NORTH-WESTERN JOURNAL OF ZOOLOGY 19 (2): 134-139
Article No.: e231306

http://biozoojournals.ro/nwjz/index.html

Molecular analysis of *Bruchophagus* species (Hym.: Eurytomidae) associated with alfalfa in Iran

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Received: 13 January 2023 / Accepted: 21 June 2023 / Available online: November 2023 / Printed: December 2023

Abstract. Alfalfa (*Medicago sativa* L.) is recognized as one of the oldest perennial forages with considerable economic importance due to its characteristic biomass yield, quality forage, adaptability to various environments, and nitrogen fixation. The alfalfa seed chalcid, *Bruchophagus* spp. (Hymenoptera: Chalcidoidea, Eurytomidae), is a key pest of alfalfa seed production worldwide. In this study, we evaluated the phylogeny of different geographically isolated populations of the alfalfa seed chalcid using cytochrome oxidase c subunit one (COI) and elongation factor 1 alpha (Ef1α). The phylogenetic tree was created based on sequenced fragments by Maximum Likelihood (ML) analyses for 17 Iranian populations. Our phylogeny study suggested that the *Bruchophagus* species associated with alfalfa in Iran are monophyletic, and the monophyly was strongly supported by bootstrap values of 80-100% under *Bruchophagus roddi* (Gussakovsky).

Keywords: *Bruchophagus roddi*, seed eater, maximum likelihood, monophyletic, phylogeny, COI, Ef1α.

Introduction

Alfalfa, *Medicago sativa* L., is the most important forage legume in the temperate regions of both the northern and southern hemispheres (Bouton 2001). The genus Medicago originated in the Caucasus, northwestern Iran, and northeastern Turkey. *Medicago sativa* consists of several perennial subspecies, both diploids, and tetraploids, which are interfertile and have the same karyotype (Quiros & Bauchan 1988).

This plant plays an essential role in nourishing the land and supplying fodder. Alfalfa roots have nitrogen-fixing properties and are sufficiently effective in weed control, used solely or with other grains. Currently, there are investigations concerning the possibility of alfalfa as a fuel for electricity generation, a source for paper manufacturing and production of industrial enzymes, and as a bioremediation system for the elimination of harmful nitrates. In addition, the possibility of using this species in low-management mixed pastures, in agriculture, or as a crop in the tropics has been considered (Bouton 2001). Increasing the cultivated area of this valuable product requires sufficient seed supply and reducing the limiting factors of its production.

Alfalfa is attacked by more than 50 arthropod pests in Iran (Khanjani 2008). The alfalfa leaf weevil (*Hypera postica* (Gyllenhal)), Tarnished plant bug (*Lygus lineolaris* (Palisot de Beauvois)), clover seed weevil (*Sitona cylindricollis* (Fahraeus)), alfalfa seed eaters (*Bruchophagus* sp.) (Khanjani & Kalafji 2003) and alfalfa caterpillar (*Colias eurytheme* (Boisduval 1852)), alfalfa webworm (*Loxostege commixtalis* (Walker 1866)), armyworms (*Spodoptera* spp.), grasshoppers, leafhoppers, *Lygus* bugs, spider mites (*Tetranychus* spp.), and various species of aphids are the most important pests of alfalfa (Edde 2022).

Alfalfa seed eaters are the most essential restrictive factors of seed production (Arbab 2001). By attacking the seeds in nature and warehouses, these insects destroy the contents, the embryos, and the seed germination capacity. The most important seed eaters of alfalfa are the alfalfa seed wasps, including *Bruchophagus roddi* (Gussakovsky) and *Bruchophagus gibbus* (Boheman) (Zerova 1995, Khanjani & Kalafji 2003, Arbab 2006). These pests are globally distributed and reported from several continents, including Asia, Africa, Europe, North America, and Oceania (CIE 1988).

In the cold and semi-cold regions of Iran, the last generation of these insects coincided with seeding the second harvest of alfalfa fields, and they can be most destructive, with damage up to 80% (Arbab 2001). According to De Barro (2001), chemical control of the seed wasps is not a practical method nor a part of its management strategy. The wasps are exposed for six months of the year to insecticides used in commercial seed production to manage other pests. However, changes in pest control management can be useful to reduce its impact on the seed crop.

Nowadays, the application of molecular studies is essential in supporting species discrimination based on morphological and phylogenetic studies. Campbell et al. (2000) used the D2 region of the 28S rDNA marker to infer the phylogeny of the entire superfamily Chalcidoidea. They found the family Eurytomidae (five species) was polyphyletic and divided into two subfamilies, Rileyinae and Eurytominae.

