Chenopodium alba</i>
35		<i>Lygaeus panderus</i> Scopoli, 1763	Kashkoiey, Ahmad Abad, Kabotar khan and Nogh	<i>Chenopodium alba</i> , <i>Medicago sativa</i>
36		<i>Melanocoryphus tristrami</i> Douglas - Scott, 1868	Kashkoiey, Ahmad Abad	<i>Chenopodium alba</i>
37		<i>Nysius cymoides</i> Spinola, 1837	All areas	<i>Pistachio vera</i> , <i>Chenopodium alba</i> , <i>Senecio vulgaris</i>
38		<i>Plinthinus longicollis</i> Fieber, 1867	Kashkoiey, Ahmad Abad	<i>Chenopodium alba</i>
39	<i>Spilostethus pandurus</i> Scopoli, 1763	All areas	<i>Pistachio vera</i> , <i>Agropyron repense</i> , <i>Chenopodium</i> <i>alba</i> , <i>Alhagi pseudalhagi</i>	
40	Nabidae Costa, 1852	<i>Nabis capsiformis</i> Germar, 1838	Ahmad Abad	<i>Medicago sativa</i> , <i>Agropyron repense</i>
41		<i>Nabis sareptanus</i> Dohm, 1862	"	"
42	Alydidae Amyot - Serville, 1843	<i>Riptortus oxianus</i> Kiritschenko, 1914	Kashkoiey, Ahmad Abad	<i>Descorania sophia</i> , <i>Agropyron repense</i> , <i>Alhagi</i> <i>pseudalhagi</i>
43	Coreidae Leach, 1815	<i>Cercinthus lehmanni</i> Kolenati, 1856	"	<i>Descorania sophia</i> , <i>Alhagi pseudalhagi</i>
44		<i>Centrocoris volxemi</i> Puton, 1878	"	"
45	Pismatidae Amyot - servile, 1843	<i>Parapiesma rotundata</i> Horvath, 1906	Ahmad Abad, Kabotar khan	<i>Chenopodium alba</i>
46	Anthocoridae Fieber, 1839	<i>Anthocoris minki pistacia</i> Wagner, 1957	All areas	<i>Pistachio vera</i> , <i>Chenopodium alba</i>

***Parapiesma rotundatum* Horvath, 1906**

Material examined: Kerman province, Rafsanjan (Kashkoiye and Ahmad Abad), 1♂, 2♀♀, April 2009

Description. Adult body length 1.85mm, width 0.85 mm, white in color. Sporadic black spots found over the body, general body looks perforated (Fig. 2). Head's width larger than its length, tylus raised and compound eyes light brown and also raised. Antennae with 4 rings, bucca with 4 links, tarsus with 2 links and a visible scutellum. Pronotum with 3 large raised trenches pointed in the side front, the frontal part wider than the posterior margins. The third middle front part of pronotum raised with 3 longitudinal lines which continue up to the pronotum. Frontal side edges of pronotum raised. Elyta light in color and extend a bit beyond the length of the body. Eight small brown spots in the connexivum region. The legs yellow, claws and the base of the tarsi brown.

There are few researches on Iranian Piesmatidae but all the data of Alydidae of Iran were catalogued by Ghahari *et al.* (2010).

Identification key of studied *Parapiesma* species (with some modifications by Heiss and Pericart, 1983)

1- Peripheral side margin of pronotum curved towards the inside, antennae long and slender, the body of the adult insect on average 2.41mm.....
..... *Parapiesma kolenatii* Hsiao et Jing

1⁻ Peripheral side margin of pronotum round2

2- Peripheral margin of pronotum round or roughly straight, frontal side margin of pronotum with 2 dark tuber, the length of the adult body on average 3mm and greenish yellow.....*Parapiesma quadratum* Fieber

2⁻ Peripheral side margin of pronotum always round, frontal side margin of pronotum raised and pointed, the length of the adult insect 2.74mm and matte white*Parapiesma rotundatum* Horvath

Analysis of Molecular Data

Four out of 46 species were chosen for molecular studies including *Agraphopus lethierry* (Rhopalidae), *Campylomma unicolor* (Miridae), *Dieuches schmitzi* (Lygaeidae), *Lamprodema maurum* (Lygaeidae) and their COI gene sequences were submitted to GenBank (NCBI) under the accession numbers of JN225416, JN225417, JN225418 and JN225419, respectively (www.ncbi.nlm.nih.gov). Nucleotide distances of the species were calculated between zero and 0.370 % based on COI gene and K2P method and the average mean of distances were 0.245%. Neighbor joining (NJ) tree showed the true bugs sequences in seven major clades (Fig. 3). COI sequences of specimens from Rafsanjan formed three single clades, two of them were placed in a clade.