Chen et al. (2004) performed the molecular phylogeny of the Eurytomidae family using four molecular markers (18s and 28s (nuDNA), rDNA 16s, and COI (mtDNA)) on 24 species of this family. Although most of the genes showed a low level of diversity and the range of analyses was highly contrasting, the authors concluded that the subfamily Rileyinae is mostly closer to Torymidae than Eurytomidae. Moreover, the species groups from the genera *Eurytoma* and *Bruchophagus* are often more closely related to other small genera than other species groups of the same genus. Hale et al. (2004) used microsatellite markers to analyze the population genetic structure of *Eurytoma brunniventris* Ratzeburg, a parasitoid of the oak gall wasps.

Most phylogenetic studies of the family Eurytomidae demonstrated that they are highly diverse, with many cryptic species, which led to the erection of new species (such as

Eurytoma cainae Lotfalizadeh and Delvare) or revalidation of old synonymized species (such as *Sycophila pistacina* (Rondani)) (Lotfalizadeh et al. 2007a, 2008b).

Lotfalizadeh et al. (2007a) showed *E. caninae* differs from *E. rosae* in the strong distances of CO1 and ITS2 (about 10% between the two taxa) that strongly support a species-level differentiation.

When comparing all available hymenopteran mitochondrial genomes (even submitted partial genome from *Ceratosolen solmsi*), Chalcidoids had dramatic mitochondrial gene rearrangement, involving not only the tRNAs but also several protein-coding genes (Xiao et al. 2011).

A preliminary phylogenetic study of the Eurytomidae using mitochondrial and nuclear genes (COI, Cytb, 16S, and D4-5 of 28S) confirmed the relationship between some genera and species groups (Lotfalizadeh et al. 2008a). Several phylogenetic studies revealed the Eurytomidae as a polyphyletic family (Munro et al. 2011, Lotfalizadeh et al. 2007b, 2008a, Delvare et al. 2014).

Delvare et al. (2019) discriminated pairs of sibling species in the three genera of Eurytomidae associated with *Asphodelus* and *Asphodeline* (Xanthorrhoeaceae) using COI and Cyt B and of the nuclear gene EF1-*a*. They established the trophic relationships of *Bruchophagus* species and their chalcidoid parasitoids. Zhang et al. (2020) confirmed the results of Lotfalizadeh et al. (2007b) concerning the close relationship between the Eurytomidae and Chalcididae families.

Despite the wide distribution of alfalfa seed chalcid worldwide, no research has been done on its global molecular phylogeny. Therefore, this research is conducted with the aim of molecular study of different populations of alfalfa seed chalcid, using mitochondrial and nuclear markers to identify the pest accurately. Considering that each pest has its own biology and control measures, the first step is to correctly identify the pest species because the presence of subspecies or even new species can affect control in the field. Considering the economic importance of alfalfa seed chalcid damage and this pest's lack of molecular information, the present research would hopefully be of great scientific value. Therefore, this research aims to identify possible species of alfalfa seed eater wasps in Iran using molecular methods.

Material and methods

Collecting

Samples were collected from July to October of 2018 and 2019 from alfalfa farms in ten provinces that are the most important alfalfa producers in Iran (Table 1).

At the end of spring and summer, once the seed capsules were almost ripened, capsules were collected from the fields and placed in paper envelopes. They were transferred to the laboratory along with the collection data, including locality, date, altitude, and latitude.

Table 1. Sample information for the associated *Bruchophagus* specimens (female and male) with alfalfa used in the molecular studies.

Locality	Code	Coordinates		Molecular markers	
		GPS	Altitude (m)	COI	EF1α
Ardabil, Meshkinshahr	Meshkinshahr G1	38° 22.7580′ N/47° 31.6360′ E	1166	+	_
East-Azarbaijan, Ahar	Ahar G	38° 26.6290′ N/47° 13.9960′ E	1275	+	+
	Ahar R2	38° 26.6290′ N/47° 13.9960′ E	1275	+	+
East-Azarbaijan, Tabriz	Tabriz G1	38° 0833′ N/46° 2833 E	1390	+	_
	Tabriz G2	38° 0833′ N/46° 2833 E	1390	+	+
Fars, Dalin	Dalin G2	52° 1235′ N/30° 0591′ E	1500	+	+
Fars, Kavar	Kavar R1	29° 16′ N/52° 41′ E	1589	+	_
Fars, Sepas	Sepas G1	29° 3700′ N/52° 3200′ E	1500	+	+
Hamedan, Hamedan	Hamedan G1	34° 80′ N/48° 52′ E	1741	+	+
Khorasan Razavi, Mashhad	Mashhad, G1	36° 305729′ N/59° 578370′ E	1050	_	+
Kordestan, Saggez	Saggez G1	36° 2453′ N/46° 2753′ E	1532	+	+
	Saggez G2	36° 2453′ N/46° 2753′ E	1532	+	+
Kerman, Jopar	Jopar G1	30° 0575′ N/57° 1108′ E	1872	+	+
	Jopar G2	30° 0575′ N/57° 1108′ E	1872	+	+
Markazi, Arak	Arak G1	34° 08′ N/49° 7′ E	1743	+	+
West-Azarbaijan, Khoy	Khoy G3	38° 38.8930′ N/44° 57.2140′ E	1146	+	+
Zanjan, Zanjan	Zanjan G2	36° 6667′ N/48° 4833′ E	1640	+	+

The seeds were kept in ventilated containers in laboratory conditions and were examined daily to monitor adult emergence. The emerging samples were directly frozen in the microtubes at -20 °C for molecular analysis. Morphospecies were separated using morphological characters of antenna, metasoma, and wing venation under an Olympus™ SZH stereomicroscope.

Molecular study

DNA extraction

We examined specimens from 17 different localities populations (Table 1), using the mitochondrial and nuclear genes, cytochrome oxidase c subunit one (COI) and elongation factor 1 alpha (Ef1α), respectively. At least two specimens for each population were used, including female and male (if present). Total genomic DNA was extracted using Dellaporta's method (Dellaporta et al. 1983). Individual wasps were placed in 1.5 ml microcentrifuge tubes containing 375µl isolation (extraction) buffer (500mM NaCl, 100 mMTris-HCl [pH 8], 50mM EDTA [pH 8], 0.2% 2-mercaptoethanol, 15 µl Proteinase K). They were entirely powdered by a homogenizer, and 25 µl SDS 20% was added to each tube. The homogenate was vortexed briefly and incubated at 65°C for 60 min. Proteins were removed using chloroform isoamyl alcohol (24:1) and centrifugation for 20 min at 13000 rpm. The supernatant was transferred to a clean tube, and 150 µl cold isopropanol was added and vortex mixed. After precipitation

for about 60 min at -20 °C, extracts were re-centrifuged for 30 min at 4°C and 13000 rpm. The proteins were cleaned with 500 µl absolute ethanol and centrifuged for 10 minutes at 4°C and 13000 rpm. Finally, DNA was pelleted by centrifugation and resuspended in 30 µl of distilled water.

PCR amplification, purification, and sequencing of CO1 and Ef1a

Amplification involved the primer set for COI as follows: forward, LCO1490F, 5′–GGTCAACAAATCATAAAGATATTGG–3′, and reverse, HCO2198pucR, 5′–AAACTTCAGGGTGACAAAAAA TCA–3′ (Coeur et al. 2014). The primers for Ef1alfa were as follows: forward, F2-557F, 5′–GAACGTGAACGTGGTTATCACCAT–3′, and reverse, F2-1118R, 5′–TTACCTGAAGGGGAAGACGGAG–3′ (Brady et al. 2006).

PCR reactions (25 µl) contained 12 µl master mix (Sina clone), 0.5 µl MgCl2 (50Mm), distilled water 6 µl, 1µl of each primer, and 1 µl DNA extract (0.5x SolutionQ, for COI) and were performed in a PTC-200 thermocycler. DNA was denatured for 3 min at 94°C, followed by 40 cycles of 1 min each at 94°C, 1:30 min annealing at 50°C and 1 min extension at 72°C. DNA was finally extended for 7min at 72°C after amplification (Coeur et al. 2014). We used the same conditions for the amplification of Ef1alfa to optimize the reactions, with the following changes: denaturation for 3 min, 35 amplification cycles, 30 s denaturing at 94°C, and 1 min annealing at 58°C. PCR products were separated on a 1.5% agarose gel. Purified DNA fragments were directly sequenced from both directions using an automated sequencer. Primers used for amplification served as sequencing primers. Sequencing was done with the Sanger method (XI 3130 analyzer). Then, the sequences were checked and edited by Finch TV® 1.4.0 software.

After editing and ensuring the correctness of each sequence, the sequences were uploaded to the website of the National Center for Biotechnology Information (submit/genebank/www.ncbi.nlm.nih.gov), in which an accession number was assigned to each uploaded sequence after verification.

To ensure the correctness of the sequences of the investigated regions and their compatibility with closely related species, each sequence is individually in the blast section of the World Gene Bank website at the address (http://blast.ncbi.nlm.nih.g). After sequence blast, several COI and Ef1α sequences of the gene bank were used to compare with the sequences obtained from these two genes in the present study in the phylogeny tree, *Eurytoma gigantean* was used as the out-group in all analyses. The names of species and their accession numbers are given in table 2.