The phylogenetic trees produced by other analysis showed the same overall topology as the NJ tree (maximum parsimony analysis and minimum evolution trees are not shown). Phylogenetic relationships analysis based on the COI sequence, using the neighbor joining method, showed four clades: the first clade including

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Campylomma unicolor from Iran and the species of the family Miridae; the second clade including *Agraphopus lethierryi* from Iran and the species of the families Rhopalidae and Pentatomidae; the third clade contained the species of the family Corixidae; the fourth clade contained *Lamprodema maurum* and *Dieuches schmitzi* from Iran and the other species of the family Lygaeidae (Fig. 3).

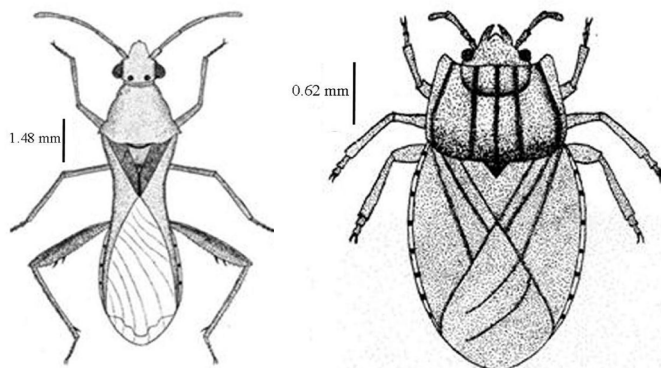


Fig. 1. *Riptortus oxianus* Kiritshenko, 1914. Fig. 2. *Parapiesma rotundata* Horvath, 1906.

Horvath, 1906. The results obtained from NJ method showed the COI sequences in the studied specimens can facilitate the separation of the species. Based on the relationships between these members, 10 tribes were clearly distinguished from each other as well as two infraorders, Cimicomorpha and Pentatomomorpha. This is the first effort to determine the DNA barcode for the Iranian true bugs in pistachio orchards. Blast analysis showed *Campylomma unicolor* with 99% bootstrap placed near to *Campylomma verbasci*. COI sequences were relatively similar between the two species *C. unicolor* and *C. verbasci* and the difference were 0.002.

DISCUSSION

In the present survey, a total 46 species from 37 genera and 17 subfamilies and 10 families were collected. Because of the limitation in collecting true bugs from all orchards and areas of the region, the pistachio true bugs orchards are not limited to these species. Hence, there must probably be much more species unstudied in pistachio orchards. Because of the difficulties in morphological species identification and accuracy of the identification tasks, molecular identification of bugs were also carried out for some species as a step for future surveys to be followed.

In conclusion, COI appears to be a good candidate marker and can be particularly suitable in combination with the sequencing of additional genes or when biological and morphological characteristics are also studied to supplement COI data. While using COI sequences as a barcode region, a threshold of differences must be existed between different species. So, the difference 0.002 between these two species verifies that COI sequences have confronted challenges and limitations as barcode of studied true bugs.

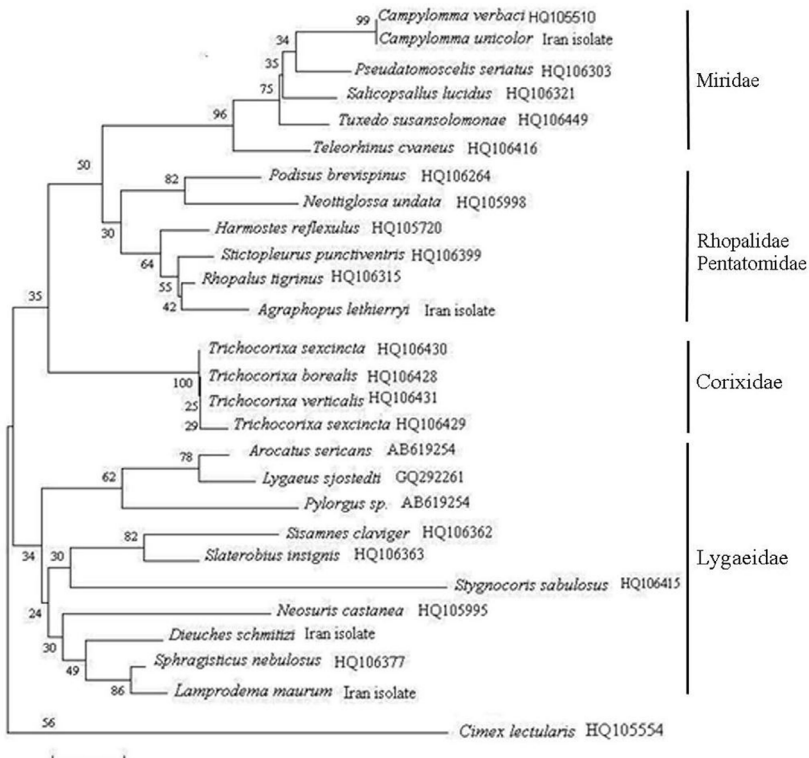


Fig. 3. Phylogenetic relationships among various species of true bug based on sequence of COI gene resulted from NJ method.

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