Table 2. Gen Bank accession numbers of studied species in this research.

Species	Gen Bank accession numbers		Reference
	Ef1α	COI	
Bruchophagus caucasicus (Zerova, 1992)	JQ756902.1	JQ756607.1	Cruaud et al. (2013)
Eurytoma gigantea (Walsh, 1870)	GQ410753.1	GQ374673.1	Heraty et al. (2013)
Bruchophagus sp. 4 LB-2019 isolate GDEL1638	MK030103.1	_	Askew et al. (2019)
Eurytoma steffani (Claridge, 1959)	MK030107.1	_	Askew et al. (2019)
Eurytoma ochraceipes (Kalina, 1970)	MK030105.1	_	Askew et al. (2019)
Sycophila sp. 2 JHX-2007 isolate M15I1	FJ438277.1	_	Xiao et al. (2010)
Bruchophagus gibbus (Boheman, 1836).	_	MH878927.1	Askew et al. (2019)
Bruchophagus ononis (Mayr, 1878)	_	AY317223.1	Chen et al. (2004)

To compare the sequences with each other and check the variable gene regions, the amount of distance, gap, and display of all the sequences aligned, the studied sequences for each gene were stacked separately using the online software MAFFT v. 7 (Kuraku et al. 2013, Katoh et al. 2019) (https://mafft.cbrc.jp/alignment/server/).

To prepare multigene alignment of the sequences, the sequences related to the individual genes were included using Mesquite v. 2.75 (Maddison & Maddison 2011). The concatenated sequences were saved as .fasta files.

The phylogenetic trees were obtained based on maximum likelihood inference using RAxML software in the program raxmlGUI 1.3 (Stamatakis 2014).

Analysis

Kimura's two-parameter model of base substitution was used to calculate genetic distances with MEGA 7 software (Kumar et al. 2016). All sequences were submitted to GenBank under accession numbers OQ450355 to 450368 and OQ352578 to OQ352592 (see Appendix 1).

RAxML software runs files with extension (.phy) to infer kinship relationships. To prepare the final executable file for RAxML (Stamatakis 2014), the aligned FASTA file was converted to (.phy) format using the PAUP program (Swofford 2002).

Maximum Likelihood (ML) analyses were performed with RAxML as implemented in raxmlGUI1.3 (Silvestro & Michalak 2012), using the ML+rapid bootstrap setting and the GTRGAMMA substitution model with 1000 bootstrap replicates.

Finally, the created maps were edited using Adobe Illustrator CS5 software. The sequences obtained in this research were registered in the gene bank, and a GenBank Accession Number was received for them (Table 2).

Results

Sequence variation of partial COI and Ef1α genes

We obtained 16 sequences for COI (700 bp) and 14 for Ef1α (600 bp). Sequence data from the mitochondrial COI partial gene and nuclear gene Ef1α indicate that the *Bruchophagus* species were monophyletic, and the monophyly was strongly supported with bootstraps values between 80% to 100% (Figs 1-3). Of the 1171 nucleotide characters of the combined matrix, 1026 are constant, 103 are parsimony uninformative, and 42 are parsimony informative. Figure 1 shows a phylogram of the best ML tree (InL= -0/2409.02, Tree-Length: 0.2) obtained by RAxML.

This study confirms the monophyletic nature of the *Bruchophagus roddi* populations of Iran. In addition, all included species of the genus *Bruchophagus* (*Bruchophagus caucasicus* and *Bruchophagus* sp.) with 70% (in COI) and 96% (in Ef1α) BS were also monophyletic (Figs 3 and 2).

The COI inter-population distances range from 0 to 4.1%. The Ef1α inter-population distances range from 0 to 3.7%. The lowest genetic distance was observed between two populations of Saggez G1 and Joupar G2 (in COI), Dalin G2 and Ahar R2 (in Ef1α). The greatest genetic distance was observed between two populations of Sepas G1 and Kavar R1 (in COI), Mashhad G1 and Saggez G1 (in Ef1α).

The COI genetic distance ranges between the outgroup (*Eurytoma gigantea*) and *B. roddi* populations from Kavar R1

(13.4%) to Sepas G1 (15.8%). The Ef1α genetic distance ranges between the outgroup (*Eurytoma gigantea*) and populations from Joupar G2 3.9%) to Tabriz G2 (6.4%).

Despite the formation of small clades consisting of two or more samples from different localities of Iran, all the studied samples were assembled in a larger clade with a bootstrap of 100, which supported their monophyly and proved that they all belong to the same species.

According to Fig. 1, Ahar R2 and Joupar G1 groups were introduced as sister groups to other species with 100% and 72% BS, respectively. In our analysis, two populations (Zanjan G2 and Saggez G2) and two populations (Tabriz G2 and Sepas G1) were proved to be monophyletic with 53% and 71% BS, respectively. The monophyletic test of the sixth clade containing the Ahar G, Saggez G1, Joipar G2, Hamedan G1, and Khoy G3 populations also resulted in 56% BS. Meanwhile, the Hamedan G1 and Khoy G3 populations were concluded to be monophyletic with 65% BS.

For the Ef1α single gene tree (Fig. 2), 5 clades have been formed. Outgroup taxa have been separated from our studied groups with 100% and 64% BS. This analysis proved the monophyly of all studied B. roddi population by 86% BS.

Fig. 1 shows that 7 clades have been formed for the COI single gene tree. This analysis introduced ingroups in a common clade with 99% BS as a monophyletic group. Meanwhile, *Bruchophagus mutabilis* Nikol'skaya, 1952, was separated from other groups with 100% BS.

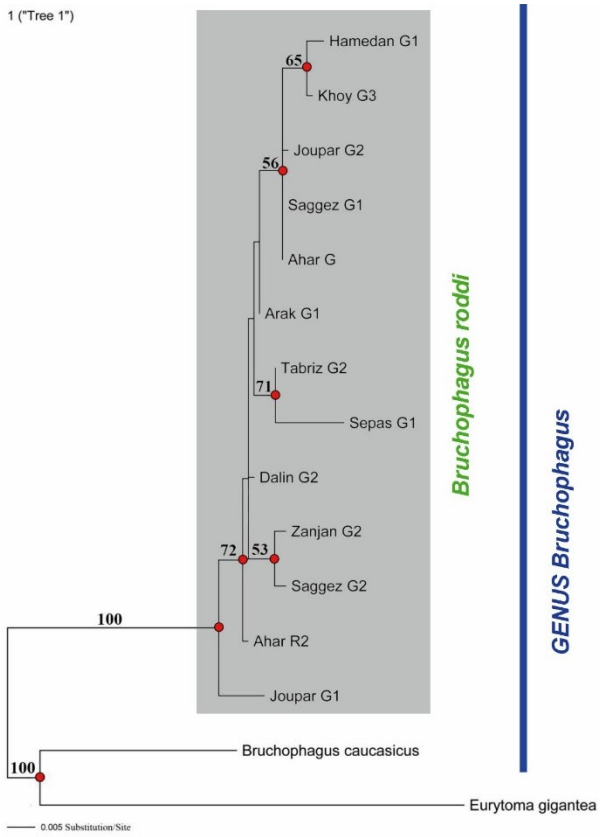

Figure 1. Phylogram of *Bruchophagus* populations obtained from *Medicago sativa* in Iran and *Eurytoma* (outgroup) relationship based on the fragments of the Ef1α and COI RaxML with 1000 bootstrap percentages (BP) replications.

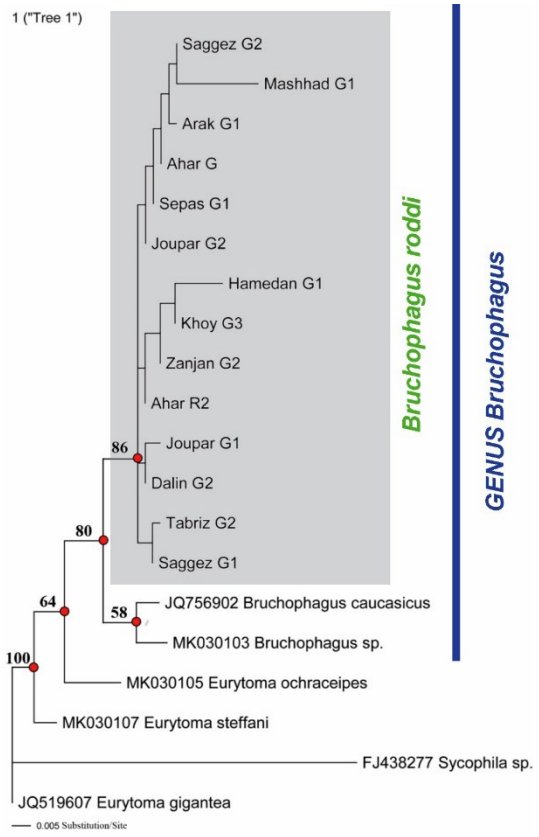

Figure 2. ML tree figuring the relationships of the *Bruchophagus* based on the fragment of the Ef1α. RAxML analysis with 1000 rbs bootstrap replicates (support >70 % are indicated below nodes).

Discussion

Like other genera and species of the family Eurytomidae, *Bruchophagus roddi* is a species that is difficult to identify morphologically, but our results provide evidence for species discrimination. Although only several populations of *B. roddi* and four species belonging to the genus *Bruchophagus* were sequenced in the present study, the phylogenetic analyses provided evidence for the monophyly of the genus. At the same time, Chen et al. (2004) evaluated the genus *Bruchophagus* as a polyphyletic group.

The monophyly of the studied population of *B. roddi* was supported by a high bootstrap ranging from 86 to 100, and two amplified markers discriminated this species from other species of *Bruchophagus* and outgroups.

The recent development of high-throughput sequencing technologies can be a powerful tool for establishing relationships among taxa. Hence, by employing different techniques, we shall soon be able to confirm the systematic placement of species.

We used two markers, COI and Ef1α, for phylogenetic study and specific discrimination of *Bruchophagus* species associated with alfalfa. Similarly, Delvare et al. (2019) used COI and CytB for *Bruchophagus* species associated with seeds

of asphodels. The obtained topology of COI and Ef1α is different for the *B. roddi* population of Iran. Likewise, Delvare et al. (2019) obtained different topologies of COI and CytB for *Bruchophagus* due to their different evolutionary history.

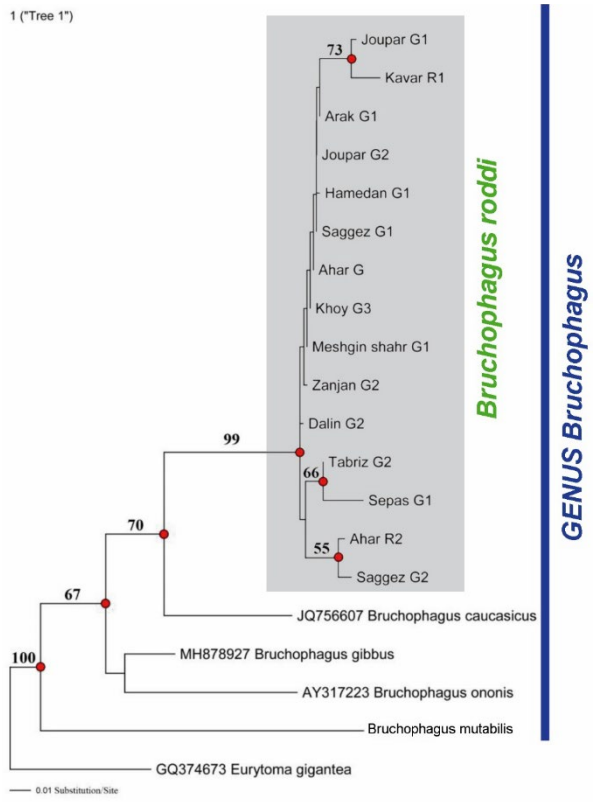

Figure 3. ML tree figuring the relationships of the *Bruchophagus* based on the fragment of the mitochondrial gene COI. RAxML analysis with 1000 rbs bootstrap replicates (support >70 % are indicated below nodes).

Our phylogenetic study confirming the monophyly of the genus *Bruchophagus* suggested that the *Bruchophagus* species associated with alfalfa in Iran are monophyletic, and the monophyly was strongly supported with the bootstrap values of 80-100%.

Acknowledgments

We thank all of our colleagues who kindly collected alfalfa seeds from different localities of Iran and supported us with materials.

References

Askew, R.R., Ribes Escola, A., Stojanova, A.M., Benoit, L., Lecomte, J., Delvare, G. (2019): Exploring insect biodiversity: the example of the parasitic Hymenoptera (Chalcidoidea and a few other Parasitica) associated with the seeds of asphodels (Xanthorrhoeaceae), with the description of nine new species (Eurytomidae, Torymidae). Zootaxa 4597(1): 1-90.

Arbab, A. (2001): Report on the research plan for the preliminary survey of alfalfa seed pests in Qazvin region. Pest and Plant Disease Research Institute, 35pp.

Arbab, A. (2006): Spatial distribution pattern of immature stages of alfalfa seed weevil *Tychius aureoles* (Col., Curculionidae) and alfalfa seed chalcid *Bruchophagus roddi* (Hym., Eurytomidae) in Qazvin alfalfa seed fields. Journal of Agricultural Sciences 12(2): 264-268.

Bouton, J.H. (2001): Alfalfa. The XIX International Gr X International Grassland Congress took place in São Pedro, São Paolo, Brazil, 11-21 February, 2001.

Brady, S.G., Schultz, T.R., Fisher, B.L., Ward, P.S. (2006): Evaluating alternative hypotheses for the early evolution and diversification of ants. Proceedings of the National Academy of Sciences of the United States of America 103: 18172-18177.

Campbell, B.C., Heraty, J.M., Rasplus, J.-Y., Chan, K., Steffen-Campbell, J.D., Babcock, C.S. (2000): Molecular systematics of the Chalcidoidea using 28S-D2 rDNA. pp: 59-73. In: Austin, A.D. and Dowton, M. (eds) Hymenoptera, Evolution, Biodiversity and Biological Control. CSIRO, Collingwood, Australia.

Chen, Y., Xiao, H.J., Huang, D.W. (2004): A molecular phylogeny of eurytomid wasps inferred from DNA sequence data of 28S, 18S, 16S, and COI genes. Molecular Phylogenetics and Evolution 31: 300-307.

CIE (1988): Distribution Maps of Pests, Map No. 500. Wallingford, UK: CAB International.

Coeur, d'A.A., Cruaud, A., Artige, E., Genson, G., Clamens, A.L., Pierre, E., Hudaverdian, A., Simon, J.C., Jousselin, E., Rasplus, J.-Y. (2014): DNA barcoding and the associated PhylAphidB@se website for the identification of European Aphids (Insecta: Hemiptera: Aphididae). Plos One 9 (6): e97620.

Cruaud, A., Underhill, J.G., Huguin, M., Genson, G., Jabbour-Zahab, R., Tolley, K.A., Rasplus, J.-Y., van Noort, S. (2013): A multilocus phylogeny of the world Sycoecinae fig wasps (Chalcidoidea: Pteromalidae). PLoS ONE 8(11): e79291.

De Barro, J. (2001): Evaluating and managing lucerne seed wasp in lucerne seed crops. A Report for the Rural Industries Research and Development Corporation. RIRDC Publication No 01/136, Australia.

Dellaporta, S.L., Wood, J., Hicks, J.B. (1983): A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter 1(4): 19-21.

Delvare, G., Gebiola, M., Zeieri, A., Garonna, A.P. (2014): Revision and phylogeny of the European species of the *Eurytoma morio* species group (Hymenoptera: Eurytomidae), parasitoids of bark and wood boring beetles. Zoological Journal of the Linnean Society 171: 370-421.

Delvare, G., Ribes Escola, A., Stojanova, A.M., Benoit, L., Lecomate, J., Askew, R.R. (2019): Exploring insect biodiversity: the parasitic Hymenoptera, chiefly Chalcidoidea, associated with seeds of asphodels (Xanthorrhoeaceae), with the description of nine new species belonging to Eurytomidae and Torymidae. Zootaxa 4597 (1): 001-090.

Edde, P.A. (2022): Field crop arthropod pests of economic importance. Science Direct, Acedemic Press, Richmond, U.S..

Hale, M.L., Acs, Z., Stone, G.N. (2004): Polymorphic microsatellite loci in *Eurytoma brunniventris*, a generalist parasitoid in oak cynipid galls. Molecular Ecology Notes 4: 197-199.

Heraty, J., Ronquist, F., Carpenter, J.M., Hawks, D., Schulmeister, S., Dowling, A.P., Murray, D., Munro, J., Wheeler, W.C., Schiff, N., Sharkey, M. (2011): Evolution of the hymenopteran megaradiation. Molecular Phylogenetics and Evolution 60(1): 73-88.

Katoh, K., Rozewicki, J., Yamada, D. (2019): MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20(4): 1160-1166.

Khanjani, M. (2008): Field Crop Pests in Iran. Bu-Ali Sina University Press No. 195, Fourth edition.

Khanjani, M., Kalafji, M. (2003): Preliminary identification of the pests of seed alfalfa fields and the study of the life cycle of the dominant damaging species in Hamedan. Tabriz Agricultural Knowledge Journal 13(2): 101-189.

Kumar, S., Stecher, G., Tamura, K. (2016): MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger dataset. Molecular Biology and Evolution 33: 1870-1874.

Kuraku, S., Zmasek, C.M., Nishimura, O., Katoh, K. (2013): A leaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Research 41(1): 22-28.

Lotfalizadeh, H., Delvare, G., Rasplus, J.-Y. (2007a): *Eurytoma caninae* sp. n. (Hymenoptera, Eurytomidae), a common species previously overlooked with *E. rosae*. Zootaxa 1640: 55-68.

Lotfalizadeh, H., Delvare, G., Rasplus, J.-Y. (2007b): Phylogenetic analysis of Eurytominae (Chalcidoidea: Eurytomidae) based on morphological characters. Zoological Journal of the Linnean Society 151: 441-510.

Lotfalizadeh, H., Delvare, G., Rasplus, J.-Y. (2008a): Eurytomidae (Hym.: Chalcidoidea), preliminary molecular study. 18th Iranian Plant Protection Congress, 24-27 August 2008, Bu-Ali-Sina university, Hamedan, Iran.

Lotfalizadeh, H., Delvare, G., Rasplus, J.-Y. (2008b): *Sycophila pistcina* (Hymenoptera: Eurytomidae): A valid species. European Journal of Entomology 105: 137-147.

Maddison, W.P., Maddison, D.R. (2011): Mesquite: A modular system for evolutionary analysis. version 2.75. URL http://mesquiteproject.org/ [accessed on 9 May 2015].

Munro, J.B., Heraty, J.M., Burks, R.A., Hawks, D., Mottern, J., Cruaud, A., Rasplus, J.-Y., Jansta, P. (2011): A molecular phylogeny of the Chalcidoidea (Hymenoptera). PLoS ONE 6(11): e27023.

Quiros, C.F., Bauchan G.R. (1988): The genus *Medicago* and the origin of the *Medicago sativa* complex. In: Alfalfa and alfalfa improvement (Hanson, A. A.,

Barnes, D. K. and Hill, R. R., eds.) Agronomy Monographs, American Society of Agronomy, Madison, WI. 29: 93-124.

Silvestro, D., Michalak, I. (2012): raxmlGUI: a graphical front-end for RAxML. Organisms Diversity & Evolution 12(4): 335-337.

Stamatakis, A. (2014): RAxML version 8: a tool for phylogenetic analysis and postanalysis of large phylogenies. Bioinformatics 30: 1312-1313.

Swofford, D.L. (2002): PAUP*: Phylogenetic analysis using parsimony (and other methods). version 4.0a165. Sinauer Associates, Sunderland, Massachusetts, USA.

Xiao, J.-H., Wang, N.-X., Li, Y.-W., Huang, D.-W. (2010): Molecular approaches to identify cryptic species and polymorphic species within a complex community of fig wasps. PLoS ONE 5(11): e15067.

Xiao, J.H., Jia, J.G. Murphy, R.W., Huang, D.W. (2011): Rapid evolution of the mitochondrial genome in chalcidoid wasps (Hymenoptera: Chalcidoidea) driven by parasitic life styles. PLoS ONE 6(11): e26645.

Zerova, M.D. (1995): The parasitic Hymenoptera - subfamilies Eurytominae and Eudecatominae (Chalcidoidea, Eurytomidae) of Palaearctics. National Academy of Sciences of Ukraine. Kiev, Ukraine: Izdatel'stvo Naukova Dumka 455 pp.

Zhang, J., Lindsey, A., Petersa, R., Heraty, J., Hopper, K., Werren, J., Martinson, E., Wolley, J., Yoder, M., Krogmann, L. (2020): Conflicting signal in transcriptomic markers leads to a poorly resolved backbone phylogeny of chalcidoid wasps. Systematic Entomology 45(4): 783-802.

Appendix 1. Accession numbers for COI and Ef1α gene sequences of Iranian populations of *Bruchophahus ruddi*.

Species	Populations	Gen Bank accession numbers	
		Ef1*a*	COI
Bruchophagus roddi	Saggez-G2	OQ450356	OQ352578
Bruchophagus roddi	Saggez-G1	OQ450357	OQ352579
Bruchophagus roddi	Ahar-G	OQ450358	OQ352580
Bruchophagus roddi	Ahar-R2	OQ450359	OQ352581
Bruchophagus roddi	Sepas-G1	OQ450360	OQ352582
Bruchophagus roddi	Zanjan-G2	OQ450361	OQ352583
Bruchophagus roddi	Arak-G1	OQ450362	OQ352584
Bruchophagus roddi	Dalin-G2	OQ450363	OQ352585
Bruchophagus roddi	Hamedan-G1	OQ450364	OQ352586
Bruchophagus roddi	Joupar-G2	OQ450365	OQ352587
Bruchophagus roddi	Joupar-G1	OQ450366	OQ352588
Bruchophagus roddi	Khoy-G3	OQ450367	OQ352589
Bruchophagus roddi	Kavar-R1	-	OQ352591
Bruchophagus roddi	Meshginshahr-G1	-	OQ352592
Bruchophagus roddi	Tabriz-G2	OQ450355	-
Bruchophagus roddi	Mashhad-G1	OQ450368	-
Bruchophagus mutabilis (out group)	Tabriz-G1	-	OQ352590